IN VITRO INHIBITION OF *LISTERIA MONOCYTOGENES* BY NOVEL COMBINATIONS OF FOOD ANTIMICROBIALS

A Thesis

by

ALEX LAMAR BRANDT

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2009

Major Subject: Food Science and Technology

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Approved by:

Chair of Committee,	T. Matthew Taylor
Committee Members,	Margaret D. Hardin
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ABSTRACT

In vitro Inhibition of *Listeria monocytogenes*

by Novel Combinations of Food Antimicrobials. (December 2009)

Alex Lamar Brandt, B.S., Texas A&M University

Chair of Advisory Committee: Dr. T. Matthew Taylor

Listeria monocytogenes is a foodborne pathogenic bacterium responsible for ~500 deaths and a financial burden of ~$2.3 billion each year in the United States. Though a zero tolerance policy is enforced with regard to its detection in cooked ready-to-eat foods, additional preemptive control alternatives are required for certain products. Among these alternatives are strategies permitting the usage of food antimicrobial combinations to control the pathogen. Research on antimicrobial combinations can provide insight into more efficient control of the pathogen, but is currently lacking.

The purpose of this study was to evaluate the *in vitro* inhibition of *L. monocytogenes* exposed to the antimicrobials ε-Poly-L-Lysine (EPL), lauric arginate ester (LAE), and sodium lactate (SL) at pH 7.3, octanoic acid (OCT) at pH 5.0, and nisin (NIS) and acidic calcium sulfate (ACS) at both pH 5.0 and 7.3. A broth dilution assay was used to determine single antimicrobial minimum inhibitory and bactericidal concentrations for *L. monocytogenes* Scott A, 310, NADC 2783, and NADC 2045. Optical density differences (Δ <0.05 at 630 nm) were used to denote inhibition. Concentrations producing population decreases of $\geq$3.0 $\log_{10}$ CFU/ml after incubation were considered bactericidal.

Inhibition resulting from combinations of antimicrobials (NIS+ACS, EPL+ACS, SL+ACS, NIS+LAE, OCT+ACS, and OCT+NIS) was assessed using a checkerboard assay, and

fractional inhibitory concentrations (FIC) were determined. FIC values were plotted on isobolograms and were used to create FIC indices (FIC_I). Isobologram curvature was used to classify combinations as synergistic, additive, or antagonistic. From FIC indices, interactions were defined as antagonistic ($FIC_I > 1.0$), additive ($FIC_I = 1.0$), or synergistic ($FIC_I < 1.0$).

Strain-dependent factors had a bearing on MIC and MBC values for NIS and EPL. At pH 7.3, NIS+ACS displayed synergistic inhibition, NIS+LAE and EPL+ACS demonstrated additive-type interactions, and the SL+ACS pairing was unable to be defined. At pH 5.0, interpretation of the OCT+NIS interaction also presented challenges, while the OCT+ACS combination resulted in synergistic behavior.

Additional studies are needed to validate *in vitro* findings on surfaces of ready-to-eat meats. Future *in vivo* studies should investigate the ability of synergistic combinations (NIS+ACS and OCT+ACS) to control the pathogen. Better characterizations of inhibitory mechanisms should also be performed.

DEDICATION

This thesis is first and foremost dedicated to my dear parents who have always encouraged me to strive for excellence and who have endured the trials and tribulations right along with me. Your love has always been a source of strength and hope in times of hardship. Secondly, I dedicate this thesis to those who have helped me along the way: labmates, friends, teachers, professors, and extended family members. The support, advice, and comfort you have given me over the years will never be forgotten.

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I would also be remiss if I did not thank the National Pork Board for funding this project. Their financial assistance, which is donated by U.S. pork producers, allowed us to conduct this research. I would also like to thank all those companies and individuals who supplied donations of products and technical advice to ensure our project was a success. These companies include the Mionix Corporation, Ecolab Inc., Purac America Inc., A&B Ingredients, and the Chisso Corporation. Individuals include Mr. Gil Bakal, Dr. Maurice Kemp, Dr. Scott Burnett, Dr. Tim Gutzmann, Dr. Peter Bodnaruk, Dr. Hakan Benli, Dr. P. Michael Davidson, and Dr. Jill Moser. The generosity and support that you all provided was greatly appreciated.

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NOMENCLATURE

2x TPB	Double-Strength Tryptose Phosphate Broth
ACS	Acidic Calcium Sulfate
APC	Aerobic Plate Count
ATP	Adenosine Triphosphate
CDC	U.S. Dept. of Health and Human Services Centers for Disease Control and Prevention
CEC	Commission of the European Communities
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
DI H_2O	Sterile Deionized Water
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EPL	ε-Poly-L-Lysine
FDA	U.S. Dept. of Health and Human Services Food and Drug Administration
FFDCA	Federal Food, Drug, and Cosmetic Act of 1938
FIC	Fractional Inhibitory Concentration
FIC_I	Fractional Inhibitory Concentration Index
FSIS	U.S. Dept. of Agriculture Food Safety and Inspection Service
GRAS	Generally Recognized As Safe
HACCP	Hazard Analysis and Critical Control Points
HAMO	Highly Acidic Metalated Organic Acid
HCl	Hydrochloric Acid

IC_{50}	Median Inhibitory Concentration
InlA	Internalin A
InlB	Internalin B
IU	International Units
IUPAC	International Union of Pure and Applied Chemistry
LAE	Lauric Arginate Ester
LPM	Lithium Chloride Phenylethanol Moxalactam Agar
MAP	Modified Atmosphere Packaging
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
MPN	Most Probable Number
MOX	Modified Oxford's Medium
NADC	National Animal Disease Center
NIS	Nisin
OCT	Octanoic Acid
OD630	Optical Density at 630 nm
ΔOD630	Change in Optical Density at 630 nm
OBC	Optimal Bactericidal Combination
OIC	Optimal Inhibitory Combination
PI	Propidium Iodide
PLEP	Post-Lethality Exposed Product
RNA	Ribonucleic Acid
RTE	Ready-To-Eat
SL	Sodium Lactate

SP.	Species (Singular)
SPP.	Species (Plural)
SSOP	Sanitation Standard Operating Procedure
SUBSP.	Subspecies
SYTO®13	Sytox Green Fluorescent Nucleic Acid Stain
TBARS	Thiobarbituric Acid Reactive Substances
TBHQ	*tert*-butylhydroquinone
TEM	Transmission Electron Microscopy
TPA	Tryptose Phosphate Agar
TPB	Tryptose Phosphate Broth
TSA	Tryptic Soy Agar
UVM	University of Vermont Medium
V/V	Per Volume Basis
W/W	Per Weight Basis

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Listeria monocytogenes is a foodborne pathogenic bacterium that poses a major challenge to the safety of the global food supply. One of the primary courses of action to control its presence in foods is to use approved antimicrobials that can either kill the pathogen or limit its growth. Though antimicrobials are validated to be effective when used alone, it has been suggested that using them in combination may provide enhanced inhibition of *L. monocytogenes* and may help prevent development of resistance. Thus, current research has taken up antimicrobial pairing as a major focus in terms of efforts to control the pathogen.

The overall goal of this research was to investigate the growth inhibition and bactericidal response of *L. monocytogenes* that resulted from the pathogen's exposure to several novel antimicrobial combinations. Though this research focused primarily on the *in vitro* activity of the antimicrobials pairs, hopes are that the information collected in these studies may be applicable to future *in vivo* studies that examine the use of these novel combinations as a means to control *L. monocytogenes* within foods subjected to varied environmental and intrinsic conditions.

Chapter II of this thesis provides a review on *L. monocytogenes* and the effects of the foodborne illness that it causes, while Chapter III supplies a review of the six food antimicrobials used in this research. Chapters IV and V present the data from two studies that involved the exposure of *L. monocytogenes* to two different sets of food antimicrobial combinations at two different pH conditions. Lastly, Chapter VI summarizes the research by providing conclusions about the activity of the antimicrobial pairs and suggested inhibitory mechanisms.

This thesis follows the style of the Journal of Food Science.

CHAPTER II

LISTERIA MONOCYTOGENES

Introduction

Since the publication of the first detailed description of *L. monocytogenes* in 1926, volumes of literature have been produced to provide a better understanding of the organism's taxonomical classification, reservoirs, physiology, pathogenesis, and effects on animals and humans. In the early years of its investigation many regarded the bacterium as one of minor significance to the general public and "of little or no economic importance" (Gray and Killinger 1966). However, in the early 1980's the link between *L. monocytogenes* and foods was established, piquing the interest of society (Ryser and Marth 2007). At that point, the efforts dedicated to understand the organism, control its presence in foods, and advance the epidemiology of its consequential disease intensified, and have remained at the forefront of food microbiological research ever since (Ryser and Marth 2007). Presently, research is conducted worldwide to control *L. monocytogenes*; such research will likely continue into the future, as the organism continues to challenge the efforts of scientists, physicians, and food producers.

History

Murray and others (1926) were the first to describe the bacterium with a high degree of detail after culturing it from the omentum, blood, and ascitic fluid of diseased laboratory rabbits and guinea pigs). The organism was originally named *Bacterium monocytogenes* because of the large mononuclear leucocytosis that developed due to its presence (Murray and others 1926), but its genus was renamed *Listeria* by J. H. Harvey Pirie (1940) in 1940. Thereafter, many reports of the widespread nature of listeriosis, the disease caused by *L. monocytogenes*, were compiled, and bacteriologists began to realize the scope of the illness and the ubiquity of the causative

organism (Murray 1955). Descriptions of meningitis, septicemia, and granulomatosis infantiseptica (a syndrome characterized by the formation of abscesses in multiple internal organs of the newborn), as well as isolation from numerous countries around the world, brought about the realization that the organism was not harbored by a single reservoir in a particular continent or region, nor were humans exempt from infection and transmission (Burn 1936; Murray 1955). Gray and Killinger (1966) published a comprehensive review on *L. monocytogenes*, noting that much information about the bacterium evaded scientists, with little known about the sources of the disease and why it displayed variable pathogenic activity across different host species. Later studies that were aimed at identifying possible environmental sources of *L. monocytogenes* concluded that decaying plant materials and soils were the likely origins of the organism within the environment and that any transmission originated there (Welshimer and Donker-Voet 1971; Weis and Seeliger 1975).

In the 1980s however, multiple outbreaks were investigated and found to be associated with several different types of food (Schlech and others 1983; Fleming and others 1985; Linnan and others 1988). As a result of the outbreaks, the general public became much more aware that foods were also key transmission vehicles for the organism, and began to increase demand for better detection and control of *L. monocytogenes* in products they consumed daily (Gill 1988). Thus, the role of the Unites States government in mitigating foodborne *L. monocytogenes* contamination escalated during the 1980s (Kozak 1986; Lecos 1986), culminating in the declaration of the pathogen as an adulterant in certain food products. By the early 1990s, it was acknowledged that *L. monocytogenes* was a major issue to be dealt with in the food processing environment and that development of technologies designed to control it were needed (Farber and others 1989b; Farber and Peterkin 1991; Tompkin and others 1992).

Several advances in the understanding of *L. monocytogenes* physiology, pathogenicity and process control have been reported in recent decades (Tompkin and others 1999), despite occurrence of multiple outbreaks of foodborne listeriosis (Dalton and others 1997; CDC 1999b; Olsen and others 2005; Gottlieb and others 2006). Among these advances was an extensive risk assessment that was completed in 2003 to evaluate the hazards associated with the pathogen and identify the foods most commonly implicated for contamination (FDA/FSIS 2003). In addition, it was reported that incidence of *L. monocytogenes* declined from seven to nearly three cases per million of population in the United States from 1986 to 2006; a testament to the coordinated efforts undertaken by multiple groups in all facets of food production and technology (Klontz and others 2008). Observed reductions in incidence, combined with a better understanding of the organism's pathogenic disposition, may already be a glimpse of future improvements that may significantly enhance control of the pathogen's presence in foods.

Microbiology

The shape and Gram reaction of *L. monocytogenes* was first described in the publication of Murray and others (1926), where the researchers noted that the bacterium was a Gram-positive bacillus. A facultative anaerobe, it is classified as a regular non-sporulating Gram-positive rod (Holt and others 1994). It ranges from 1-2 µm in length, 0.5 µm in width, and has rounded ends which make it diphtheroid-like in appearance (Murray and others 1926). It is also non-acid fast (Murray and others 1926), cytochrome oxidase negative, catalase positive, and carries out hemolysin-mediated β-hemolysis on blood agar (Farber and Peterkin 1991). Cells can be arranged in either a side-by-side parallel fashion or in a thread-like manner depending upon the culture (Murray and others 1926). Colonies grown on agar plates tend to be bluish-gray in appearance when viewed by reflected light (Gray and Killinger 1966), and can produce a characteristic blue-green sheen when viewed by obliquely transmitted light (Henry 1933).

In addition to *L. monocytogenes*, the *Listeria* genus also contains *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. murrayi*, *L. grayi*, and the animal pathogen *L. ivanovii* (Holt and others 1994). Species can be differentiated by examining carbohydrate fermentation profiles and ability to lyse blood agar (Bille and others 1992; Rocourt and others 1983). The genetic composition of the *Listeria* genus causes it to be classified within the *Clostridium-Lactobacillus-Bacillus* branch (Jay and others 2005). Members of this branch typically have a less than 50 mol % of guanine (G) + cytosine (C) pairs in their DNA (Jay and others 2005), with *L. monocytogenes* falling into the 36-42% range (Rocourt and Buchrieser 2007). *Listeria* species also exhibit similarities in genetic material composition and carbohydrate utilization to members of the *Brochothrix* and *Lactobacillus* genera, respectively (Jay and others 2005).

Growth of *L. monocytogenes* can occur at low temperatures (Junttila and others 1988), which prompts some to classify the organism as a psychrotroph. It is capable of survival from - 0.4-50 °C, but optimum temperatures for the organism are considered to be from 30-37 °C (Low and Donachie 1997). Production of flagella occurs optimally at ambient temperatures (20-25 °C); peritrichous flagella are synthesized and yield a distinctive tumbling motility (Peel and others 1988). At temperatures above this range, and within its optimum growth temperature span, flagella synthesis is decreased, and limited motility is displayed (Gray and Killinger 1966).

When incubated aerobically at 30 °C in tryptic soy broth with 0.6% yeast extract, most strains of the pathogen are capable of growth within the pH range 4.5-7.0, with little growth occurring below pH 4.0 (Parish and Higgins 1989). However, temperature can affect pH requirements, as growth of the pathogen in brain heart infusion broth in the presence of acidulants at 30 °C only requires a pH of 4.3 to commence, while at 4 °C the pH must be higher (pH 5.0-5.7) for growth to occur (Farber and others 1989a). *L. monocytogenes* is also fairly osmotolerant and has been shown to survive for up to 259 days at 4 °C in commercial cheese

brines with NaCl concentrations up to 23.8% (Larson and others 1999). However, this tolerance decreases proportionately with both increases in NaCl concentrations (Hudson 1992) and with increases in the growth temperature (Sorrells and Enigl 1990).

Evaluation of the carbohydrate utilization by *L. monocytogenes* along with other *Listeria* species was performed by Pine and others (1989). Based on observations, it was noted that *L. monocytogenes* utilizes the monosaccharides glucose and rhamnose under both aerobic and anaerobic conditions, and that neither xylose nor galactose are able to support growth of the pathogen under any type of atmosphere. Disaccharide utilization is limited to lactose, which supports growth of the bacterium only under aerobic conditions (Pine and others 1989). Sucrose is unable to support growth of any *Listeria* species regardless of atmospheric composition (Pine and others 1989). Utilization of glucose by *L. monocytogenes* yields trace to large amounts of acetic, isobutyric, isovaleric, phenylacetic and isocaproic acids, a variety of alcohols, and lactic, 2-hydroxybutyric, 2-hydroxyvaleric and 2-hydroxyisocaproic acids (Daneshvar and others 1989). Another interesting characteristic of *L. monocytogenes* and all *Listeria* species is the ability to hydrolyze esculin and produce 6,7-dihydroxycoumarin, a compound that can react with available iron (Fe^{3+}) to produce a black precipitate (Fraser and Sperber 1988). This particular trait has proven useful in the development of several different types of differential media, namely Fraser Broth, PALCAM medium, and Oxford's medium, which utilize this mechanism to identify the presence of *Listeria* species (Fraser and Sperber 1988; Van Netten and others 1989; Curtis and others 1989).

Listeriosis

Murray (1955) characterized listeriosis in humans and animals, describing traits of the disease that were observed in a wide variety of species. He noted that the majority of documented human infections entailed conditions that included encephalitis and meningitis,

granulomatosis infantiseptica, septicemia, mononucleosis, and conjunctvitis (Murray 1955). Present day listeriosis complications are much the same, as meningitis, spontaneous abortion, and septicemia remain the primary conditions associated with human *L. monocytogenes* infections (Vázquez-Boland and others 2001). However, recent reports of febrile gastroenteritis have also shown that the pathogen is capable of producing the typical gastrointestinal complications associated with foodborne illnesses (Salamina and others 1996; Dalton and others 1997; Aureli and others 2000). Because of the nature of its pathogenesis, pregnant women, neonates, immunocompromised individuals, and the elderly are at highest risk for infection (Cherubin and others 1991).

Human perinatal listeriosis usually involves mild influenza-like symptoms as well as diarrhea, abdominal cramps, and lower back pain in the pregnant mother (McLauchlin 1990; Svare and others 1991). However, the more deleterious effects can include stillbirth and premature delivery of the fetus (Lorber 1997) with nearly 20% of infections resulting in one of the two outcomes (Mylonakis and others 2002). Neonatal infections can occur *in utero* resulting in early-onset neonatal listeriosis; postnatal infection results in late-onset listeriosis (Painter and Slutsker 2007). Early-onset neonatal listeriosis can result in the production of lesions in internal organs such as the liver, lungs, kidney, spleen, and brain (Banerji and Noya 1999), fever, respiratory distress, and neurologic deformities in the infant (Painter and Slutsker 2007). Late-onset neonatal listeriosis is mainly characterized by meningitis and is believed to be the result of *L. monocytogenes* transmission from the birth canal and other maternal sites or from the postnatal environment (McLauchlin 1990). Both types of neonatal infections are considered highly fatal with estimated mortality rates near 20-30% (Boucher and Yonekura 1984; McLauchlin 1990; Lorber 1997).

Among non-pregnant adults, encephalitic and meningitic manifestations of *L. monocytogenes* are the most commonly reported, accounting for nearly 55-70% of cases (Vázquez-Boland and others 2001). In fact, *L. monocytogenes* is considered to be one of the predominant agents of bacterial meningitis (Durand and others 1993). A recent study noted that within a group of 80 culture-positive cases of bacterial meningitis, *L. monocytogenes* was responsible for at least 12.5% of all cases, making it the second most common causative organism behind *Streptococcus pneumoniae* (Hussein and Shafran 2000). Such complications are of great concern for elderly and immunocompromised individuals, as they are the most susceptible populations to listeric meningitis due to inherent deficiencies in their immune systems (Bell and Kyriakides 2005). In addition, because *L. monocytogenes* has an affinity for brain and meninge tissue, encephalitic and meningitic infestations usually have very well-defined consequences on the nervous system (Nieman and Lorber 1980). As a result, deleterious effects such as gross lack of muscular coordination, seizures, and altered mental status can occur, as well as more minor effects such as fever and depression-like behavior (Painter and Slutsker 2007). Septicemic and bacteremic conditions without meningitis are also common forms of listeriosis presentation among non-pregnant adults (Rocourt 1990). Such infestations of *L. monocytogenes* usually entail fever, and can be accompanied by fatigue, malaise, and abdominal pain (Painter and Slutsker 2007).

Listeriosis cases that result in symptoms more commonly associated with foodborne infections, such as diarrhea and vomiting, are less common, but have been documented (Bell and Kyriakides 2005). In a 1994 chocolate milk outbreak discussed by Dalton and others (1997), the presence of high levels of *L. monocytogenes* (8.9-9.1 $\log_{10}$ CFU/ml), caused rapid onset of diarrhea and vomiting among the individuals who had consumed the contaminated milk. Other cases of *L. monocytogenes* gastroenteritis due to consumption of rice salad (Salamina and others

1996) and corn salad (Aureli and others 2000) involved presentations of similar symptoms in patients. However, such cases almost exclusively result from consumption of highly contaminated foods, occur within less than 24 hours after consumption, and are only occasionally followed by bacteremia (Bell and Kyriakides 2005).

Pathogenic Mechanism and Virulence

As evidenced by the broad range of disease complications associated with listeriosis, the pathogenicity of *L. monocytogenes* in the human body is quite extensive. The ability of the pathogen to invade cells such as fribroblasts, epithelial and endothelial cells, and dendritic cells translates into a diverse range of pathogenic activity with a capacious spectrum of consequences (Kuhn and Goebel 2007). The mechanism by which *L. monocytogenes* accomplishes invasion is phagocytic in nature and involves the use of several attachment proteins, cellular vacuoles, a propulsion mechanism, and a lytic protein (Kuhn and Goebel 2007; Gaillard and others 1987).

The first stage of internalization of the *L. monocytogenes* cell into a non-phagocytic cell (such as an epithelial cell) occurs when the bacterium attaches to the exterior of the host cell. Attachment is accomplished through the use of the Internalin A (*inlA*) protein (Gaillard and others 1991), which contains a specialized LPXTG motif and a cell wall-spanning region (Dramsi and others 1993) to allow *L. monocytogenes* cells to firmly to attach to the host cell via a reaction involving the enzyme sortase (Garandeau and others 2002). Another internalin protein, Internalin B (*inlB*), is also coded by the same operon (the *inlAB* operon) (Lingnau and others 1995), but lacks the motif and cell-wall spanning region of *inlA* (Braun and others 1997). Based on high amounts of *inl*B in supernatants of cultures (Jonquières and others 1999), it is believed that its bonds with the lipoteichoic acids of the pathogen's cell wall are weak, and cause it to be less effective than *inl*A at promoting attachment (Kuhn and Goebel 2007).

After attachment, the pathogen undergoes entry into the host cell by inducing formation of a phagocytic vacuole that allows it to internalize (Gaillard and others 1987). Once inside the cell, *L. monocytogenes* lyses the vacuole through the use of a hemolytic protein known as listeriolysin O (Geoffroy and others 1987). Unless it is able to accomplish this lysis, the bacterium is trapped within the vacuole and will become digested by the host cell (Gaillard and others 1987). However, if the pathogen is able to produce lysis, it can then enter the cytoplasm of the host cell and begin to grow and multiply (Portnoy and others 1988).

After cytoplasmic proliferation begins, *L. monocytogenes* utilizes host cell actin to form a capsule of filaments on its exterior (Tilney and Portnoy 1989). It then polymerizes these filaments into a long "comet's tail," which permits propulsion throughout the cytoplasm (Tilney and Portnoy 1989). Once it reaches the cell membrane of the host, the bacterium creates a protrusion with the actin filament tail embedded in the cytoplasm behind it (Mounier and others 1990). At this stage, it can be absorbed into the interior of a neighboring host cell and become enveloped in a double-membrane vacuole (Mounier and others 1990). Once this double-membrane vacuole is lysed by listeriolysin O, the bacterium enters the cytoplasm of the new cell and proliferates once again; thereby it continues to infect other cells while evading the immune system of the host (Tilney and Portnoy 1989).

Though this pathogenic mechanism is well understood, the differences in the potency of virulence that are observed between different strains of *L. monocytogenes* remain to be completely explained (Kathariou 2002). Some have suggested, and exhibited, that temperature affects the production of the internalin proteins (Lingnau and others 1995) and listeriolysin O (Buncic and others 1996), and thus can decrease pathogenicity of *L. monocytogenes* cells. Others have exhibited that multiple lineages of *L. monocytogenes* actually exist and possess genetic differences that dispose them to variations in pathogenic potential (Wiedmann and others 1997).

One of these major genetic differences is a greater tendency of some strains to possess premature stop codons for the internalin proteins, which produce truncated *inlA* and *inlB* with no activity (Nightingale and others 2005; Nightingale and others 2007; Van Stelten and Nightingale 2008). This sort of observation is of particular interest to the food industry as some isolates from foods have been shown to have an attenuated virulence due to the presence of these particular mechanisms (Nightingale and others 2005).

Incidence in the Environment and Biofilms

As noted by the work of Welshimer and Donker-Voet (1971) and Weis and Seeliger (1975), *L. monocytogenes* is widely distributed in the environment, in the soil, and in plants and animals. Welshimer and Donker-Voet (1971) suggested that because it was present in the decaying vegetation of soil in non-agricultural areas at certain seasons of the year, animal feces deposition was not the sole source of the pathogen. Weis and Seeliger (1975) later confirmed that the organism is saprophytic in nature and can thus be contracted via a variety of routes of contamination. Nonetheless, though such studies have shown that decaying vegetation and the soil are the primary habitats for *L. monocytogenes* in the environment, a variety of animals serve as reservoirs of the pathogen (Murray 1955) and deposit it in their feces (Skovgaard and Morgen 1988). Indeed, more than 40 species of animals have been implicated as carriers of the microorganism over the span of six continents (Low and Donachie 1997). Finally, *L. monocytogenes* often resides in sewage and effluent sources and thus can be distributed onto agricultural land through the spraying of fecal sludge (McCarthy 1990).

Although much research has been completed to detail the diversity of strains of *L. monocytogenes* in food processing environments, little is known about the transmission from natural to food processing environments (Sauders and Wiedmann 2007). Some have implicated raw food ingredients and their high levels of *L. monocytogenes* contamination as the primary

mode of transmission of the organism into facilities (Samelis and Metaxopoulos 1999). Yet while others have shown that transfer from raw to processed products is minimal, and that humans are likely the responsible vehicle (Nesbakken and others 1996). Regardless of the vehicle of transmission, once established in the food processing environment, *L. monocytogenes* can attach to and colonize virtually any surface including stainless steel, glass, polypropylene, and rubber (Mafu and others 1990). As many factors required for growth of *L. monocytogenes* in the environment (cool, damp areas and decaying nutrients) may be found in food processing facilities, the organism is capable of surviving months or even years within a food processing plant after its introduction from the surroundings (Tompkin and others 1999). Thus, machines, peelers, doors (Tompkin 2002), framework, floor sealants, drains (Lado and Yousef 2007), and conveyor belts (Wenger and others 1990) can all become potential environmental niches of *L. monocytogenes* in the food plant. The same is true for the retail foodservice environment where equipment and materials such as slicers, refrigerator handles, food preparation surfaces, and other utensils can harbor the pathogen and cause cross-contamination (Lianou and Sofos 2007).

The main characteristic that enables *L. monocytogenes* survival on inanimate surfaces is its ability to develop a biofilm (Lado and Yousef 2007). Biofilms are defined as matrix-enclosed bacterial populations which are adherent to each other and to surfaces and interfaces (Costerton and others 1995). Most biofilm formation entails the production of a complex exopolysaccharide matrix, which allows the bacteria to aggregate and condition their own microenvironments (Costerton and others 1995). *L. monocytogenes* biofilm formation begins with planktonic cells being deposited on the exterior of a particular material by means of hydrophilic interactions or by clinging to the surface using flagella. Once this occurs, synthesis of fibrils and exopolymers allow the organism to fully adhere to the surface. Colonization and multiplication follows resulting in the formation of multiple layers of cells. As the biofilm continues to grow and

mature, horizontal cell distribution becomes more homogeneous, cell proliferation continues, and capillary water channels form to impart a three dimensional structure to the biofilm (Lado and Yousef 2007). Once fully mature, the biofilm can generate large amounts of planktonic cells, which can easily contaminate foods and food handling equipment when released. Once established, *L. monocytogenes* biofilms are considerably harder to remove than planktonic cells and make food plant cleaning and sanitation less effective (Lado and Yousef 2007).

Incidence and Significant Foodborne Disease Outbreaks

Along with *Salmonella* species and *Escherichia coli* O157:H7, foodborne *L. monocytogenes* infections have had a major impact on the economy and public health of the United States. Mead and others (1999) estimated the total number of hospitalizations related to *L. monocytogenes* infections to be ~2500/year; approximately 20% of cases were fatal, with almost 99% of reported cases resulting from foodborne transmission. Such numbers have undoubtedly caused great concerns for the food industry and consumers, and have been estimated to cost the United States economy approximately $2.3 billion each year in terms of food product recalls, hospitalizations, and other complications from foodborne listeriosis cases (ERS 2000). In addition, because several food categories (dairy foods, seafood, raw vegetables, and processed meats) have been assessed as being of high risk for transmission of *L. monocytogenes*, its control demands considerable effort from multiple portions of the food industry (FDA/FSIS 2003).

In 1981 an outbreak of foodborne listeriosis involving coleslaw occurred in the coastal provinces of Canada (Schlech and others 1983). Forty-one cases of infection were found to be due to consumption of coleslaw that contained cabbage grown on a farm where raw and uncomposted manure from listeriosis-laden sheep had been used as a fertilizer (Schlech and others 1983). In addition to seven adult listeriosis cases, of which 2 were fatal, 15 of 34 perinatal

cases resulted in mortality of the fetus or newborn infant (Schlech and others 1983). Yet, even as the coleslaw was narrowed down as the exclusive source of the outbreak strain, little alarm was raised as to the need for precautionary measures in consumption of similar food products (Schlech and others 1983). Soon thereafter, 49 cases of listeriosis due to consumption of pasteurized milk were reported in Boston (Fleming and others 1985). Seven cases occurred in infants and fetuses; 42 involved immunocompromised adults (Fleming and others 1985). Fourteen fatalities occurred during the course of the outbreak, which took place within only about one month's time (Fleming and others 1985). In 1985 in Los Angeles, Mexican-style soft cheese was implicated as the means of transmission in an outbreak of foodborne listeriosis; 142 cases and 48 deaths were ultimately attributed to this outbreak (Linnan and others 1988; CDC 1985). The attention generated by the outbreak prompted the U.S. government to take quick action and subsequently led to the consideration of the pathogen as an adulterant in FDA-regulated ready-to-eat food products (Shank and others 1996). In addition, the outbreak spawned development of new regulatory practices in the production of certain foods, enforcement of plant visitations and inspections, establishment of state and national surveillance systems, and grounds for listeriosis being a reportable disease in certain states (Linnan and others 1988). Several other notable outbreaks occurred during the late 1980s and early 1990s involving a variety of foods as vehicles (Schwartz and others 1989; Riedo and others 1994; Dalton and others 1997). Recent outbreaks in North Carolina and Texas involving queso fresco cheese (CDC 2000a; CDC 2001; CDC 2003; CDC 2005), an Oregon outbreak involving cheese made from pasteurized milk (CDC 2006), and a skim milk transmitted outbreak in Massachusetts (CDC 2007) have played a pivotal role in the continual recognition of dairy foods as sources of the pathogen and continue to keep strict regulations in place for their production.

Prior to the late 1980s processed meats were not recognized as vehicles for transmission of foodborne listeriosis. In 1989 a single case of listeriosis attributed to consumption of contaminated turkey frankfurters was identified (CDC 1989). Since the initial case, multiple outbreaks of foodborne listeriosis have occurred which have involved processed meat(s) as the vehicle of transmission. In 1998 a multi-state *L. monocytogenes* disease outbreak involving frankfurters and a variety of processed meats produced by a particular Michigan firm occurred, resulting in 101 cases (15 fatalities) across 22 states (CDC 1998b; CDC 1999b; Olsen and others 2005). Since then, a Colorado hot dog outbreak (CDC 1998a), a multistate outbreak in paté (CDC 1999a) and three multistate outbreaks in 2000, 2002, and 2005 all involving turkey deli meat (CDC 2000b; Olsen and others 2005; CDC 2002a; CDC 2002b; Gottlieb and others 2006; CDC 2005) have demonstrated the risk that *L. monocytogenes* poses to processed meats. In addition, occurrence of at least 175 Class I and voluntary recalls involving processed meats between 1994 and 2006 reiterates the challenge the pathogen presents to the processed meats sector of the food industry (Farber and others 2007).

Because of these and other outbreaks, processed ready-to-eat (RTE) meats have a high level of risk in terms of transmission of the pathogen (Farber and others 1989b; FDA/FSIS 2003). Factors such as availability of nutrients, product processing procedures, and storage conditions predispose processed meats to cross-contamination with and survival of *L. monocytogenes*. It has been found that *L. monocytogenes* can grow quite well on RTE meats such as cooked roast beef and frankfurters, and even better in products such as ham, bologna, bratwurst, sliced chicken, and sliced turkey (Glass and Doyle 1989). The moderate acidity of such products (pH 5.6-6.5) corresponds with the optimal pH conditions for growth of the pathogen; this, along with increased moisture percentages and significant content of protein and carbohydrate, contributes to survival and proliferation of the organism (Glass and Doyle 1989).

Although most RTE meat processes include a cooking step that is designed to kill any *L. monocytogenes* present in the raw product, contamination often results from exposure of the product to the plant environment between this step and the final packaging of the product (FSIS 2003a). Since it is common practice to perform further mechanical processing and handling of foods such as deli meats and frankfurters in this interim, contamination from the food plant environment, equipment, and additional handling may occur (Aberle and others 2001). Several studies have verified that post-process contact with the environment is the primary source of *L. monocytogenes* contamination. Bunčić (1991) showed that *L. monocytogenes* was absent in hot smoked sausages sampled immediately after the cooking process, but that nearly 21% of surface samples from the same sausages were positive for *L. monocytogenes* following handling and packaging. In tests for presence of *L. monocytogenes* in the internal portion of frankfurters and in purged fluid, Wang and Muriana (1994) showed that the pathogen was solely present in the exudate. Thus, it can be said that control of the pathogen's presence in post-lethality handling areas through good sanitation standard operating procedures (SSOPs) are of key importance to prevent contamination of product exteriors during handling (Tompkin 2002).

In addition to the concern associated with post-lethality contamination, the practice of storing processed RTE meats for extended periods of time (e.g. longer shelf life) at refrigeration temperatures, coupled with the bacterium's ability to grow, albeit slowly, at such temperatures (Junttila and others 1988; Glass and Doyle 1989), increases the risk for this class of products. If present, *L. monocytogenes* can proliferate on the product during this storage time, and will grow well under the low oxygen conditions of vacuum packaging (Beumer and others 1996). The key issue with this increase in numbers is that many RTE products are typically consumed without any heating by the consumer (Lianou and Sofos 2007). Consequently, food processor-directed prevention of RTE meat contamination has been a focus of federal regulatory agencies such as

the United States Food and Drug Administration (FDA) and the United States Department of Agriculture Food Safety and Inspection Service (FSIS), and has led to the passage of many regulations on the matter. Most recently, retail preparation and processing of RTE foods, particularly deli items such as salads and slicing of deli meats has gained additional focus.

Control and Regulations

The current position of the United States government with regard to *L. monocytogenes* and foods consists of a strict "Zero Tolerance" policy toward the presence of the pathogen in all cooked RTE products (Klontz and others 2008). Section 402(a) (1) of the 1938 Federal Food, Drug, and Cosmetic Act (FFDCA) prohibits the presence of any poisonous or deleterious substance in foods that may render the food injurious to health. Similarly, section 402(a) (4) of the FFDCA prohibits the preparation, packaging, or holding of a product under unsanitary conditions that can allow it to become contaminated with filth or render it injurious to health. The FDA was the first agency to enact the "Zero Tolerance" policy toward *L. monocytogenes*, in part as a response to the 1985 Mexican-style soft cheese outbreak in Los Angeles (Klima and Montville 1995). After the occurrence of the outbreak, the FDA began an enhancement of its dairy plant inspection program, intensified surveillance of dairy products, and stepped up training for dairy inspectors and other officials (Kozak 1986). Later in 1985, FDA made a decision to consider the microbe an adulterant in cooked RTE foods falling under its jurisdiction and to request recalls of RTE products that were not in compliance (Klontz and others 2008).

Although no outbreaks had previously been linked to meat and poultry products, beginning in December 1985 the FSIS began to follow the example of the FDA and started to investigate the incidence of *L. monocytogenes* in products under their jurisdiction (Jinneman and others 2007). After acknowledging the possibility for such foods to be contaminated by the pathogen (Shank and others 1996), FSIS again followed the example of the FDA, and began to

intensify the scope of its testing and monitoring programs for the bacterium by implementing a testing plan specific for *L. monocytogenes* in 1987 (FSIS 1989). However, the agency did not implement a "Zero Tolerance" policy *per se* until the 1989 case of the turkey frankfurters contaminated with *L. monocytogenes* (CDC 1989). Immediately after the occurrence of the listeriosis case, the agency amended its policies to include "Zero Tolerance" of the pathogen in all cooked RTE foods that it controlled, and to encourage voluntary recalls of entire lots whose tests indicated presence of the pathogen (FSIS 1989).

Though both agencies publicize their policies under the term of "zero tolerance," a more correct term for the policy is one of "zero detection" (Klontz and others 2008). Indeed, the FDA method involves a 4 h pre-enrichment and subsequent 48 h enrichment of a 25 g sample of food in 225 ml Buffered *Listeria* Enrichment Broth, with streaking done at 24 and 48 h on one of the agars approved for selective and differential isolation of the organism (Modified Oxford's Medium (MOX), PALCAM, or Lithium Chloride Phenylethanol Moxalactam (LPM) agar) (Hitchins 2003). This allows for a detection level of only 1 colony forming unit (CFU) per 25 g or 0.04 CFU/g. Thus, the attainment of results that ensure a level of 0 CFU of the pathogen per gram of food is not possible through the use of the agency-prescribed methods. The same is true for the USDA method of detection, which involves the 22 h primary enrichment of a 25 g food sample in University of Vermont (UVM) Broth. This is followed by a 26 h secondary enrichment of 0.1 ml of UVM culture in Fraser Broth and streaking on MOX Agar with further incubation of the Fraser Broth up to 48 h (FSIS 2008). Because the method also utilizes a 25 gram sample, the detection limit is also 0.04 CFU/g and cannot theoretically reach the 0 CFU/g level of assurance implied by the "zero tolerance" policy term. Although the FDA and FSIS continue to staunchly support their approach to handling the pathogen, the need for a strict "Zero Tolerance" policy for *L. monocytogenes* has been continually brought into question. Petitions

have been brought forward by several food industry interest groups in recent years that are aimed at relaxation of the restrictions (FDA 2004b). Most petitions have implored the FDA take a risk-based approach to the matter and revise its policy to consider allowing up to 100 CFU/g of *L. monocytogenes* in foods that do not support its growth (FDA 2004b). A risk-based approach is already widely used for the pathogen in other countries, such as the European Union and Canada, where up to 100 CFU/g is allowed in some foods that are not considered to be of high risk (CEC 2005). Though both the FDA and FSIS still maintain their "zero tolerance" policy for the pathogen based on rationale from their 2003 risk assessment (FDA/FSIS 2003), the possibility of amending this policy is currently under review (FDA 2008a).

In addition to testing programs mandated for RTE foods that fall under its jurisdiction, the FSIS maintains an additional policy for prevention of post-process contamination in products that are exposed to the environment after receiving their process step for microbial lethality (FSIS 2003a). Examples of such post-lethality exposed products (PLEP) include frankfurter products and sliced deli meats (FSIS 2003a). In its final rule on the control of *L. monocytogenes* in certain RTE meat and poultry products, FSIS mandated that all producers of PLEP products must incorporate one of three post-lethality strategies to control contamination and outgrowth of the pathogen on the product (FSIS 2003a). Alternative 1 involves the use of a post-lethality treatment (which can involve the application of an antimicrobial compound) that reduces or eliminates the pathogen on the product coupled with an antimicrobial agent or process that is designed to limit or suppress the growth of the pathogen throughout the product's shelf life (FSIS 2003a). Alternative 2 involves the use of a post-lethality treatment (which again can involve the application of an antimicrobial compound) that reduces or eliminates the pathogen *or* an antimicrobial agent or process that limits or suppresses pathogen growth (FSIS 2003a). Alternative 3 is the least stringent of all three programs, and involves the control of the pathogen

through the development and use of sanitation measures only (FSIS 2003a). It must be noted, however, that although stringency of control *decreases* with the ascending number of the alternative, the stringency of verification activities by FSIS *increases* in the same fashion (FSIS 2003b). Because of the more frequent use of Alternatives 1 and 2 in large volume food plants, the application of antimicrobial compounds as a measure of control for *L. monocytogenes* is commonplace in the food industry. In its Amendment 19 of Directive 7120.1, FSIS lists a number of approved compounds that can be used in processed meat applications (FSIS 2009). Details on the use of several of these antimicrobials and their effects on *L. monocytogenes* will be discussed in the next chapter and are thus precluded from discussion within this section.

CHAPTER III

FOOD ANTIMICROBIALS

Introduction

By definition, any chemical substance is classified as a food antimicrobial if it is added to or inherently present in foods, packaging, contact surfaces, or the processing environment, and is able to either inhibit or eliminate pathogenic or spoilage microorganisms (Davidson and others 2005; FSIS 2003a). Though much of the traditional use of antimicrobials has revolved around inhibiting the growth of spoilage microorganisms and preventing the deterioration of foods, the topics of current research on antimicrobials and their application have also included a focus on preventing the presence and growth of foodborne pathogens (Davidson and others 2005). This new outlook has driven much scientific development, yielding novel antimicrobials with diverse modes of action. However, this same shift in perspective has caused many to look at centuries-old antimicrobials like organic acids and salts in a different way and to re-approach commonly used materials with new purposes in mind. The promises afforded by constant innovation, coupled with new approaches to current interventions, have led many to believe that research in antimicrobial application will produce solutions necessary to meet the challenges that pathogens commonly pose to foods in the present day (Davidson and Branen 2005).

Nisin

Though Rogers and Whittier (1928) were the first to suggest the presence of a lactic acid bacterium-synthesized substance capable of inhibiting bacterial growth, H. R. Whitehead (1933) is credited with being the first to isolate and further investigate the properties of the inhibitory material. This complex compound, believed to be a protein, was inadvertently discovered during investigation of problems with a starter culture used for the manufacture of cheddar cheese

(Whitehead 1933). Whitehead concluded that strains of *Streptococcus lactis* (now *Lactococcus lactis* subsp. *lactis*) isolated from the starter culture produced this particular compound, inhibited growth of other strains, and subsequently led to a decrease in acid production (1933). Mattick and Hirsch (1944; 1946; 1947) recorded inhibition in lactic acid bacteria cultures due to the presence of the same substance previously described by Whitehead. Further characterizations led them to conclude that the compound was produced exclusively by members of the Group N *Streptococci* (Mattick and Hirsch 1947). As a result, they named the compound nisin (derived as an acronym from Group **N** *Streptococcus* **I**nhibitory **S**ubstance), and assayed its antimicrobial activity against other groups of organisms (Mattick and Hirsch 1947). These assays proved that nisin had a great amount of *in vitro* activity against certain pathogens and that it was also quite effective against other microbes *in vivo* (Mattick and Hirsch 1947). These growth inhibition properties of nisin sparked much interest in its production and usage as a food preservative, and consequently led to its first commercial preparation in 1953 (Adams 2003).

Nisin is still extensively produced in its commercially available form and is added to a plethora of foods as an antimicrobial. Furthermore, as it possesses no toxic effects upon consumption (Frazer and others 1962) and is produced by microorganisms that are considered safe for human consumption, nisin is generally considered to be a natural food preservative (Adams 2003). It is currently approved for use in at least 80 countries worldwide and a commercial fermentate containing 2.5% nisin (w/w) was approved as generally recognized as safe (GRAS) in 1988 (FDA 1988; Adams 2003). Initially approved for use in foods as a control measure for prevention of *Clostridium botulinum* spore formation and toxin production in certain pasteurized cheese spreads (FDA 1988), nisin is currently used to control the growth of a variety of spore-forming organisms in a diverse group of food products ranging from processed cheese, dairy products, canned food, dressings and sauces, beer and other alcoholic products, and fruit

juices (Delves-Broughton and others 1996). It also meets the criteria for use as an antimicrobial to combat *L. monocytogenes* in processed meat and poultry products, and is approved for use at different levels in a variety of applications as per the FSIS Directive 7120.1 Amendment 19 (FSIS 2009).

Nisin and other bacterially-synthesized antimicrobial polypeptides with similar properties are generally classified as bacteriocins; nisin is categorized as a Class I bacteriocin or lantibiotic due to its membrane-active nature and the presence of several unusual and post-translationally modified amino acid residues that allow the formation of five lanthionine rings in the mature polypeptide (Klaenhammer 1993). Other Class I bacteriocins produced by lactic acid bacteria include the lantibiotics lacticin and carnocin (Klaenhammer 1993). The formation of a mature nisin molecule (Figure 1) involves several steps. Nisin contains the residues of 34 amino acids and has a molecular weight slightly over 3.5 kDa (Gross and Morell 1971). After formation of the 34 residue polypeptide in the ribosome, threonine and serine residues are enzymatically converted to didehydroalanine and didehydroaminobutyric acid (Kupke and Götz 1996). Within this intermediate structure, the interaction between double bonds and sulfur atoms from cysteine sulfhydryl groups causes the formation of five ring structures within the molecule (Gross and Morell 1971; Adams 2003). After the rings form, a portion of the C-terminal end is cleaved off and mature nisin is released (Adams 2003).

Because of its three positively-charged lysine residues and histidine residue, nisin is polycationic (Thomas and Delves-Broughton 2005). Nisin can exist in two forms based on the substitution of an amino acid at the 27 position; nisin A has a histidine residue while asparagine is present in nisin Z (Mulders and others 1991). Nisin is highly soluble at low pH (pH ~2.0), with decreasing solubility as pH is increased to pH 8.0-12.0 (Liu and Hansen 1990). Thus, nisin

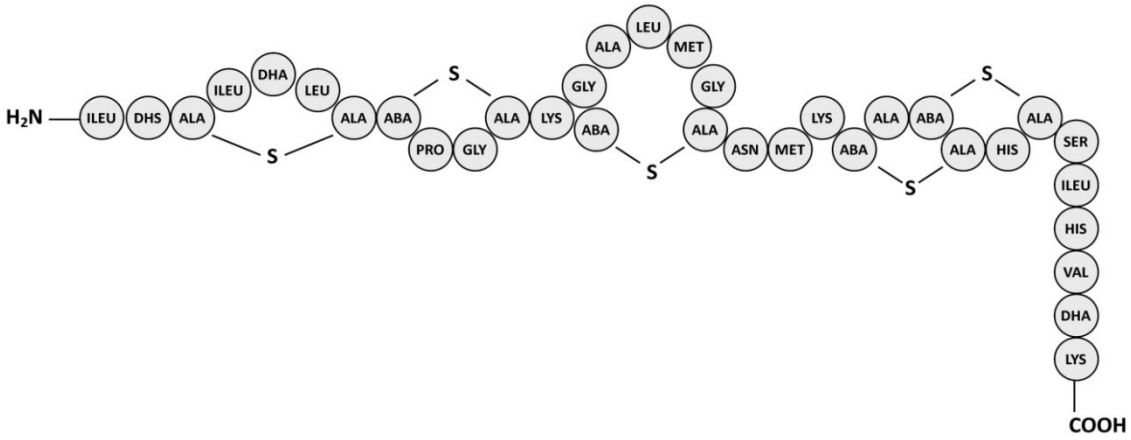

Figure 1-Mature Nisin A molecule.
Adapted from Gross and Morell (1971).

preparation procedures often involve the dissolution of the compound in a slightly acidic solution before it is applied or tested (Cleveland and others 2002).

Through the exposure of nisin to *Staphylococcus cohnii*, *Bacillus subtilis*, *Micrococcus luteus*, and *Streptococcus zymogenes*, researchers were able to find that nisin strongly decreased the membrane potential of cells and caused the efflux of certain amino acids (Ruhr and Sahl 1985). They concluded that nisin was able to disrupt the function of the bacterial membrane and that this particular region of the cell was its target for attack (Ruhr and Sahl 1985). Henning and others (1986b) later verified the hypothesis that nisin interacts with phospholipids in the cytoplasmic membrane and demonstrated that the presence of murein, previously believed to be a target for nisin, was of minimal importance for nisin activity. Further observation by Henning and others (1986a), who noted that emulsifiers in foods affected nisin activity, led to additional agreement that phospholipids are the target for the bacteriocin.

Through macroscopic conductivity measurements Sahl and others (1987) determined that nisin's activity was voltage-dependent and that it formed transient multi-state pores in the range of 0.2 to 1 nm in diameter. These pores subsequently induce release of cellular materials such as ions, amino acids, and adenosine triphosphate (ATP) from the cytoplasm (Moll and

others 1997), and lead to a disruption in the proton motive force of the organism (Bruno and others 1992; Okereke and Montville 1992). Further investigation of the mechanism by which pores are formed has shown that nisin actually interacts with lipid II, a membrane-bound cell wall precursor molecule (Wiedemann and others 2001), and in doing so forms a ring of four lipid II molecules and eight nisin molecules that embeds into and forms a hole in the phospholipid bilayer of the cell membrane (Breukink and others 1999; Breukink and de Kruijff 2006). Because nisin interacts with the oligosaccharide cores of lipopolysaccharides in the outer membrane of Gram-negative bacteria (Stevens and others 1992) it cannot reach the cell membrane of such organisms, and is ineffective against them unless alterations of the lipopolysaccharide layer are accomplished through the use of other compounds (Stevens and others 1991; Branen and Davidson 2004). Thus, nisin is most effective against Gram-positive foodborne pathogens such as *L. monocytogenes*, *C. botulinum,* and *Bacillus cereus*, as well as the spores of the two latter organisms (Thomas and Delves-Broughton 2005).

Benkerroum and others (1988) were among the first to test the sensitivity of *L. monocytogenes* to nisin *in vitro*, and found that although minimum inhibitory concentration (MIC) values varied among strains, nisin was quite inhibitory to the pathogen and effectively halted growth at levels between 740 and 100,000 IU/ml. Likewise, Harris and others (1991) showed that *in vitro* applications of nisin at a concentration of 10 µg/ml were able to decrease *L. monocytogenes* numbers by 6.0 to 7.0 $\log_{10}$ CFU/ml. Ukuku and Shelef (1997) displayed similar results with nisin being effective in decreasing survivors after a contact time of 30 minutes at levels of 240 IU/ml and 600 IU/ml. A recent study showed that instantaneous addition of 200 IU/ml of nisin into buffered brain heart infusion broth at 10 °C decreased initial *L. monocytogenes* Scott A numbers by 4.0 $\log_{10}$ CFU/ml, and that outgrowth was even more

effectively inhibited when the initial addition of 200 IU/ml was combined with slow additions of 200 IU/ml and 500 IU/ml of nisin over time (Chi-Zhang and others 2004).

It has been shown that nisin resistant mutants of *L. monocytogenes* may be isolated with a relatively high frequency (Harris and others 1991; Davies and Adams 1994). The resistance mechanism utilized by these mutants is believed to involve changes in membrane fluidity by alteration of the fatty acid profile of constituent phospholipids (Ming and Daeschel 1993; Mazzotta and Montville 1997; Crandall and Montville 1998). In fatty acid profile examinations, mutants examined in these studies showed a higher tendency to contain straight chain fatty acids in their cytoplasmic membrane and thus decrease the potential for nisin to embed within the bilayer (Ming and Daeschel 1993; Mazzotta and Montville 1997). Other factors, such as the presence of divalent cations (Crandall and Montville 1998), and decreases in proton motive force and membrane potential due to acid tolerance responses (Bonnet and others 2006), have also been implicated as reasons for decreased nisin sensitivity in the pathogen. Likewise, nisin-inactivating enzymes (nisinases) produced by certain *Bacillus* spp. have also been shown to degrade the bacteriocin and render it ineffective (Jarvis 1967; Jarvis and Farr 1971).

To combat such resistances, applications involving combinations of nisin with environmental factors and other antimicrobials are most common, and will be discussed in later sections. However, the individual *in vivo* effect of nisin on *L. monocytogenes* in processed meats has been investigated, and mainly involves its use as a component of casings or dipping solutions. When included into edible zein coatings on RTE chicken at 1000 IU/g, nisin was able to decrease initial *L. monocytogenes* populations by 1.2 $\log_{10}$ CFU/g and 1.6 $\log_{10}$ CFU/g with ethanol- and propylene glycol-solubilized zein, respectively (Janes and others 2002). Growth of only 1.2 $\log_{10}$ CFU/g and 0.98 $\log_{10}$ CFU/g in RTE chicken with ethanol- and propylene glycol-solubilized zein containing nisin, respectively, was observed after storage at 4 °C after 24 days

(Janes and others 2002). When incorporated into zein casings for turkey frankfurters, nisin has been shown to decrease numbers of *L. monocytogenes* by 1.5 $\log_{10}$ CFU/g over 28 days at 4 °C (Lungu and Johnson 2005). Likewise, incorporation of nisin into the cellulose casings of pork-turkey-beef frankfurters has been shown to decrease *L. monocytogenes* populations by ~0.9 $\log_{10}$ CFU/package over the course of 15 days at 4 °C with growth following thereafter (Luchansky and Call 2004). Controls without nisin showed increases of *L. monocytogenes* after only 5 days at 4 °C (Luchansky and Call 2004). A 6400 IU/ml dipping solution of nisin applied to turkey frankfurters was able to decrease *L. monocytogenes* counts by 2.1 $\log_{10}$ CFU/g and 1.8 $\log_{10}$ CFU/g after 7 days at 4 °C and 10 °C, respectively (Sivarooban and others 2007). The same 6400 IU/ml dipping solution also limited growth of *L. monocytogenes* to 1.1 $\log_{10}$ CFU/g and 1.4 $\log_{10}$ CFU/g after 28 days of storage at 4°C and 10°C, respectively (Sivarooban and others 2007). Similarly, a dipping solution of 5000 IU/ml nisin from Nisaplin® was able to impart immediate reductions of *L. monocytogenes* by ~2.4 and ~2.6 $\log_{10}$ CFU/cm^2 in ham and bologna samples, respectively, and was even more effective when used in combination with organic acids (Geornaras and others 2005). Thus, nisin is an effective antimicrobial for processed meats and is easily able to meet FSIS standards that require reductions within certain periods of time.

Sodium Lactate

Unlike nisin, which is only produced by a specific group of microorganisms, the various forms in which lactic acid can exist are some of the most widely distributed compounds in nature (Shelef 1994). As such, sodium lactate, the sodium salt form of the compound, is easily produced by neutralizing the acid with an appropriate base. The presence of sodium lactate within foods is considered natural and intrinsic (Samelis and Sofos 2003), especially in dairy and muscle foods where lactic acid bacteria produce large amounts of the acid during fermentation. Though its use as a food preservative in processed meat products has escalated in recent years, it

was primarily incorporated into meat products as a flavoring agent before its antimicrobial properties were discovered. Inclusion of the compound in formulations promotes desirable sensory characteristics in the product such as enhanced meat flavor, enhanced color retention, increased juiciness, improved water-holding capacity, and elevated product yields (Doores 2005), as well as deterrence of microbial spoilage (Papadopoulos and others 1991b). In addition, preservation of fresh meat flavor and decreases in warmed-over flavor notes due to suppressed lipid oxidation in beef top rounds have also been attributed to sodium lactate (Papadopoulos and others 1991a). By regulation, sodium lactate can be added to meat and poultry products at levels up to 4.8% of the final weight (FSIS 2000).

Sodium L-Lactate can be derived from the neutralization of lactic acid, commercially produced by the controlled fermentation of refined sucrose or other carbohydrates, followed by crystallization as calcium lactate and acidification by an appropriate inorganic acid (Morgan and Goodman 1939). It is commercially available in a 60% w/w aqueous solution at a neutral pH and is usually applied to food products as such (Shelef 1994). Because of its nature as the salt of a weak organic acid, lactate can form a buffer in the range of pH values near to its pK_a of 3.86 (Samelis and Sofos 2003). As a result, the molecule primarily exists in its disassociated form at pH values above its pK_a and in its undisassociated form at pH conditions below its pK_a. However, it is widely believed that the molecule's acid form is the more microbiologically active of the two.

The mechanisms by which lactate and other organic acid salts inhibit bacterial growth have been studied in detail, but uncertainty still remains as to the exact mode or modes of action. Several have noted that the ability of sodium lactate to depress water activity may have an effect on microbial growth (Chen and Shelef 1992; Shelef 1994). Nonetheless, many regard the uncoupling hypothesis proposed by Freese and others (1973) to be the primary mode of action.

Freese and collaborators suggested that organic and lipophilic acids acted on cells by being absorbed through the membrane in their associated forms and acidifying the interior of the cell upon disassociation within the cytoplasm (1973). The theory that the undissociated acid penetrates the membrane most easily has been substantiated in a number of cases (Cramer and Prestegard 1977; Eklund 1983; Chu and others 1987), and it has been suggested that the acidification which occurs can have deleterious consequences (Baird-Parker 1980). However, the resulting pH imbalance that disrupts the proton motive force of the cell, inhibits its growth, and hinders its ability to transport materials across the membrane is regarded as the primary consequence of uncoupling (Salmond and others 1984). The validity of this hypothesis was tested by Hunter and Segel (1973), who investigated the uptake of several organic acids in *Penicillium chrysogenum*. Their results showed that the ionized form of the acid was unable to penetrate the membrane and that absorption of the associated form was the primary means of uptake. They also were able to show that once within the cell, the disassociation of the acid decreased cellular pH and caused depletion of adenosine triphosphate within the cell. Observations from similar organic acid treatments of *Shigella flexneri* (Baskett and Hentges 1973) and *E. coli* (Salmond and others 1984) displayed that the phenomenon observed in fungi also yielded the same effect in bacteria. Buchanan and others (1993) confirmed this mechanism showing that the *in vitro* effectiveness of lactic acid/sodium lactate buffers on *L. monocytogenes* was correlated with environmental pH and disassociation of the acid.

Though uncoupling is widely accepted as a predominant means of inhibition by organic acids, questions have been raised as to whether passage through the membrane, disassociation in the cytoplasm, and inhibition of material transport is the sole mechanism. Eklund (1980) noted that although the pH gradient is disrupted, the cell membrane electric potential should still be sufficient to drive uptake of certain substances. He also noted that although the uncoupling

concept held true for paraben molecules, it was unable to fully explain the action of weak organic acids (Eklund 1980). Thus, he claimed that regarding transport inhibition as the sole mechanism of organic acid inhibition was flawed (Eklund 1980). Cherrington and others (1990) exposed *E. coli* to 5 mM propionic acid and 10 mM formic acid and noted that upon exposure the rates of RNA, DNA, protein, lipid, and cell wall synthesis immediately decreased. Thus, they concluded that a deviation from coordinated macromolecular synthesis and inhibited DNA replication resulting in reduced cell division were also potential mechanisms of inhibition for organic acids (Cherrington and others 1990). Alakomi and others (2000) observed that lactic acid exerted a permeabilizing effect on the outer membrane of *E. coli* O157:H7, *Pseudomonas aeruginosa*, and *Salmonella enterica* Serovar Typhimurium. Through a fluorescent-probe uptake assay, 5 mM lactic acid was determined to be a much better membrane permeabilizer than either ethylenediaminetetraacetic acid (EDTA) or hydrochloric acid (HCl), and was found to increase the lytic action of sodium dodecyl sulfate in both *E. coli* O157:H7 and *S.* Typhimurium (Alakomi and others 2000). Considerably higher amounts of lipopolysaccharide were also liberated when *S.* Typhimurium was treated with lactic acid opposed to EDTA and HCl (Alakomi and others 2000). From these observations, they postulated that such actions of the acid may serve as a potentiator of other antimicrobial substances. Koczoń (2009) further characterized potential organic acid mechanisms with three benzoic acid derivatives used against the yeast *Pichia anomala*. Through his observation of fluctuating levels of the organic acid salts in the medium, he was able to conclude that a molecular pumping system was activated in the yeast cells, and possibly led to increased energy expenditures that caused cell inhibition (Koczoń 2009). Thus, inhibition by mechanisms other than the cytoplasmic acidification and pH gradient disruption of uncoupling may also contribute to the inhibitory action of sodium lactate and similar compounds, and may provide other means for synergism with other compounds.

Although organic acids and their salts are effective in controlling the growth of pathogens, continued exposure of microorganisms to low levels of acid derivatives can lead to development of an acid tolerance response or acid resistance (Samelis and Sofos 2003). Acid tolerance responses of bacteria result when they are briefly exposed to sublethal pH either during the exponential growth phase (O'Driscoll and others 1996; Jordan and others 1999) or the stationary phase (Lee and others 1994; Buchanan and Edelson 1996) and develop induced adaptations to the conditions. Acid resistance, however, is acquired over a much greater length of time through frequent exposure to acids (Samelis and Sofos 2003). It is a portion of the generalized stress resistance that cells continually express when entering the stationary phase (Cheville and others 1996; Lin and others 1995; Davis and others 1996) and represents the intrinsic ability of an organism to survive at lethal pH, especially in the stationary phase (Jordan and others 1999). The development of these adaptations is usually accompanied by production of specific stress-protective proteins, which can also decrease sensitivity to other stresses such as heat (Farber and Pagotto 1992; Leyer and Johnson 1993). As such, the virulence of certain organisms can become enhanced (O'Driscoll and others 1996; Buncic and Avery 1998), and many stresses that the human body normally poses on the organism, such as the acid of the stomach and the attack of macrophages, may be evaded (Gahan and Hill 1999; Ricke 2003). Thus, development of acid tolerance or resistance in pathogenic organisms is a major issue to the food industry (Sheridan and McDowell 1998). To combat this, cooperative effects between sodium lactate and the use of physical processes and/or other antimicrobials to control *L. monocytogenes* have been investigated. Some of these instances will be discussed in later sections.

Though sodium lactate is highly effective at delaying growth of spoilage microorganisms and prolonging shelf life (Brewer and others 1991; Brewer and others 1995;

Papadopoulos and others 1991b), it has also been shown to be highly effective against foodborne pathogens including *L. monocytogenes.* Shelef and Yang (1991) showed that concentrations of sodium lactate at 10% were able to inhibit *L. monocytogenes* growth by at least ~3.0 $\log_{10}$ CFU/ml in tryptic soy broth after 24 h at 35 °C as compared to unexposed controls. In addition, 4% lactate was generally able to suppress growth of the pathogen in sterile comminuted beef for at least 18 days at 5 °C. Unda and others (1991) showed that including 2% sodium lactate in brines used for microwave-ready beef roasts was more effective at inhibiting *L. monocytogenes* than traditional phosphate ingredients. Zeitoun and Debevere (1991) demonstrated that a 10% application of lactate along with MAP storage at 6 °C was able to inhibit outgrowth of the pathogen on fresh chicken legs for 2 days and that application of 2, 5, and 10% lactate plus modified atmosphere packaging (MAP) could result in shelf life extensions of 2, 3, 4, and 11 days, respectively. Several other noteworthy investigations of sodium lactate's effect on *L. monocytogenes* are summarized in Table 1.

Table 1-Studies highlighting the *in vivo* and *in vitro* effectiveness of sodium lactate against *L. monocytogenes.*

Product/Medium	Conditions	Effect	Reference
Cooked strained beef	Meat moisture contents of 25 to 85% and incubation at 20 °C	4% Sodium lactate suppressed growth of *L. monocytogenes* in moisture contents above 55% and inhibited growth in moisture ranges of 25 to 55%	(Chen and Shelef 1992)
Raw and cooked ground beef	Aerobic storage at 4 °C	1.8% Sodium lactate was better at inhibiting growth of *L. monocytogenes* than was sodium erythorbate; APC increases were also less pronounced in samples treated with lactate	(Harmayani and others 1993)

Table 1-Continued

Product/Medium	Conditions	Effect	Reference
Concentrated GYS broth	Incubation at 20 °C and pH 6.5 for 7 days	Minimum inhibitory concentrations of sodium lactate for five *L. monocytogenes* strains ranged from 804 to 982 mM	(Houtsma and others 1993)
Sliced bologna type sausage	Vacuum-packaged storage at 5 °C and 10 °C for 35 days	2% Sodium lactate suppressed *L. monocytogenes* growth for 28 days at 5 °C; 2% sodium lactate combined with glucono-δ-lactone suppressed growth for 35 days at both 5 °C and 10 °C	(Qvist and others 1994)
Pork liver sausage	Incubation at 20 °C for 10 days and storage at 5°C for 50 days	*L. monocytogenes* was able to add only 1.4 $\log_{10}$ CFU/g at 20 °C when in the presence of 4% sodium lactate as compared to 5 $\log_{10}$ CFU/g in controls; At 5 °C controls allowed for 4.5 $\log_{10}$ CFU/g of growth, while samples with 3% sodium lactate added only 0.9 $\log_{10}$ CFU/g	(Weaver and Shelef 1993)
Cooked, quartered beef top rounds	Storage at 10 °C for up to 28 days	Sodium lactate at 3 and 4% was able to limit *L. monocytogenes* growth by 0.9 and 5.1 $\log_{10}$ MPN/cm^2 respectively, after 28 days of storage; 2% lactate samples were not significantly different than controls	(Miller and Acuff 1994)
Cold-processed (smoked) salmon	Vacuum-packaged storage at 5 °C and 10 °C for up to 50 days	2% Sodium lactate in combination with 3% sodium chloride completely inhibited growth of *L. monocytogenes* after 50 days at 5 °C; 3% sodium lactate in combination with 3% sodium chloride inhibited growth of the pathogen for 35 days at 10 °C	(Pelroy and others 1994)

Table 1-Continued

Product/Medium	Conditions	Effect	Reference
Sliced cooked turkey bologna	Vacuum-packaged storage at 4 °C for 100 days	2% Sodium lactate was able to suppress growth of *L. monocytogenes* by at least 3 $\log_{10}$ CFU/g as compared to controls after storage for 100 days	(Wederquist and others 1994)
Salt-free, sterile comminuted beef emulsion	Aerobic storage for 30 days at 5 °C	Sodium lactate concentrations of 1.8% and 2.5% were able to limit growth of *L. monocytogenes* to ~1.0 $\log_{10}$ CFU/g over the course of 30 days	(Mbandi and Shelef 2001)
Pork frankfurters	Vacuum-packaged storage at 4 °C for 120 days	Sodium lactate at 6% was bacteriostatic toward *L. monocytogenes* for the entire 120 days; 3% sodium lactate prevented pathogen growth for at least 70 days	(Bedie and others 2001)
Beef bologna	Aerobic storage for 45 days at 5 °C	2.5% sodium lactate was able to keep growth of an *L. monocytogenes* mixture to ~1.0 $\log_{10}$ CFU/g for 45 days; *L. monocytogenes* Scott A only grew 1.3 $\log_{10}$ CFU/g over the same period of time	(Mbandi and Shelef 2002)
Pork-turkey-beef frankfurters	Vacuum-packaged storage at 4.5 °C for 60 days	Frankfurters formulated with either 3.0% or 3.5% sodium lactate inhibited growth of *L. monocytogenes* for the entire 60 days of storage	(Glass and others 2002)
Pork frankfurters	Vacuum-packaged storage at 4 °C for up to 8 weeks	Samples with 3.3% sodium lactate had antilisterial effects that were the same as seen in 0.05 to 1.0% of potassium sorbate with much lower TBARS values in the meat product	(Choi and Chin 2003)

Table 1-Continued

Product/Medium	Conditions	Effect	Reference
Pork frankfurters	Vacuum-packaged storage at 10 °C for 40 days	Frankfurters formulated with 1.8% sodium lactate in the formulation were able to produce significantly ($P < 0.05$) growth of *L. monocytogenes* compared to controls	(Barmpalia and others 2004)

As a result of its effective antimicrobial properties, and its desirable sensory effects, sodium lactate is one of the most widely used and efficacious choices for control of pathogens in processed meats. Thus, developing effective control measures that utilize sodium lactate as a component of a control process are desirable.

ε-Poly-L-Lysine

Among all the classes of biodegradable polymers, polyamino acids are one of the most important due to their specialized applications in biological systems, such as drug delivery (Shih and others 2006). In the late 1970s, researchers from Japan isolated a polyamino acid during their regular screening of Dragendorff positive substances from a *Streptomyces albulus* strain (Shima and Sakai 1977). Unlike other polypeptides, hydrolysis of the molecule yielded a sole amino acid, L-lysine, as the product (Shima and Sakai 1977). This observation suggested that the substrate molecule was a poly-L-lysine, a compound previously synthesized but never been found to accumulate within microbial populations (Shima and Sakai 1977). Molecular structure investigations concluded that it consisted of about 25 to 30 monomers of lysine that were primarily joined by an ε-amino and α-carboxyl linkage of the amino acids (Shima and Sakai 1981a; Shima and Sakai 1981b). Thus the name ε-Poly-L-Lysine (EPL) was adopted for the molecule and is still used today.

Natural production of EPL is a unique ability of bacterial organisms in the taxonomic family *Streptomyces*, and an ergot fungus, *Eipchloe* sp. strain MN-9 (Nishikawa and Ogawa 2002). Commercially, the compound is produced using *S. albulus* subsp. *lysinopolymerus*, grown under aerobic conditions to allow separation and purification of polymer from cell mass (Hiraki and Suzuki 1999). Furthermore, by keeping the growth medium near pH 4.0 cells are induced to increase EPL production, and the slightly acidic pH helps in retention of the polymerized molecule (Kahar and others 2001). Once produced, the compound is then separated from the growth medium via centrifugation or filtration, and is further purified by passage through an ion exchange chromatography column, neutralization, decolorization, evaporation, and precipitation in an ethanol/diethyl-ether mixture (Shih and others 2006).

The pure form of EPL that is produced can be employed for a variety of uses due to its water soluble, thermally stable, and biodegradable nature (Yoshida and Nagasawa 2003). A synthetically produced cousin, α-Poly-L-Lysine, is similar in structure, but possesses toxic activity. Thus, EPL is preferred for use in biological systems and is heavily employed by the biomedical industry as a drug delivery carrier, endotoxin remover, and biosensor (Shih and others 2006). The electronics industry also uses EPL for biochips and bioelectronics (Shih and others 2006). However, due to its antimicrobial activity, its utility as a food preservative and additive has continued to draw the most attention (Yoshida and Nagasawa 2003).

As a food additive, EPL has been shown to be safe for human consumption (Hiraki and others 2003), and is considered to be a natural food ingredient due to its production by *S. albulus* (Shih and others 2006). The FDA approved its use in the United States in 2004 as an antimicrobial agent used exclusively for cooked and sushi rice at levels up to 50 mg/kg (FDA 2004a). Conversely, the compound has had approved status for a number of years in Japan and is currently used there as an antimicrobial agent within a variety of food products. For

decontamination of fish sushi and sliced fish, it is usually applied as a dipping solution with concentrations in the 1000-5000 ppm range (Hiraki and others 2003). In nimono, a common Japanese dish, it is currently used at levels up to 500 ppm, and is used at levels of 10 to 500 ppm in dishes such as boiled rice, noodle soup stocks, noodles, other soups, cooked vegetables, Japanese beef steak, potato salad, steamed cakes, and custard cream (Hiraki and others 2003).

The biochemical mechanism by which the compound is able to inhibit bacterial growth has not been extensively studied, but is believed to stem from its polycationic structure. The repeating strand of positively-charged α-amine side groups is believed to cause release of lipopolysaccharide from the outer membrane in Gram-negative bacteria and allow binding to the cytoplasmic membrane of both Gram-negative and Gram-positive microorganisms (Yoshida and Nagasawa 2003). Shima and others (1984) were the first to propose this mode of action for EPL, when they suggested that the compound stripped off the outer membrane of *E. coli* and then electrostatically adsorbed to the plasma membrane. This adsorption, they believed, led to the abnormal distribution of the cytoplasm that they had observed through electron microscopy, which had in turn led to physiological damage in the cell. In addition, sensitization of the cell membrane to 10 to 100 fold increases of absorption of hydrophobic antibiotics (such as novobiocin and erythromycin) and loss of 20 to 30% of lipopolysaccharide from the outer membrane, has also been observed when bacteria are exposed to slightly shorter poly-L-lysine molecules (of about 20 residues) and is thought to be due to the same mechanism (Vaara and Vaara 1983a; Vaara and Vaara 1983b; Vaara 1992). Bactericidal effects have also been shown to be correlated with the chain length of the poly-L-lysine molecule, with those that are longer than 20 units in length and up to 50 units in length being significantly bactericidal (Vaara and Vaara 1983b). Others have also shown that trans-membrane passage of polycationic poly-L-arginine molecules, which are very similar in structure to EPL, is possible, and once inside the cell, may

cause depolarization of the cytoplasmic membrane or even cell lysis (Conte and others 2007). Chitosan, a derivative of the N-deactylation of chitin, shares the generic polycationic structure with EPL, and has been shown to have very similar effects on cells (Rabea and others 2003; Raafat and others 2008). As with EPL analogs, chitosan depolarizes the cell membrane (Raafat and others 2008) and permeabilizes it to small cellular components (Fang and others 1994). The mode of action of chitosan has been postulated to be due to its electrostatic interactions with teichoic acids, which may lead to extraction of membrane lipids such as lipoteichoic acid and the disabling and disruption of the cell membrane functions such as membrane-bound energy generation pathways (Raafat and others 2008). Because EPL and chitosan share these similarities, investigations of such cellular targets might provide insight into the mode of action of EPL. Finally, development of resistance to EPL is believed infrequent due to its simple ionic interactions-based mechanism. However, factors such as ionic strength of the surroundings can affect the electrostatic charge reactions needed for initial contact of polycations with the membrane (Zasloff 2002). In addition, rare cases of production of EPL-degrading enzymes have also been reported and may play an overlooked role in deactivating the compound (Kito and others 2002a; Kito and others 2002b).

As with the biochemical mechanism, the general body of published information about the inhibitory effect of EPL on groups of microorganisms is considerably limited. Shima and others (1982) first investigated the antimicrobial properties of the substance by exposing T4 and T5 bacteriophages to varied concentrations of the compound, and found that the polymer was effective at inactivating both types of phages and was even more effective when coupled with certain cations. Likewise, studies on the inhibitory activity of the compound against a diverse array of bacteria, yeasts, and molds demonstrated that the compound has a broad spectrum of action and is highly effective at low levels (~1-8 µg/ml) for both Gram-positive and Gram-

negative bacteria (Shima and others 1984). Although yeasts showed some susceptibility, it was generally considered that both yeasts and molds were much less affected by exposure to the compound than were bacteria. Delihas and others (1995) also probed the effects of the compound on a group of bacteria and found that, in disagreement with Shima and others, *Mycobacterium tuberculosis* was actually more sensitive to the activity of EPL than *E. coli*, requiring only 0.36 µg/ml EPL to exhibit a 50% reduction in cell viability after 2 h, whereas *E. coli* required levels of up to 5.8 µg/ml. Their additional observation of differences in susceptibility between *Staphylococcus epidermidis* and *Streptococcus salivarius* (two Gram-positive organisms) suggested that the compound is able to act on the cell with little regard for the envelope type possessed by the organism, which is in agreement with its accepted biochemical mode of antimicrobial action (Delihas and others 1995). *In vivo* applications that focus on incorporating the compound into food matrices are undoubtedly needed, as well as studies on interactions of the compound with other food antimicrobials. The use of EPL in combination with other antimicrobials to produce an enhanced effect has been investigated, but these aspects of its usage will be discussed later.

Lauric Arginate Ester

The surface active properties of N^{α}-acyl amino acids have led to their usage in a variety of applications within the cosmetic and biomedical industries (Infante and others 1984). L-arginine, N^{α}-lauroyl ethylester, also known as lauric arginate ester (LAE), belongs to this particular class of compounds, and was first synthesized in Spain in the early 1980s (Infante and others 1984). Though originally intended to be an ingredient in the formulations of cosmetics, the observed antimicrobial capabilities of the compound (Infante and others 1984; Infante and others 1985) as well as its synthesis from natural precursor molecules, sparked its usage within

food systems as a multi-factorial food additive that could be used for both its emulsification and antimicrobial properties (McKellar and others 1992).

With regard to structure, LAE follows the generic N^{α}-acyl amino acid configuration (Figure 2). It consists of the 12 carbon saturated fatty acid laurate esterified to the α-amine of the cationic amino acid arginine, and has an ethyl ester moiety from the esterification of ethanol to the carboxyl functional group of the arginine. The commercial production of the molecule and its applications in food have been patented through European and International patents (Contijoch and others 2006; Urgell and Seguer 2003), and is primarily conducted by a company in Spain (Bakal and Diaz 2005). The production process is broken into two steps and begins with the reaction of ethanol with the arginine molecule in a catalyst-containing ethanol solution to form the ester (Contijoch and others 2006). After this, ethanol is removed and an oily intermediate product is isolated (Contijoch and others 2006). This is followed by the second step, which involves reaction of this intermediate product with lauric acid chloride for 5 to 10 h in an aqueous environment of neutral pH to favor the amidization (Contijoch and others 2006).

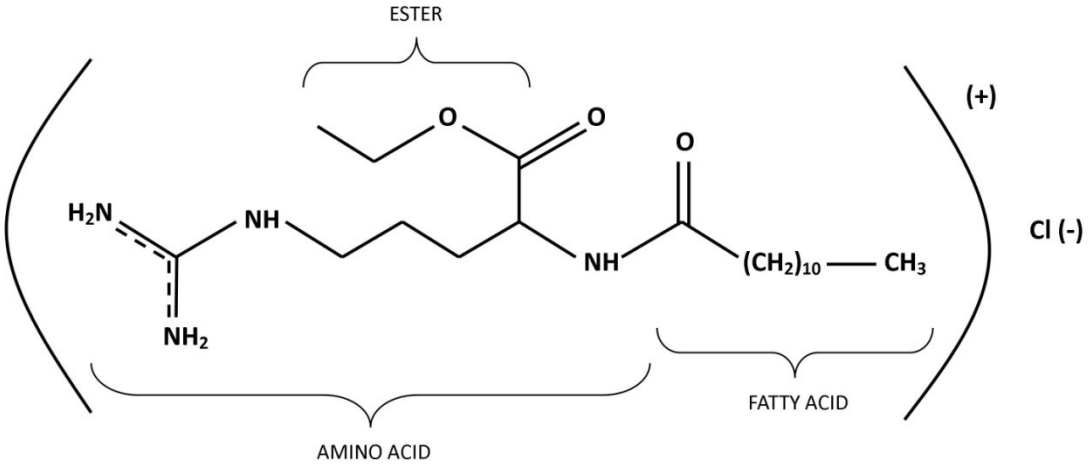

Figure 2-Structure of L-arginine N^{α}-lauroyl ethylester (lauric arginate ester).
Adapted from Urgell and Seguer (2003).

Metabolism of LAE in the body occurs very rapidly upon ingestion and separates the compound into its constituents of arginine and lauric acid (Ruckman and others 2004). Toxicity tests of LAE in rats and rabbits showed that the compound is non-toxic even when fed at concentrations of 50,000 ppm (Ruckman and others 2004). Based on this information, LAE is considered to be safe for human consumption by the FDA and is approved for use at levels of up to 200 ppm in meat and poultry foods (FDA 2005). The use of the compound in such foods is primarily as an antimicrobial, where it is included into the product's formulation or is used as a rinse aid (FSIS 2009). As a result, it is also regulated by FSIS, who holds the same standard of application as is allowed by the FDA's GRAS approval (FSIS 2009).

The mechanism by which LAE inhibits microorganisms has not been widely studied, but published data indicates a mode of action in line with other cationic surfactants. The positive charge of LAE is believed to favor association with the polar surface of the bacterial membrane and its surfactant properties are believed to cause disruption of the membrane (Infante and others 1985). In addition, it has been shown to maintain activity over a broad spectrum of acidity (pH 3-7), which is further evidence of this mechanism (Bakal and Diaz 2005). Rodríguez and others (2004) confirmed postulations about the similarity of its action to that of analogous molecules. Using transmission electron microscopy (TEM) and flow cytometry, it was noted that LAE-treated *S.* Typhimurium cells showed extensive disruption in both their outer and cytoplasmic membranes. Similar cytoplasmic membrane damage was observed in TEM images of LAE-treated *S. aureus* cells (Rodríguez and others 2004). In contrast to *S.* Typhimurium, *S. aureus* cells showed more extensive interior damage with formation of mesosome-like structures, white spots, multi-septated cells, and clear zones in their cytoplasms. Examination of fluorescence microscopy images after SYTO®13/PI flow cytometry exposure in the *S.* Typhimurium cells revealed that nearly 97% of the cells had damaged membranes after 24 h of exposure to LAE,

evidenced by uptake the differential fluorescent stain propidium iodide (PI) (Rodríguez and others 2004). *S. aureus* fluorescent microscopy images also demonstrated extensive damage of the cell membrane with over 56% of cells being stained by uptake of PI after 24 h of exposure to LAE. In addition, it was noted that within 30 min of exposure to LAE, 43% of the *S. aureus* population already displayed cell membrane damage, while an additional 21% displayed partial injury. Finally, the most noteworthy observation was that the membrane disruption observed occurred without cell lysis. Thus, the antimicrobial mechanism of LAE may not require cell lysis, and may only involve disturbance of membrane potential and structure, much like the mechanism of ε-Poly-L-Lysine.

Studies investigating susceptibility of microorganisms to different LAE applications are limited, and until recently, much of the knowledge about the spectrum of LAE's antimicrobial activity has come from early studies and confirmatory tests done for patenting. Several recent patents and articles have stated that LAE has considerably diverse activity with bacteria, yeasts, and molds all being susceptible to its inhibitory function (Contijoch and others 2006; Seguer and others 2006; Bakal and Diaz 2005). Infante and others (1984) were the first to analyze the antimicrobic potential of LAE in tryptic soy broth (TSB) at 37 °C against species of *Staphylococcus*, *Bacillus*, *Micrococcus*, *Citrobacter*, *Escherichia*, and *Pseudomonas*. In all, the MIC of LAE for Gram-positive cocci was at or below 13 µg/ml; MICs for Gram-negative bacilli ranged 26 to 107 µg/ml. In another study, antimicrobial testing in Müeller-Hinton agar yielded similar trends with Gram-positives having slightly lower MICs (2-16 µg/ml), while Gram-negative bacteria required higher concentrations for inhibition (MICs: 8-128 µg/ml) (Infante and others 1985). Rodríguez and others (2004) found that LAE at 32 µg/ml was inhibitory to *S.* Typhimurium and 8 µg/ml was inhibitory to *S. aureus*.

With regard to application of LAE in food matrices, much of the research in this area has surfaced within the past several years. A study investigating the use of LAE as a Sprayed Lethality In Container™ intervention to combat *L. monocytogenes* in table brown hams was one of the first studies to test the antimicrobial against the pathogen *in vivo* (Luchansky and others 2005). Researchers artificially inoculated hams with *L. monocytogenes* and placed them in vacuum packages containing either 5% or 10% solutions of LAE as in-package purge/fluid. Hams were sealed and stored at 4 °C, and surviving *Listeria* were enumerated after 24 h. Addition of 2 ml of a 5% LAE solution resulted in *L. monocytogenes* population decreases of 3.3 $\log_{10}$ CFU/ham, and decreases as much as 6.5 $\log_{10}$ CFU/ham when 8 ml of 5% LAE or any volume of 10% LAE was added. Extended storage (60 days, 4 °C) yielded similar effects with 5% LAE producing decreases of up to 5.5 $\log_{10}$ CFU of *L. monocytogenes* per ham (Luchansky and others 2005).

A more recent study investigated the susceptibility of *L. monocytogenes* to LAE when applied to vacuum-packaged frankfurters stored at 4 °C using a method similar to the previously described Sprayed Lethality In Package™ treatment (Taormina and Dorsa 2009). Differences in *L. monocytogenes* reductions as a function of the combination of LAE with liquid smoke extract, volume of LAE solution added, inoculum levels, and inoculation method (dip or spot inoculation onto the frankfurter) were evaluated. Liquid smoke in combination with a 5000 ppm LAE solution did not produce greater reduction than LAE alone. Counts of dip inoculated *L. monocytogenes* from the 7.0 $\log_{10}$ CFU starting point were significantly reduced by 1.4 $\log_{10}$ CFU/package (LAE only) and 1.8 $\log_{10}$ CFU/package (LAE + Smoke) ($P < 0.05$) after the usage of 3 ml of the antimicrobial. Similar reductions were observed in samples dip inoculated with 3.0 $\log_{10}$ CFU/package as with those dip inoculated with 7.0 $\log_{10}$ CFU/package. Volume of the antimicrobial solution used was found to have little effect on the activity of LAE, with all

volumes in the range of 3-4.5 ml yielding reductions of 1.8-2.2 $\log_{10}$ CFU/package in frankfurters that had been inoculated at 7.0 $\log_{10}$ CFU through dip inoculation (Taormina and Dorsa 2009). Spot inoculation on frankfurters yielded a slightly higher initial load of *L. monocytogenes* (difference of 0.47 $\log_{10}$ CFU/package) than dip inoculation, but had little effect on the reductions due to LAE, with dip inoculated *L. monocytogenes* being reduced by 1.7 and 1.8 $\log_{10}$ CFU/package and spot inoculated *L. monocytogenes* being reduced by 1.4 to 2.0 $\log_{10}$ CFU/package by sprays of 2.5 ml of a 5000 and an 8000 ppm LAE solution, respectively.

Another recent study examined the antimicrobial effects of LAE on *L. monocytogenes* in frankfurters alongside potassium lactate and sodium diacetate (Martin and others 2009). The study, which was aimed at validating post-lethality standards for LAE, concluded that 2.0, 2.5, and 3.0 ml of a 2.5% solution of LAE stock were able to reduce the *L. monocytogenes* counts by nearly 1.3, 1.3, and 1.4 $\log_{10}$ CFU/ml, respectively, after 12 h at 6 °C when used in a prepackaging application (Martin and others 2009). These results validated the 1 log cycle reduction in *L. monocytogenes* necessary to achieve compliance with the immediate lethality stipulation posed by FSIS. Researchers observed that using the same LAE working solution on frankfurters that were formulated with either 1.8%/0.13% or 2.1%/0.15% combinations of potassium lactate/sodium diacetate yielded a > 2 log cycle reduction in the pathogen at 12 h of storage in both formulations. Also, both formulations in combination with the LAE solution yielded ≤ 2 log cycles' increase in *L. monocytogenes* over the entire course of the 156 day shelf-life of the frankfurters, meeting the growth suppression stipulation enforced by FSIS (Martin and others 2009). As a result, it was determined that the combination of LAE with potassium lactate and sodium diacetate could yield better inhibition than using either alone and could be an effective antimicrobial hurdle treatment.

Although the usage of such combinations of LAE with other antimicrobials certainly helps to overcome any decreased sensitivity exhibited by a pathogen, no incidences of innate resistance to LAE have been documented amongst foodborne bacterial pathogens. This is believed to be due to its cationic structure and surfactant-based mechanism, which is difficult for the target bacterium to overcome. However, this same mechanism that lends LAE its activity has also been shown to render it inactive in several cases. For instance, it has been shown that LAE can electrostatically bind to pectin, a normal component of foods, and form an inactive complex with the biopolymer (Asker and others 2009). In addition, its tendency to precipitate out of solution at high ionic strength and pH levels above 4.5 has also proven to be an obstacle to its usage within food products, and represents a challenge yet to be overcome in implementing it into food systems (Asker and others 2009).

Acidic Calcium Sulfate

As one of the more recent additions to the assortment of food antimicrobials, acidic calcium sulfate (ACS), formed by mixing calcium, an organic acid, and the sulfate anion with acid and base, has been gaining popularity in use as a pathogen intervention. It has shown exceptional potential in combating foodborne pathogens in high throughput operations, not only because of its ease of use and effectiveness, but also because of its environmental friendliness (Lamb 2002). In addition, the compound is highly acidic to bacteria but will not harm human or animal tissue; this has made many eager to experiment with its application in their food products. Thus, with its growing popularity and diverse scope of action, the incorporation of ACS interventions into food processes may become much more common and may be a good solution for processors to use in achieving pathogen control goals.

From its precursor solution known as $Safe_2O$, ACS is produced in its commercially available RTE:01® form by mixing an organic acid with other ions to form a complex acidic

solution. Solutions that are produced in this way have been termed highly acidic metalated organic acids (HAMOs), and the production of such solutions and their components has been patented several times (Kemp and others 2003; Kemp and others 2005a; Kemp and others 2005b). As with other HAMOs, the fundamental components of ACS are an organic acid or its salt form, a metal base (which can be excluded in some occasions), and a strong acid which is able to regenerate the acid form of the organic acid after reactions take place (a strong binary acid, oxyacid, or acidic complex of Group II metal ions can all be used for this purpose) (Kemp and others 2003). For the RTE:01 form of ACS, lactic acid serves as the organic acid, with calcium hydroxide as the metal base, and sulfuric acid as the regenerating acid (Kemp and others 2005a), all of which are considered to be GRAS (FDA 2008d; FDA 2008b; FDA 2008e). Production is initiated by adding water to a lactic acid solution, followed by addition of calcium hydroxide with agitation (Kemp and others 2003). In the second stage, water is added again, sulfuric acid is added, the mixture is agitated, and the solution is filtered (Kemp and others 2003). The resulting product, which is ready for food use, has a pH of 2 or less, a density of 1.06 to 1.10 g/ml, and a clear to slightly cloudy appearance.

In terms of food applications, ACS is primarily used as a pH control agent in the water of spray or dip applications that are used to decontaminate the exterior of a food product (FSIS 2009). Thus, investigations of its use have primarily focused on meat, poultry, and seafood. Indeed, many internal and published studies have looked at its ability to decontaminate cooked chicken breast (Ananth 2003), frankfurters (Anonymous 2004b; Nuñez de Gonzalez and others 2004), roast beef (Anonymous 2004a), ground beef (Zhao and others 2004), cooked turkey products (Centrella 2005), and commercially prepared hams (Luchansky and others 2005). In addition, because FSIS does not limit producers in the amount of ACS that can be present in the final product, its maximum application rate is at the discretion of the food manufacturer, which

allows processors to apply the amount necessary to achieve validated lethality of the target pathogens in their food product. To date, all studies on ACS have produced results that favor the inclusion of the solution into raw product treatments and post-lethality processes, and several have even focused specifically on combating *L. monocytogenes* in RTE meats.

In a study conducted to validate the reductions in *L. monocytogenes* due to ACS treatments in cooked chicken breasts, the HAMO was found to be considerably effective (Ananth 2003). Most Probable Number (MPN) estimation of *L. monocytogenes* (as determined through the USDA enrichment and detection method), along with lactic acid bacteria and aerobic plate count numbers, were assayed over a 40 day period of storage of the chicken breasts at 4.4 °C. The effectiveness of ACS was tested by applying a 20 second spray of a 1:2 ACS:distilled water solution to chicken breast samples that had been inoculated with 100 CFU/serving of *L. monocytogenes*. Considerably lower MPN values of *L. monocytogenes* in ACS-treated samples, as opposed to water-treated and non-treated samples, were observed beginning on Day 0 and continued throughout the shelf life until day 40, where estimated levels of *L. monocytogenes* were 1.1×10^6 average MPN/g for non-treated samples, 3.8×10^4 average MPN/g for water-treated samples, and $< 8.0 \times 10^1$ average MPN/g for samples treated with the 1:2 ACS solution.

Likewise, investigations of ACS's effectiveness on reducing *L. monocytogenes* in sliced cured and uncured cooked turkey meat showed that, according to FSIS standards, the compound is effective as both a post-lethality treatment and an antimicrobial agent in such products (Centrella 2005). Approximately 4.0 $\log_{10}$ CFU/ml of *L. monocytogenes* was applied to the product at the outset of the study, and a 1:3 ACS:sterile water solution was applied via dip application. *L. monocytogenes* was immediately (after 1 hr of storage at 3.3 to 4.4 °C) reduced on cured samples by 1.4 $\log_{10}$ CFU/g and was suppressed to an only 1.0 $\log_{10}$ CFU/g increase over the course of the 75 day shelf life of the product. In uncured samples, the ACS was able to

induce an immediate post-process lethality of 1.5 $\log_{10}$ CFU/g and actually produced a 0.7 $\log_{10}$ CFU/g reduction in the pathogen over the course of 60 days of shelf life. Though it did not meet the ideal situation of a 2 log cycle immediate reduction, the immediate decrease in *L. monocytogenes* that was afforded by the ACS allowed it to be classified as a post-lethality treatment in such products. In addition, due to its ability to suppress outgrowth during the product shelf life, ACS also met FSIS Alternative 1 standards for being an antimicrobial agent that could be used for such products.

Multiple studies have also shown that the compound is efficacious at abating *L. monocytogenes* growth in frankfurters. In one study, ACS was used to treat artificially inoculated (~5.3 $\log_{10}$ CFU/package of *L. monocytogenes*) frankfurters that were either in their in-casing or final-product forms, and either did or did not contain a potassium lactate/sodium diacetate mixture (Anonymous 2004b). The study tested a variety of ACS treatments with application method (dip tray or spray application), time of exposure, and temperature of solution as variable factors. Within every category of frankfurter (in-casing, final-product, with or without potassium lactate/sodium diacetate) several different ACS treatment conditions were able to produce *L. monocytogenes* lethalities of greater than 2.0 log cycles at the outset and suppressions of growth that resulted in less than a 1 log cycle increase over the course of 12 weeks of storage at 4.4 °C, thus validating its usage as a post-lethality treatment and antimicrobial for these products as well. The second study also reflected effectiveness of ACS (containing both lactic and propionic acid) when applied as a post-process dipping solution to reduce *L. monocytogenes* populations in frankfurters that either did or did not contain potassium lactate (Keeton and others 2002; Nuñez de Gonzalez and others 2004). Dipping frankfurters in a 1:2 ACS:water solution for 30 seconds kept *L. monocytogenes* counts below the minimum detection level for the full 12 weeks of storage at 4.5 °C, was slightly more effective than dipping in solutions of potassium lactate or

lactic acid, and also kept aerobic plate counts (APCs) at the limit of detection for the duration of product storage (12 weeks) (Keeton and others 2002; Nuñez de Gonzalez and others 2004).

For ACS-treated sliced roast beef artificially-inoculated with a beginning population of 2.5 $\log_{10}$ CFU/piece (3 cm x 3 cm x 0.8 cm) of *L. monocytogenes*, an internal study demonstrated that sufficient on-contact lethality levels of the pathogen were achieved and outgrowth of the pathogen was limited to within FSIS standards during the course of storage (8 weeks) (Anonymous 2004a). Roast beef slices were inoculated with a five strain cocktail of *L. monocytogenes*, treated by a 30 second dip in 1000 ml of a 1:2 ACS:water solution, and vacuum packaged for up to 8 weeks of storage at 4 °C. At the outset of the experiment, samples treated with a 30 second dip of ACS exhibited a 2.2 log cycle reduction in *L. monocytogenes* as compared to controls; populations of the pathogen remained at or below the 0.2 $\log_{10}$ CFU/piece level that was achieved upon contact with the solution. In so doing, ACS treatments also proved to be valid for use in achieving Alternative 1 standards. In a separate study, commercially prepared hams were spot-inoculated with a 2 ml aliquot of *L. monocytogenes* to achieve a 7.0 $\log_{10}$ CFU/ham starting population of *L. monocytogenes* on ham surfaces (Luchansky and others 2005). At a 1:2 ACS:water concentration, as little as 2.5 ml of the solution placed into the package was able to produce short-term (24 h) post-lethality reductions of 1.2 $\log_{10}$ CFU/ham when hams were placed into vacuum packaging, submerged in 88 °C water, and stored at 4 °C. Larger volumes (4.5 ml and 6.5 ml) were able to generate even greater reductions of 1.4 and 2.5 $\log_{10}$ CFU/ham, respectively. Over the course of the 60 day shelf life, samples from hams treated with 4 ml of the same concentration of ACS yielded only 0.5 $\log_{10}$ CFU/ham increases from the Day 0 starting point, and higher volumes (6 ml and 8 ml) were able to actually reduce the initial populations by 0.5 and 1.3 $\log_{10}$ CFU/ham by the end of the shelf life period that was tested (Luchansky and others 2005).

Little work has been published detailing the antimicrobial mechanism(s) of ACS. Acidic calcium sulfate contains lactic acid as its primary ingredient, thus it is generally accepted that its mode of action is essentially that of cellular acidification or uncoupling, previously described. Like other organic acids and derivatives, ACS likely behaves according to the model proposed previously. Thus it is believed to inhibit microbial growth by allowing lactic acid to pass through the cytoplasmic membrane in its associated form and cause deregulation of cellular metabolism through energy expenditure to expel the dissociated proton and maintain pH homeostasis (Freese and others 1973; Lamb 2002). As lactic acid is supposedly regenerated by the presence of the sulfuric acid, the lethal effects of ACS may be enhanced by continual regeneration of the protonated acid (Kemp and others 2003).

However, such increases in acid exposure may also lead to much higher chances of development of acid tolerance in those cells that survive ACS treatment; this concern has been a subject of research conducted with HAMOs. Beuchat and Scouten (2004) investigated the effectiveness of lactic acid, acetic acid, and ACS at reducing viability of *E. coli* O157:H7 in ground beef, as well as the ability of exposed microorganisms to develop acid tolerance. Although no significant differences in acid tolerance were observed from one acidulant to another, ACS seemed to induce some acid tolerance. This conclusion was derived from the observation that control cells grown on agar in the absence of ACS showed significantly different (α =0.05) susceptibility when inoculated into ground beef compared to *E. coli* cells which had been previously grown on agar acidified to pH 4.5 using ACS; controls grew to 6.7 $\log_{10}$ CFU/g, whereas ACS-exposed cells grew to 6.8 $\log_{10}$ CFU/g (Beuchat and Scouten 2004). Thus, even though ACS may be initially effective at reducing pathogens, the development of acid tolerance in survivors may prove to be an undesirable side effect of its use. Yet, acidic calcium sulfate does have potential to be used as an individual pathogen intervention for meat

and poultry products or as a component of a combination of interventions. Its efficacy in reducing pathogen populations, the flexible regulations regarding usage levels, and its minimal environmental impacts are all favorable factors. However, research on the chemistry of the solution and the effects of its use should be continued.

Octanoic Acid

Though early twentieth century reports are some of the first to scientifically document the potential for fatty acids and their soaps to act as antibacterial agents (Lamar 1911), it is generally considered that the antimicrobial value of such compounds was recognized well before any modern documentation of their effects (Kabara and Marshall 2005). A great deal of research conducted prior to the 1950s documented that fatty acids can have a wide array of antimicrobial activity (Nieman 1954). At present, it is known that such compounds are effective against both Gram-positive and Gram-negative bacteria alike (Kabara and others 1972), and are active against other types of microorganisms, including enveloped viruses (Thormar and others 1987) and yeasts (Viegas and others 1989). Thus, fatty acids are normal components in antibacterial, antifungal, and insecticidal products, and are widely used within sanitizers and disinfectants (Kabara and Marshall 2005). In addition, since they are naturally occurring and have little to no toxicity associated with their consumption, fatty acids and their derivatives have been consistently considered to be natural antimicrobial food additives (Kabara and Marshall 2005). As such, they are a popular choice among individuals in the food industry when pathogen control measures need to be met with such stipulations in mind. Finally, their ease of production and disposal are favorable aspects, and lead many to regard them as ideal for certain products or processes.

The eight-carbon fully saturated (8:0) fatty acid, International Union of Pure and Applied Chemistry (IUPAC) name octanoic acid and common name caprylic acid, has shown

promise for use as an antimicrobial intervention for foodborne pathogens. Octanoic acid is classified as a medium chain fatty acid (6-12 carbons), and retains moderate water solubility (0.068g/100g water at 20 °C). Because it is found naturally in bovine milk at levels ranging from 1-3% of the total fatty acid composition (Jensen 2002), in the oil of coconuts (Ghosh and Bhattacharyya 1997; Wang and others 1993), and as a byproduct of ethanolic fermentation by yeasts such as *Saccharomyces cerevisiae* (Viegas and others 1989), there is no need to synthetically produce the compound, and it is primarily derived from the saponification and distillation of the coconut oil (Richter and others 1993). Thus, as a direct derivative of foods, it has enjoyed approved status from the FDA for a number of years (FDA 2008c). It is commonly applied to foods in a commercially available solution known as Octa-Gone™, which also contains propylene glycol, citric acid, sodium citrate, polysorbate 20, and polysorbate 80. This product is approved for use in meat and poultry products as an antimicrobial agent, and can be used at levels up to 400 ppm of octanoic acid by weight of the final product (FSIS 2009). Solutions of Octa-Gone™ are commonly applied as surface treatments to RTE meats after processing, and can even meet Alternative 1 standards for post-lethality treatments of such products (Burnett and others 2007).

As noted, the antibacterial activity of octanoic acid and other fatty acids has been well documented over time, and has been shown to be equally effective for both Gram-positive and Gram-negative organisms in both *in vitro* and *in vivo* applications. For instance, with regard to Gram-negative microorganisms, the fatty acid has been found to be effective at low levels for reducing populations of both *E. coli* and *Shigella sonnei* when added to a growth medium (Nakamura and Zangar 1968). In this particular case, not only was the acid effective at killing both organisms after 24 h of exposure to levels as low as 0.3%, but it was also found to be more efficacious than acetic or propionic acid (Nakamura and Zangar 1968). Likewise, in another *in*

vitro study, the inhibitory potential of octanoic acid against *E. coli* grown in brain heart infusion broth was demonstrated, with 2-4% (w/v) of the compound being able to reduce initial populations of 7 $\log_{10}$ CFU/ml to below detection limits after 24 h of incubation at 37 °C (Hismiogullari and others 2008). Moreover, octanoic acid and caproic acid have been shown to inhibit glucose utilization by *E. coli*, and accordingly have a much more pronounced effect against the organism as compared to short chain and long chain fatty acids (Marounek and others 2003). Similar results have also been demonstrated for several *Salmonella* serovars, with glucose utilization being dramatically reduced by exposure of cells to octanoic acid, and median inhibitory concentrations (IC_{50}) of low (0.75 to 1.17 mg/ml) levels of octanoic acid observed in all serovars tested in the experiment (Skřivonová and others 2004).

Comparable results to those obtained *in vitro* have also been observed when the compound is used *in vivo*. *E. coli* O157:H7 cells grown in either acidic or buffered bovine rumen fluid were inhibited through the addition of octanoic acid, as shown in studies aimed at minimizing carriage of the pathogen in the gastrointestinal tract of cattle through its application as a pre-slaughter dietary supplement (Annamalai and others 2004). Exposure to 35 or 50 mM octanoic acid in rumen fluid maintained at a pH of 5.6 reduced *E. coli* by at least 6.5 $\log_{10}$ CFU/ml. In buffered rumen fluid at pH 6.8, the reduction was the same for samples treated with 50 mM octanoic acid, but was nearer to only a 1 log cycle decrease for 35 mM octanoic acid after the same length of time for exposure (Annamalai and others 2004). Similar *in vivo* decreases in *E. coli* O157:H7 have been reported by using 50 mM octanoic acid in whole milk, where the pathogen was decreased by at least 2.7, 5.0, and 8.6 $\log_{10}$ CFU/ml over the course of 24 h of incubation at 4 °C, 8 °C, and 37 °C, respectively (Nair and others 2004). Continuing the incubation to 48 h decreased pathogen populations even further, with reductions of 6.1 and 6.0 $\log_{10}$ CFU/ml observed for the incubation temperatures of 4 °C and 8 °C, respectively.

Octanoic acid is equally effective against Gram-positive microorganisms and has been shown to have great efficacy on mastitis-causing *Streptococci* and *L. monocytogenes*. A study focused on the antimicrobial efficacy of the compound against three species of mastitis-causing *Streptococcus* found that when the organisms were incubated in sterile whole milk in the presence of either 50 mM or 100 mM octanoic acid, reductions of nearly 6.0 $\log_{10}$ CFU/ml were observed after just 6 h of incubation at 39 °C (Nair and others 2005). In addition, even more rapid (within 1 minute) reductions of the organisms by 5.0 $\log_{10}$ CFU/ml were observed when the higher concentrations of 100 mM octanoic acid were used, as opposed to the lower 50 mM concentration. With regard to *L. monocytogenes*, the compound has also been shown to have low MICs, ranging from 0.69 to 3.49 mM depending on the *L. monocytogenes* strain being exposed to the substance under *in vitro* conditions (Kinderlerer and Lund 1992). The findings of this particular study also showed that the degree of disassociation of the acid plays a major role in its efficacy against the pathogen, with less disassociated solutions (at pH 5.0) being much more effective and having lower MIC values than slightly more alkaline solutions (pH 5.5). Similar *in vitro* MIC values have also been recorded in a more recent study, where three strains of *L. monocytogenes* grown for 18 h in tryptic soy broth at 37 °C yielded octanoic acid MIC values of 5 or slightly above 5 mM (Nobmann and others 2009).

In vivo exposure of *L. monocytogenes* to octanoic acid has also yielded evidence of reductions, though not quite as profound as those observed *in vitro*. A previously mentioned study observed the activity of the compound against *L. monocytogenes* incubated in whole milk. Samples treated with 50 mM octanoic acid and incubated at 37 °C for 6 h showed reductions in the pathogen's viability at levels of at least 6.3 $\log_{10}$ CFU/ml (Nair and others 2004). Additionally, by comparison to controls, the 50 mM concentration was able to suppress pathogen growth by 1.2 and 2.9 $\log_{10}$ CFU/ml after incubation at 8 °C for 24 and 48 h, respectively.

However, at even lower incubation temperatures (4 °C) and lower octanoic acid concentrations (25 mM), this inhibitory effect was not as pronounced, and thereby showed that the limitations of its effectiveness on the pathogen are dependent on the conditions of the environment and the concentration at which the compound is used.

Another *in vivo* application of the fatty acid against *L. monocytogenes* investigated its efficacy in whole muscle and comminuted RTE meat and poultry products, and subsequently led to the validation of its effectiveness for use as a post-lethality treatment for the pathogen (Burnett and others 2007). Oven-roasted and oil-browned whole muscle turkey breast, whole muscle cured ham, whole muscle roast beef, and comminuted and formed roast beef were inoculated with a five strain cocktail of the pathogen and exposed to octanoic acid by means of contact with solutions that were placed into the final packaging material. Solutions of 1% octanoic acid were acidified to pH 2.0 with phosphoric acid or to pH 4.0 with citric acid and were applied to final packaging in order to achieve a target application rate of 1.9 ± 0.5 ml per 100 cm^2 of the product. Packages were then heat shrunk using a conventional (2 sec, 93 °C) or a modified (7 sec, 93 °C for all products except oil-browned turkey which was 13 sec) hot water shrinkage process to compare effects on the survival of the pathogen. Products were then stored at 5 °C for 24 h before enumerations were completed. On the oil-browned turkey breast, significant ($P \leq 0.05$) reductions in the pathogen were observed, with the citric acid-acidified solution producing decreases of 1.1 and 1.9 $\log_{10}$ CFU/sample for the conventional and modified shrinkage process, respectively, as opposed to 0.9 and 1.5 $\log_{10}$ CFU/sample for the phosphate-acidified solution. This same trend was also exhibited for all other products where the citric-acid acidified solution coupled with conventional and modified shrink processes produced respective reductions of 2.8 and 3.0 $\log_{10}$ CFU/sample in oven roasted turkey breast, 2.9 and 3.3 $\log_{10}$ CFU/sample in cured ham, 1.7 and 2.5 $\log_{10}$ CFU/sample in whole muscle roast beef, and 1.6

and 2.0 $\log_{10}$ CFU/sample in comminuted roast beef, which were greater than the reductions observed with the usage of the phosphoric acid-acidified solutions. Thus, as a result of these >1 log cycle reductions of *L. monocytogenes* by use of the fatty acid solutions, the compound was able to meet the standards of FSIS Alternative 1 and be approved for use as a post-lethality treatment for such products.

As can be deduced by observing the broad range of organisms affected by exposure to octanoic acid, the mechanisms by which it and other fatty acids are able to inhibit growth are likely nonspecific in nature (Kabara and Marshall 2005). Of primary importance is the principle of uncoupling, whereby the fatty acid behaves as an organic acid, passing through the membrane as an associated and slightly hydrophobic molecule, disassociates in and acidifies the cytoplasm, and causes depletion of energy (Freese and others 1973). Demonstrations of the pH dependency of the acid's effectiveness in several studies (Kinderlerer and Lund 1992; Annamalai and others 2004) strongly suggest that penetration and acidification is a very important factor in its inhibitory scheme. Observations that medium chain fatty acids like octanoic acid are more effective at inhibiting bacterial growth than are shorter chain fatty acids (Nakamura and Zangar 1968) and longer chain fatty acids (Wang and Johnson 1992), may also be indicative of such a mechanism of action. However, additional inhibitory mechanisms of octanoic acid have also been suggested. For example, the membrane destabilization characteristic of surfactants observed by medium and longer chain fatty acids may also produce an inhibitory effect (Greenway and Dyke 1979). In addition, manipulation of the activities of membrane-based enzymes in yeasts as a result of exposure to octanoic acid, suggests that the fatty acid may even be able to cause disruptions of cellular metabolism by causing deviations in the normal function of such cell components (Viegas and Sá-Correia 1991). At present, documentation of resistance development by foodborne bacterial pathogens has yet to occur (Kabara and Marshall 2005).

However, as with all food derived molecules, interactions between the fatty acids and other components of a food matrix can reduce effectiveness *in vivo*, and can cause drastic changes in the expected functionality of such compounds. The importance of *in vivo* validations is crucial to avoid such situations, and to ensure the maximum activity of the fatty acid when it is incorporated in foods as an antimicrobial treatment.

Despite these minor challenges that accompany using octanoic acid in foods, the compound maintains good potential overall. Indeed, the overall effectiveness of octanoic acid in reducing target microorganisms, its identity as a natural antimicrobial, and its bio-friendly nature balance with the challenges of its incorporation into foods and food processes, and continue to make the antimicrobial a favorable prospect for continued and possibly increased future use.

Overcoming Antimicrobial Resistance

Though published incidences of bacterial resistance to food antimicrobials can be found intermittently throughout the literature, the relatively infrequent nature of such occurrences is supportive of the belief that the phenomenon is not a major concern to public health in the present day (Doyle 2006). However, trends of increased resistance to therapeutic antibiotics, increased reliance on antimicrobials and sanitizers for control measures, and evidence that microbial stress responses can produce tolerances to antimicrobials have all generated concerns about the eventual diminution of food antimicrobial potencies (Davidson and Harrison 2002). These trends, coupled with limited knowledge of the specific cellular targets of food antimicrobials have led many to regard resistance development as one of the foremost challenges to the future of food safety (Doyle 2006).

As noted in several of the preceding sections, microorganisms possess a wide array of measures to endure the action of antimicrobials. Modifying cell surface permeability (Ishikawa and others 2002), efflux of biocides (McMurry and others 1980; Levy 1992), resistance-

producing genetic mutations and acquisitions (McBain and Gilbert 2001), plasmid-conferred resistance (Russell 1985; Russell 1997), and tolerances developed from stress responses (Abee and Wouters 1999) are all means employed by bacterial species to decrease their sensitivity to antimicrobic agents. As variations exist in the extent to which these physiological mechanisms are employed by cells (Turner and others 2000; Russell 2003), inconsistencies in responses to antimicrobials can result, and can lead to further problems when determining their effects for challenge studies and Hazard Analysis and Critical Control Points (HACCP) validations (Leyer and Johnson 1993). As a result, the mitigation of resistance development is a desirable goal that food technologists should devote great efforts toward achieving.

Though microorganisms have survival mechanisms at their disposal, several simple strategies can be employed to augment the effect of antimicrobials and minimize tolerance or resistance development (Davidson and Harrison 2002). For instance, avoiding repeated exposure of microorganisms to sub-lethal levels of antimicrobials can decrease the potential for adaptation (Ricke 2003; Koutsoumanis and Sofos 2004). Also, using combinations of antimicrobials with process and environmental controls (such as in the concept of hurdle technology) can present multiple stress points for the microorganism to combat (Leistner 1994; Leistner and Gorris 1995; Leistner 2000). Combinations of two or more antimicrobials with different mechanisms, such as a membrane permeabilizer and an organic acid, can create an ideal situation where the microorganism must struggle to handle simultaneous attacks on multiple cellular targets (Sofos and others 1998; Ricke and others 2005; Kabara and Marshall 2005). All of these strategies are effective, and are widely accepted as potential solutions to resistance. Consequently, innovative applications of existing antimicrobials, rather than discoveries of novel compounds, is believed to be the most reliable means by which tolerance and resistance challenges will be overcome in the future (Kabara and Marshall 2005). Research in the field has shifted away from a focus on

the development of novel antimicrobials toward a greater emphasis on employing hurdle technologies and antimicrobial pairings to combat resistance. These two concepts will be the focus of discussion for the remainder of this section. The distinction must be made that hurdle technologies primarily focus on employing combinations of environmental factors, known as "hurdles" (e.g. temperature, water activity, pH, oxidation/reduction potential, preservatives, and competitive flora), with each other and with antimicrobials, while antimicrobial combinations only entail the paring of two or more antimicrobials for use in minimal processing measures or eventual incorporation into hurdle technology.

Though the practice of combining preservative factors has been carried out for centuries, the reinvention of this principle in the 1970s as the hurdle effect (Leistner 1978) reinvigorated interest in the implementation of such practices in the food industry of today (Leistner and Gould 2005). Upon its reinvention, the hurdle effect was mainly intended to be used as an empirical concept for developing processing condition strategies (Leistner and Gould 2005). However, with the continued incorporation of more scientific principles into the concept, hurdle technologies, which involved the methodical use and control of multiple processing factors, were soon derived (Leistner 2000). The design and application of such technologies has continued to be an area of major interest in research (Leistner and Gorris 1995), and the use of the technical knowledge gained from the hurdle concept has become widespread in many countries around the globe (Leistner and Gould 2005).

The fundamental idea behind the hurdle concept involves the recognition of hurdles used in food processes as the basic procedures to inactivate foodborne pathogens (Leistner and Gorris 1995). Contrary to the idea that intense individual applications of these factors leads to better pathogen mitigation, the hurdle concept promulgates the notion that using multiple hurdles of less intensity is the most efficient manner to achieve food safety and stability (Leistner 1992).

The reasoning behind such strategies stems from the concept that when these hurdles are combined, continued challenges are presented to microorganisms throughout the duration of a food process, resulting in the inability of microorganisms to overcome each hurdle and survive (Leistner 1992). To date, over 60 potential hurdles, which do not include any of the hundreds of antimicrobials, have been identified for food processes (Bøgh-Sørensen 1994). With the great number of hurdles available, custom designs of hurdle combinations can be made to meet the needs of specific food processes.

Most original hurdle technologies employed simpler hurdles such as temperature, water activity, and pH adjustments (Leistner 1994). However, in recent years, combinations of non-thermal processes such as pulsed-electric fields, high hydrostatic pressure, high-intensity ultrasound, ultraviolet light, oscillating magnetic fields, and ionizing radiation have been gaining attention (Ross and others 2003). Also, combinations of hurdles with antimicrobials have increased in popularity. For instance, it has been shown that simple factors such as water activity, temperature, and pH can affect the activity of nisin in *L. monocytogenes* and have the potential for usage in hurdle effect mechanisms in controlling the organism (Boziaris and Nychas 2006). In addition, incorporating 3% lactate/diacetate into frankfurters along with post-process irradiation treatments of 1.8 kGy and 2.6 kGy has been shown to decrease initial *L. monocytogenes* numbers by ~3.0 $\log_{10}$ and ~5.0 $\log_{10}$ CFU/frankfurter, respectively, with continued suppression of the pathogen during the entire 8 weeks of vacuum-packaged storage (Knight and others 2007a). Furthermore, the combination has been shown to have minimal effects on sensory qualities (Knight and others 2007b). Likewise, combining ultraviolet light (1.0 J/cm^2), flash pasteurization (0.75 sec steam at 121 °C), lactate (1.13%), and diacetate (0.07%) in frankfurters has been demonstrated to decrease *Listeria innocua*, a surrogate for *L. monocytogenes*, by 3.2 $\log_{10}$ CFU/g (Sommers and others 2009). Increased levels of ultraviolet

light (4.0 J/cm^2) and flash pasteurization (3 sec steam at 121 °C) produced even greater decreases in numbers of the surrogate at 3.9 $\log_{10}$ CFU/g (Sommers and others 2009). It has also been shown that high pressure processing (400 MPa for 5 min) in concert with nisin (100 IU/ml) and *tert*-butylhydroquinone (TBHQ) (100 ppm) can reduce *L. monocytogenes* numbers by approximately 7.3 $\log_{10}$ CFU/ml (Chung and others 2005).

The concept that combining antimicrobials with complementary activities can produce synergistic effects is not a new idea (Klein and Kimmelman 1947; Eagle and Fleischman 1948). However, it has only been recently that the approach has generated interest in the food sector (Sofos and others 1998; Ricke and others 2005). The consensus is that by using combined food antimicrobial agents a greater spectrum of activity becomes available to combat pathogenic or spoilage microorganisms (Vigil and others 2005). The inhibitory mechanisms of paired antimicrobials, and the synergism that results, rely on the principle of simultaneous attacks at multiple cellular targets and is distinctive from hurdle technology in this way. To combat the effects of resistance, many combinations of the antimicrobials discussed in the preceding sections have been carried out to enhance their activities. For instance, recent studies that have focused on combining nisin with other antimicrobials have found that better effects are achieved by combining the bacteriocin with other antimicrobials as opposed to using it alone against target pathogens. Combinations of nisin with lactate and polyphosphate (Buncic and others 1995), sucrose fatty acid esters (Thomas and others 1998), garlic extract (Singh and others 2001), ethylenediaminetetraacetic acid (Branen and Davidson 2004), and lactoferrin (Murdock and others 2007) have all demonstrated increased ability of nisin to inhibit the growth of *L. monocytogenes* when the pathogen is exposed to multiple antimicrobials. Thus, such interactions may be the key to overcoming the nisin resistance mechanisms of *L. monocytogenes* and maintaining the sensitivity of the organism to the bacteriocin.

Likewise, pairing lactate derivatives with other antimicrobials has been shown to result in enhanced activities of the combination. For example, lactic acid in combination with both monolaurin and nisin has also been shown to have synergistic effects against *L. monocytogenes* (Tokarskyy and Marshall 2008). Based on the results of the study the researchers postulated that by using lactic acid, monolaurin was able to achieve better incorporation into the membrane and allow for enhanced activity of nisin (Tokarskyy and Marshall 2008). A similar pairing of lactate and diacetate with LAE also showed improvements over using each compound individually (Martin and others 2009), which could have also been due to increased incorporation of the LAE into the membrane due to the lactate. Thus, combining lactate with such antimicrobials could be the solution for decreasing acid tolerance development and other factors that are involved with decreased resistance to derivatives of organic acids.

Furthermore, combinations involving EPL have shown signs of synergism. Geornaras and Sofos (2005) investigated the effect of the compound on *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. monocytogenes* alone and in combination with either lactate, diacetate, lactic acid, or acetic acid in broth at either 4 °C or 24 °C. At 24 °C, EPL at 0.02%, 0.05%, and 0.02% alone was able to decrease numbers of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* from 2.0, 2.2, and 2.0 $\log_{10}$ CFU/ml, respectively, to below detection limits (1.3 $\log_{10}$ CFU/ml). Combinations of EPL (0.02% and 0.03%) with diacetate (0.25%) and EPL (0.01% and 0.02%) with acetic acid (0.1 %) produced more effective inhibition ($p < 0.05$) than using the compound alone for *E. coli* and *S.* Typhimurium. Najjar and others (2007) investigated the *in vitro* activity of the polymer on *L. monocytogenes* and *B. cereus*, and noted that the compound interacted with nisin A to produce synergistic inhibition (FIC value of 0.56) for *B. cereus* and additive inhibition (FIC value for 0.86) for *L. monocytogenes*. Their observations

suggested that using two membrane-active agents destabilize the membrane even further than individual usages and lead to better inhibition and a mitigation of resistance.

It seems worthwhile for researchers to investigate antimicrobial combinations further and to better understand their mechanisms so that they can be made more efficient. By accomplishing this, food processors will not only have more effective means of controlling pathogens, but will also be able to minimize the concerns of consumers about maintaining the integrity of antimicrobials that are currently available. As a result, research in the area should continue into the near future, and should aim at achieving these particular goals.

Antimicrobial Susceptibility Tests

The ultimate goal of assaying the activity of food antimicrobials is to have them work under *in vivo* conditions within a food system. However, the starting point for understanding the expected effects of a food antimicrobial usually entails some sort of preliminary *in vitro* susceptibility test to determine its limitations and optimal conditions for use. From Koch's tests of the mercuric chloride susceptibility of *Bacillus anthracis* (Koch 1881), to the complex automated tests of today, antimicrobial susceptibility tests have constantly evolved over the past century to accommodate new sets of experimental designs and novel analytes. Fleming's discovery of penicillin in the mid 1920s (Fleming 1929) and the desire to understand its functionality were instigating forces behind development of susceptibility testing procedures in the decades to follow (Reddish 1929; Abraham and others 1941; Vincent and Vincent 1944; Mohs 1945; Morley 1945; Kolmer 1947; Bondi and others 1947; Bauer and others 1966). Likewise, early experiments in testing the efficacy of food-based antimicrobials yielded different techniques that could be specialized with regard to the properties of components from food systems (Walton and others 1936). Even with the diverse array of technologically assisted procedures that are used today, many of these procedures that were developed during the early

years of experimentation are still fundamental components of the methods that are currently used (Davidson and Parish 1989).

The most fundamental classifications of *in vitro* antimicrobial susceptibility assays are the diffusion methods and the dilution methods (Vigil and others 2005). Of the two, diffusion methods have likely been the most frequently used throughout the past century to qualitatively test the efficacy of a number of different antimicrobials (Vigil and others 2005). Dilution methods, while not as widely used, are more often employed by researchers and clinical personnel because of their quantitative nature (Murray and Jorgensen 1981). However, each set of methods has advantages and disadvantages to its use, and much of the consideration for selecting a particular method should take into account the nature of the analyte and the most applicable way for it to be evaluated. Other gradient-based testing procedures are available, such as the wedge system (Szybalski and Bryson 1952), spiral plating (Hill 1991), and various automated methods (Piddock 1990), but these tend to be used for more specialized purposes. Within the two main classes of methods are sub-categories of test procedures that are based on the way results are collected from the assay. Endpoint analyses involve the exposure of a microorganism to a particular compound for a specified period of time (Davidson and Parish 1989). After time has elapsed, results are collected and are indicative of the inhibitory potential of the substance for that particular length of time. In contrast, descriptive analyses often involve periodic sampling, and involve creation of multiple data points over time (Vigil and others 2005). Such analyses can be useful for purposes of constructing growth or inhibition curves to visualize a substance's effect over time. Thus, as with the method type, each data collection method has advantages and disadvantages that predispose it for use under certain conditions.

As mentioned, the most frequently used form of susceptibility testing is the diffusion method, and within this method, agar diffusion has been the type of test that has predominated.

The basic principle of the traditional agar diffusion test is the creation of a gradient of antimicrobial concentrations over the radius extending out from a central application site (Bauer and others 1966). When performed on an agar plate seeded with the test organism, the application of the antimicrobial will create a zone of inhibition extending out from the center, which can be measured (Bauer and others 1966). At the outset of its use, wells punched into agar were used as the application sites for the analyte to be examined (Reddish 1929). However, incorporation of compounds into paper disks, which were then laid on the surface of the agar to allow for diffusion, soon became the customary method (Vincent and Vincent 1944; Morley 1945; Kolmer 1947). The standard 6.5 mm filter paper disk that is still used for conducting disk assays today was introduced in a study by Bondi and others (1947), and many set of standards for the test were established by Bauer and others (1966). Presently, most of the stipulations regarding the test are governed by the Clinical and Laboratory Standards Institute, which periodically publishes a guide on its methodology (CLSI 2009b). Such stipulations state that a non-selective medium must be used, preferably one like Müeller-Hinton Agar, and that the test microorganism should be seeded onto the plate at a rate of 6.0 $\log_{10}$ CFU/ml. Disks impregnated with different concentrations of the test antimicrobial are then placed onto the surface of the agar, and plates are incubated for 16 to 24 h. Zones of inhibition form around the disks if the antimicrobial is active, and zone diameters are measured to provide an indication of the activity.

Though the agar diffusion test is very easy to conduct and is very easy to obtain results from, its main limitation is that its results are only subjective, and can only give one a generic classification of the antimicrobial's effectiveness (susceptible, intermediate, resistant) (Piddock 1990). Diffusion tests can give semi-quantitative MIC values, but dilution methods are considered to be more quantitative (Vigil and others 2005). Another limitation is that because results must be obtained after growth for a specified period of time, diffusion methods are solely

limited to endpoint analyses. In addition, because diffusion methods use water as their medium to diffuse the antimicrobial, diffusion throughout the agar matrix can be hindered for antimicrobials that have hydrophobic tendencies (Chao and others 2008). Furthermore, the requirement for growth of organisms on the surface of the agar in the presence of oxygen also limits the assay to aerobic microorganisms, as oxygen toxicity can lead to misperceptions about the activity of the analyte against anaerobic species (Johnson and others 1995).

Dilution-based susceptibility tests include both agar dilution methods and broth dilution methods, and have been in use since the time of Fleming's discovery (Piddock 1990). Though most standards for dilution procedures were developed at the same time as those for the disk diffusion assay (Fleming 1942; Rammelkamp and Maxon 1942; Schmidt and Sesler 1943; Buggs and others 1946), dilution methods focus on exposure of a test microorganism to one individual concentration of an antimicrobial, rather than a gradient of several concentrations (CLSI 2009a). Thus, in contrast to diffusion methods, antimicrobial dilution methods are more appropriate for determining quantitative data. In serially diluting an antimicrobial into inoculated agar or broth, one can determine a distinct MIC value; the lowest concentration at which the agent can kill a microorganism or inhibit its growth (Barry 1976). Such specific values cannot be obtained from diffusion tests because of the gradient effect, and thus dilution methods are at an advantage in this respect. In addition, dilution tests tend to be much more accommodating for the maintenance of special growth conditions, as they allow growth of microaerophiles, anaerobes, and slow growing microbes (Murray and Jorgensen 1981). Furthermore, use of paper disks as vehicles for antimicrobials in diffusion tests requires additional care to assure that components of disks do not hinder the activity of antimicrobials nor create antimicrobial effects themselves (WHO 1982). Thus, dilution tests do possess distinctive advantages over diffusion tests, and additional specific benefits exist between choosing either a broth dilution or an agar dilution method.

Conducting agar dilution tests involves dissolving a specific amount of antimicrobial in molten agar to achieve a uniform concentration of the analyte over the entire volume of the growth medium (Barry 1976). In setting up the assay, each agar plate contains an individual concentration, and serial twofold dilutions are made over the series of plates (CLSI 2009a). Cultures are diluted to 7.0 $\log_{10}$ CFU/ml and are spot-inoculated onto the plate surfaces in 1 to 2 µl aliquots to produce starting inocula of approximately 4.0 $\log_{10}$ CFU/ml (CLSI 2009a). Spot inoculation allows for multiple organisms to be simultaneously tested on the same plate, and is an advantage of the method (Barry 1976). MIC values are determined by observing the plate with the lowest concentration of the antimicrobial that demonstrates absence of growth (single colonies or faint hazes do not constitute growth) (Barry 1976). Though determining growth in this fashion is fairly subjective and thus puts the method at a disadvantage, contamination is easily detected and opaque materials can be supplemented in the agar (Barry 1976).

Broth dilution techniques involve the same concept of the agar dilution method with regard to serial dilution, but use liquid media rather than agar (Barry 1976). Large volumes of 1 to 10 ml of medium can be used, but most current methodologies are adapted for microtitration with volumes of 50 to 200 µl being common (Vigil and others 2005). Inocula are slightly higher than agar dilution tests with levels of approximately 5.0 $\log_{10}$ CFU/ml being common (Thrupp 1986). Samples can also be incubated under specific conditions which allow accommodations to be made for slow growing microorganisms and for anaerobes. MIC determinations are commonly done by observing turbidity of the medium due to cell proliferation (Barry 1976). Turbidity can be observed subjectively or monitored by use of a spectrophotometer to determine absorbance at a given wavelength; however the latter affords greater objectivity to observations. By calculating the change in medium turbidity from a beginning to a terminal spectrophotometer reading in an endpoint-style analysis, one can set a quantifiable difference in absorption that

defines growth (Branen and Davidson 2004; Skandamis and others 2007). For tubes or wells that display absorption changes less than this specified value, corresponding concentrations of the antimicrobial are considered inhibitory, and the lowest such concentration is considered the MIC. Likewise, one can also record the absorbance of a solution periodically over the course of incubation, correlate it with growth of the organism using a function, and produce growth curves for descriptive-type analyses (Najjar and others 2007; Tokarskyy and Marshall 2008). In addition, broth dilutions also allow for determination of minimum bactericidal concentrations (MBC), which involves the spread-plating of 10 to 100 µl aliquots of test solutions displaying inhibition at the endpoint (Vigil and others 2005). Plates which show a ≥ 99.9% decrease from the starting population for a particular organism are deemed bactericidal (Barry 1976).

Though most of these methods described are intended for evaluation of a single antimicrobial, recent trends toward using combined agents in food systems have generated a demand to assay the simultaneous effect of two food antimicrobials on an organism (Vigil and others 2005). When two antimicrobials are paired, outcomes of synergism, additivism (also referred to as indifference), or antagonism can result from their combination (Davidson and Parish 1989). Barry (1976) gives an excellent definition of synergism as an "effect observed [when] a combination is greater than the sum of the effects observed with the two drugs independently." Likewise, he defines additivism as "a combined effect [that] is equal to the sum of the effects observed when the two drugs are tested separately or equal to that of the most active drug in the combination." He also states that antagonistic behaviors between antimicrobials are characterized by a combination that "is less effective than the most active drug in the combination." The classification of an antimicrobial combination into one of these three categories is key to communicating to others how the compounds can be expected to interact, and is crucial for preventing problematic applications.

All forms of antimicrobial susceptibility tests can be used to test antimicrobials in combination, but certain modifications must be made. For instance, agar diffusion methods can be used to test two antimicrobials at once by using two strips of filter paper impregnated with antimicrobial to form an L-shape on the surface of the agar (Garrod and Waterworth 1962). Based on the shape of the zone of inhibition, one can interpret the general type of interaction observed (Barry 1976). However, as in single antimicrobial testing, this type of test lacks quantitative results. Thus, it is actually more common to modify more quantitative broth dilution techniques to evaluate the interactions.

Broth dilution tests of antimicrobial combinations most often involve a method known as the checkerboard assay to obtain results (Vigil and others 2005). Using this concept, a series of tubes, or wells on a microtiter plate, are used to create criss-cross combinations of stepwise diluted antimicrobials working down from the highest concentration of compound A in rows and working left to right from the highest concentration of compound B in columns (Figure 3). Clinical tests which use this principle customarily employ twofold dilutions when moving down rows and across columns, but because of differences in function of food antimicrobials, modifications to this standard have been done in the past and are a source of non-uniformity between studies (Davidson and Parish 1989). Once a checkerboard assay has been arranged with antimicrobials, and combinational minimum inhibitory combinations have been deciphered using the prescribed means, interpretation of the type of interaction observed can be accomplished mathematically. To do this, combination concentrations must be converted from MIC values to fractional inhibitory concentration (FIC) values for each agent in the combination (Parish and Carroll 1988). This is achieved by dividing the combinational MIC value for a compound by the

Compound A → Compound B ↓	1.000	0.500	0.250	0.125	0.063	0.000
1.000	1.000/1.000	0.500/1.000	0.250/1.000	0.125/1.000	0.063/1.000	0.000/1.000
0.500	1.000/0.500	0.500/0.500	0.250/0.500	0.125/0.500	0.063/0.500	0.000/0.500
0.250	1.000/0.250	0.500/0.250	0.250/0.250	0.125/0.250	0.063/0.250	0.000/0.250
0.125	1.000/0.125	0.500/0.125	0.250/0.125	0.125/0.125	0.063/0.125	0.000/0.125
0.063	1.000/0.063	0.500/0.063	0.250/0.063	0.125/0.063	0.063/0.063	0.000/0.063
0.000	1.000/0.000	0.500/0.000	0.250/0.000	0.125/0.000	0.063/0.000	0.000/0.000

Figure 3-Checkerboard assay schematic.
Combinations of serial dilutions are created by pairing corresponding proportions of the MIC for each antimicrobial. Adapted from Vigil and others (2005).

MIC of the compound when it is used by itself (Figure 4) (Parish and Carroll 1988). The resulting ratio is the desired FIC value.

Once FICs are obtained for each antimicrobial, addition of the two FIC values provides one with a fractional inhibitory concentration index (FIC_I) for the combination, which can then be used to interpret the type of interaction (Figure 4) (Barry 1976). Though this is a quantitative number, interpretation of FIC indices has been fairly ambiguous with different researchers suggesting different ranges of values for classifying the respective interactions (Squires and Cleeland 1985; Branen and Davidson 2004; Kumar and others 2004). However, conservative use of FIC indices continues to claim that an FIC index <1 is indicative of synergism, an FIC index ≈ 1 is characteristic of additivism (indifference), and that an FIC index >1 is indicative of antagonism (Branen and Davidson 2004).

FIC values can be even further analyzed by plotting FIC values for one antimicrobial versus the corresponding FIC for the other antimicrobial in a combination to form an FIC

$$\text{Fractional Inhibitory Concentration (FIC)} = \frac{\text{Antimicrobial A Combination Inhibitory Concentration}}{\text{Antimicrobial A Single Compound MIC}}$$

$$\text{Fractional Inhibitory Concentration Index } (FIC_I) = \text{FIC Antimicrobial A} + \text{FIC Antimicrobial B}$$

Figure 4-Equations used to calculate fractional inhibitory concentrations and fractional inhibitory concentration indices.
Based on descriptions by Barry (1976).

isobologram (Parish and Carroll 1988). Curvature of lines between the points on an FIC isobologram is then used to interpret interactions (Parish and Carroll 1988). Isobolograms whose points form a straight line with a -1 slope, are classified as additive. Those whose points form a curved line which trends toward the origin are classified as synergistic. Consequently, isobolograms with points that form a curve which trends away from the origin are classified as antagonistic. Figure 5 shows several isobolograms and their subsequent classifications.

Though such methods and interpretations have become standard for determining the interactions between antimicrobic agents, several have expressed concerns about their validity (Lambert and Lambert 2003; Odds 2003). Indeed, some have claimed that current methods have no tolerance for varied dose responses of antimicrobials in a combination and that identical dose response assumptions may falsely identify a synergistic interaction, when one, in fact, does not exist (Lambert and Lambert 2003). Others have also communicated concerns with the reproducibility of the test method, stating that current numbers of replicates are inadequate, and that agreement between replications should be considerably high before an interpretation is accepted (Rand and others 1993). However, the truth is that without advanced software to

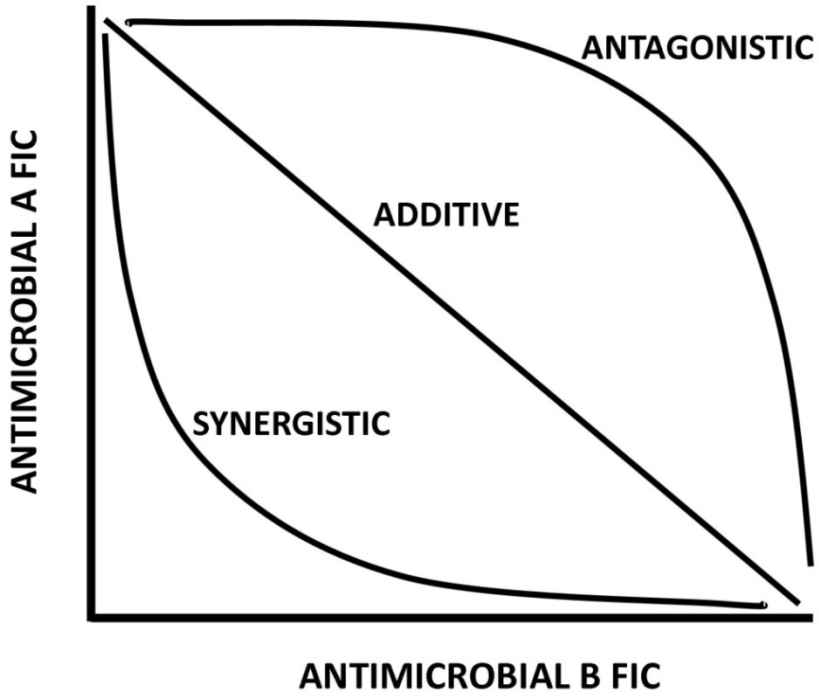

Figure 5-Interpretation of antimicrobial interaction from isobologram analysis.
Adapted from Parish and Carroll (1988).

produce models using the data that are collected, these types of tests are currently limited to these procedures, as these are the most current and uniform approved methods available.

Though *in vitro* methods to assess antimicrobial interactions have been in place for several decades, research that has used them to classify interactions between food antimicrobials is lacking. Results from such research can provide insight into more efficient use of antimicrobials that have compatible activities which produce enhanced inhibition of foodborne pathogens such as *L. monocytogenes*. Although some combinations of the antimicrobials mentioned in this review have been tested *in vitro* against *L. monocytogenes*, the aim of this research was to apply these *in vitro* methods to classify interactions of new and different combinations between them to better understand their inhibition of the pathogen.

CHAPTER IV

IN VITRO INHIBITION OF *LISTERIA MONOCYTOGENES* BY FOOD ANTIMICROBIAL COMBINATIONS TESTED AT PH 7.3

Introduction

At present, the use of food antimicrobials in processed meat and poultry products is a key component to control of *L. monocytogenes* (FSIS 2003a; Lado and Yousef 2007). Bacteriocins such as nisin, surfactants such as lauric arginate ester (LAE) and monolaurin, polycationic molecules such as ε-Poly-L-Lysine (EPL) and chitosan, and organic acids such as lactic acid, acetic acid, and octanoic acid are widely used as interventions in food manufacturing processes. Yet, even though a good understanding of the levels of antimicrobials and conditions needed to produce inhibition or death of the pathogen exists (Lado and Yousef 2007), several challenges are associated with their use. For instance, adaptations can develop which allow cells to counteract the activity of antimicrobials through resistance mechanisms (Davidson and Harrison 2002; Doyle 2006). Likewise, the development of undesirable sensory effects, as well as impediments associated with cost of incorporating new processes, are additional factors to be considered (Davidson and Branen 2005). Also, because knowledge on specific antimicrobial mechanisms and interactions is lacking (Davidson and Harrison 2002), food processors are generally confined to the use of a small scope of validated treatments with limited efficiency. However, the use of antimicrobial pairings that involve the interaction of compounds that have complimentary or enhancing activities on one another can produce a multi-pronged attack on microbial pathogens and make use of antimicrobials more effective and efficient. Thus, the investigation of antimicrobial combinations which can offset such challenges and produce more efficient control of *L. monocytogenes* in food systems, are readily welcomed.

The primary objective of this study was to investigate the inhibition of four *L. monocytogenes* strains that resulted from exposure to nisin (NIS), Sodium L-Lactate (SL), ε-Poly-L-Lysine (EPL), lauric arginate ester (LAE), and acidic calcium sulfate (ACS) *in vitro* at pH 7.3. A growth medium pH of 7.3 was used in order to minimize acid stress on the organism so that inhibition was primarily a function of antimicrobial stress. Antimicrobial susceptibility was tested using a broth dilution microassay to determine the minimum inhibitory concentration of each individual antimicrobial. Bactericidal responses of each antimicrobial were also assessed after completion of the incubation period to determine minimum bactericidal concentrations.

A second objective was to assess the efficacy of antimicrobial combinations for the *in vitro* inhibition of *L. monocytogenes* growth at pH 7.3. *L. monocytogenes* strains were exposed to nisin combined with ACS, nisin combined with LAE, EPL combined with ACS, and sodium lactate combined with ACS through use of a checkerboard broth dilution microassay. Inhibitory activity was assessed and optimal inhibitory combinations of each antimicrobial pairing were determined. As with single antimicrobial testing, bactericidal responses were also determined for combinations of antimicrobials that produced growth inhibition.

A final objective of the study was to determine the types of interaction types that resulted from each antimicrobial pairing. Using optimal inhibitory combinations derived from the combinations assay and MICs, fractional inhibitory concentrations were calculated. From these values, fractional inhibitory concentration index values (FIC_I) and isobolograms were constructed and interactions were classified as synergistic, additive, or antagonistic in nature. It was determined whether interactions resulted in enhanced activity, no change in activity, or reduced activity by using the antimicrobials in a pair as opposed to using them separately. Using this information and suggested modes of antimicrobial action, postulations pertaining to possible reasons for increased or decreased activity were also made for each pairing.

Materials and Methods

Bacterial Culture Preparation and Maintenance

Listeria monocytogenes Scott A (clinical isolate), 310 (goat cheese-associated outbreak isolate), National Animal Disease Center (NADC) 2783 and NADC 2045 were obtained from the Center for Food Safety culture collection (Department of Animal Science, Texas A&M University, College Station, TX). All strains were biochemically confirmed as *Listeria monocytogenes* using the API® *Listeria* identification system (bioMérieux, Inc., Hazelwood, MO) according to manufacturer instructions. Thereafter, cultures were maintained on Tryptic Soy Agar (TSA; Becton Dickinson and Co., Sparks, MD) slants at 5 °C. In order to prevent potential development of antimicrobial resistance via repeated sub-culturing, working cultures were obtained by transferring a loopfull of culture from TSA slants to 10 ml of Fraser Broth (Becton Dickinson and Co.) and incubating aerobically for 24 h without agitation at 35 °C. After confirming a *Listeria* species correct phenotype (esculin hydrolysis) via medium blackening (Fraser and Sperber 1988), a loopfull of culture was transferred to 10 ml Tryptose Phosphate Broth (TPB; Becton Dickinson and Co.), incubated aerobically for an additional 24 h at 35 °C without agitation. Cultures from Fraser Broth were streaked for isolation on Tryptose Phosphate Agar (TPA; Becton Dickinson and Co.) plates at the time of TPB inoculation and incubated at 35 °C for 48 h to confirm culture purity.

Inoculum Preparation

Overnight (24 h) cultures of each *L. monocytogenes* strain in TPB were serially diluted in 9.9 ml volumes of double-strength TPB (2x TPB) to achieve a final inoculum concentration of approximately 5.0 $\log_{10}$ CFU/ml. Inocula were enumerated by diluting to approximately 3.0 $\log_{10}$ CFU/ml in sterile 0.1% Peptone (Becton Dickinson and Co.), and then spread-plating 100 µl on both TPA and PALCAM Agar (Becton Dickinson and Co.). Plates were aerobically incubated at

35 °C for 48 h prior to enumeration. Use of PALCAM Agar served to ensure that cells from the inoculum displayed a correct phenotype for *Listeria* species (medium blackening via esculin hydrolysis) (Van Netten and others 1989), while simultaneous enumeration on TPA plates confirmed inoculum purity.

Test Antimicrobial Preparation

A 250.0 µg/g stock solution of active nisin was prepared by dissolving a mass of 0.1000 g powdered nisin fermentate (NIS; Sigma-Aldrich, St. Louis, MO, 2.5% w/w nisin) in 10.0 ml sterile 0.02 M hydrochloric acid (HCl; Thermo-Fisher Scientific, Waltham, MA) according to previously reported methods (Rogers and Montville 1991). The solution was boiled in water for 4 minutes to aid in dissolution of nisin (Wolf and Gibbons 1996; Taylor and others 2008). Stock solutions of 2500.0 µg/g and 5000.0 µg/g active ε-Poly-L-Lysine were prepared for single antimicrobial testing and combinations antimicrobial testing by dissolving 0.0500 g and 0.1000 g Save-ory® PL-25 (EPL; Chisso America, Inc., Rye, NY; 50% w/w EPL) in 10.0 ml sterile distilled water (DI H_2O), respectively. Safe$_2$O® RTE:01 (ACS; Mionix Corp., Rocklin, CA; saturated Acidic Calcium Sulfate solution), CytoGuard LA (LAE; A&B Ingredients, Fairfield, NJ; 10% w/w Lauric Arginate Ester), and Purasal® S (SL; Purac America, Inc., Lincolnshire, IL; 60% w/w Sodium L-Lactate) were all dissolved in sterile DI H_2O to obtain working solutions. After preparation, 100 µl of stock solutions were spread-plated on TPA and incubated at 35 °C for 48 h to ensure that solutions were free of microbial contamination. Antimicrobials, active agent, concentration of initial stock solution, and manufacturers are presented in Table 2.

Single Antimicrobial Inhibition Assay

A broth dilution microassay (Barry 1976) was used to determine the strain-specific minimum inhibitory concentration for each antimicrobial. Strains were exposed to NIS at 6.25, 3.13, 1.56, 0.78, and 0.39 µg/g active nisin. Working nisin solutions were prepared by dissolving

Table 2-Experimental antimicrobials, active agents, and manufacturer for tests at pH 7.3.

Antimicrobial	Active Agent	Stock Concentration	Manufacturer	Headquarters
Nisin	Nisin	2.50% w/w	Sigma-Aldrich, Inc.	St. Louis, MO
Save-ory®	ε-Poly-L-Lysine	50% w/w	Chisso America, Inc.	Rye, NY
$Safe_2O$® RTE:01	Acidic Calcium Sulfate	100% v/v	Mionix Corp.	Rocklin, CA
CytoGuard LA	Lauric Arginate Ester	10% w/w	A&B Ingredients, Inc.	Fairfield, NJ
Purasal® S	Sodium L-Lactate	60% w/w	Purac America	Lincolnshire, IL

500 µl of the 250 µg/g stock solution in 10.0 ml of DI H_2O to generate the stock solution needed to deliver the initial 6.25 µg/g concentration, with dilutions thereafter consisting of 2500 µl of the previous solution dissolved into 5.0 ml of DI H_2O. Acidic calcium sulfate was tested at 50.00, 25.00, 12.50, 6.25, and 3.13 ml/L. Working solutions of ACS were generated by dissolving 1000 µl $Safe_2O$® RTE:01 in 10.0 ml DI H_2O to obtain the solution required to deliver 50.00 ml/L, with dilutions of 2500 µl of the previous solution dissolved into 5.0 ml DI H_2O afterwards. ε-Poly-L-Lysine was delivered at 50.00, 25.00, 12.50, 6.25, and 3.13 µg/g active. The respective solutions were produced by dissolving 400 µl of the 2500 µg/g solution into 10.0 ml DI H_2O, with serial dilutions of 2500 µl of the previous solution into 5.0 ml DI H_2O following. Sodium L-Lactate activity was assessed at 50.00, 25.00, 12.50, 6.25, and 3.13 mg/g active ingredient. Corresponding solutions were prepared by dissolving 1289 µl of Purasal® S into 10.0 ml DI H_2O, with 2474, 2488, 2493, and 2497 µl of the corresponding previous solution

dissolved into 5.0 ml DI H_2O thereafter. LAE activity was assessed at 50.00, 25.00, 12.50, 6.25, and 3.13 µg/g. Working solutions of LAE were prepared by dissolving 965 µl of CytoGuard LA in 10.0 ml DI H_2O to obtain a primary working stock, 1000 µl of primary stock dissolved in 10.0 ml DI H_2O to generate a secondary working stock, and 1000 µl of the secondary working stock dissolved in 10.0 ml DI H_2O to obtain the solution needed to deliver 50.00 µg/g. Thereafter, solutions were obtained by dissolving 2499, 2500, 2500, and 2499 µl of the previous solution in 5.0 ml of DI H_2O to deliver the correct amounts for testing. As noted, concentrations of working solutions were devised so as to deliver the correct concentration of test antimicrobial to the test wells once all additions were made. Sterile barrier tips and serological pipettes were always used in making dilutions so as to minimize potential for cross-contamination.

Microtiter plates (96 wells; 300 µl capacity) (Microtest™, Becton Dickinson and Co.) were used for all broth dilution assays. Equivalent volumes of test antimicrobial solution (125 µl) and serially diluted *L. monocytogenes* inoculum in 2x TPB (125 µl) were aseptically loaded into test wells. Cells were diluted and inoculated in 2x TPB to allow the correct content of nutrients in the final sample well upon delivery. Sterile barrier tips were used to load solutions into the wells so as to minimize potential for cross-contamination. Figure 6 shows the individual antimicrobial testing setup for the microtiter plate. Appropriate negative controls containing only antimicrobial solutions and sterile 2x TPB were built in for baseline adjustment of experimental wells. Positive controls containing sterile water and inoculum in 2x TPB were also completed. Immediately following plate preparation, optical density of the test wells at 630 nm (OD630) was measured at 0 h using an EL800 absorbance microplate reader (BioTek® Instruments, Inc., Winooski, VT). Optical density at 630 nm was used in order to minimize absorption of light by the growth medium and optimize light scattering by cells (Burton and Kaguni 1997). Microplates were incubated aerobically for 24 h at 35 °C before obtaining a 24 h OD630 reading.

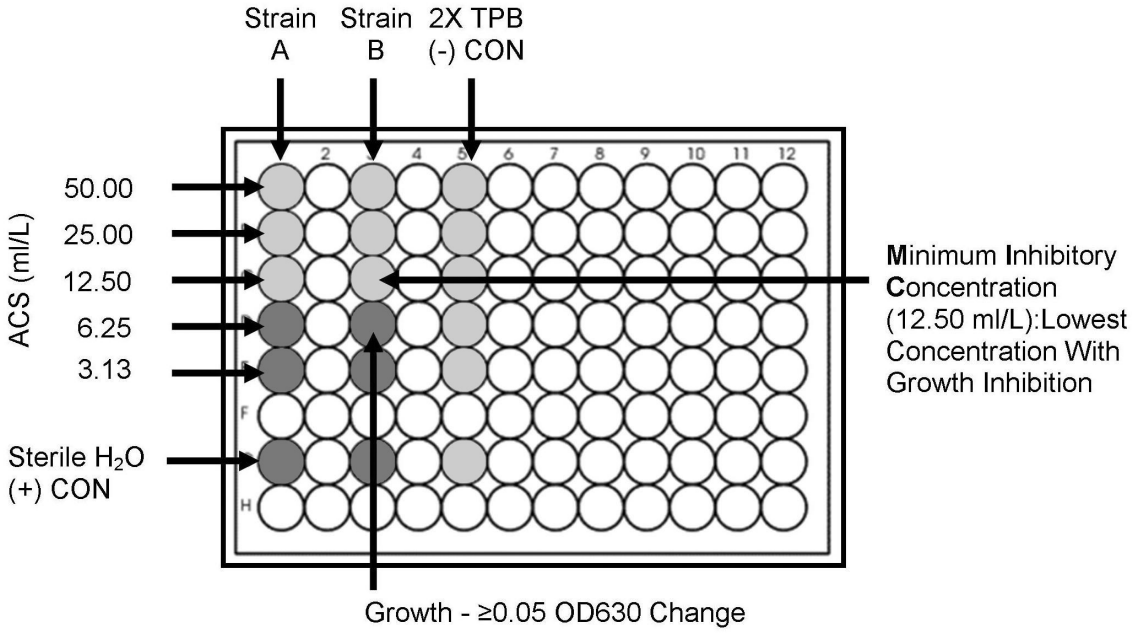

Figure 6-Broth dilution microplate setup and interpretation for tests at pH 7.3.

Antimicrobial inhibition testing of each antimicrobial at all concentrations was replicated at least twice for each strain. Concentrations of antimicrobial that produced a <0.05 change (Δ) in OD630 from 0 h to 24 h following appropriate baseline adjustment over duplicate replication were classified as inhibitory (Branen and Davidson 2004). The MIC for each antimicrobial was defined as the lowest concentration of antimicrobial that produced <0.05 ΔOD630 after 24 h incubation following subtraction of the baseline OD630 values of the negative controls from the treatment well OD630 values (Figure 6).

Antimicrobial Combinations Susceptibility Testing

The response of *L. monocytogenes* to four combinations of antimicrobials: NIS combined with ACS (NIS+ACS), EPL combined with ACS (EPL+ACS), NIS combined with LAE (NIS+LAE) and SL combined with ACS (SL+ACS) was evaluated using a checkerboard broth dilution microassay. Minimum inhibitory concentrations previously determined from single antimicrobial susceptibility tests were used as starting concentrations for the combination

assays. Serial (1:2) dilutions of antimicrobials were made from these starting concentrations so as to produce a 5X5 checkerboard of combinations between the two antimicrobial compounds. Concentrations of working solutions were again devised so as to provide the correct concentration of test antimicrobial when finally delivered to the test wells. Sterile barrier tips and sterile pipettes were used in making dilutions and in plate loading so as to minimize potential for cross-contamination.

For the NIS+ACS combination, *L. monocytogenes* strains were exposed to 6.25, 3.13, 1.56, 0.78, and 0.39 µg/g active NIS in combination with 12.50, 6.25, 3.13, 1.56, and 0.78 ml/L ACS. The first working solution for nisin was created by dissolving 833 µl of the 250 µg/g nisin stock solution in 10.0 ml DI H_2O, with dilutions of 2500 µl of the previous solution into 5.0 ml DI H_2O for the remainder of the solutions. The first solution of ACS was created by dissolving 625 µl of Safe$_2$O® RTE:01 in 10.0 ml DI H_2O, with dilutions of 2500 µl of the previous solution into 5.0 ml of DI H_2O for all solutions thereafter. For the EPL+ACS combination, all strains were exposed to 12.50, 6.25, 3.13, 1.56, and 0.78 µg/g active EPL in combination with 12.50, 6.25, 3.13, 1.56, and 0.78 ml/L ACS. A primary working stock solution of EPL at 500 µg/g was prepared by dissolving 1000 µl of the 5000 µg/g EPL stock solution in 10.0 ml DI H_2O; 833 µl of this solution was transferred to 10.0 ml to produce the first solution of EPL, with volumes of 2500 µl of previous solutions being diluted in 5.0 ml of DI H_2O thereafter. Solutions of ACS were produced in the same way as for the NIS+ACS combination. For the SL+ACS combination, strains were exposed to 50.00, 25.00, 12.50, 6.25, and 3.13 mg/g of active SL in combination with 12.50, 6.25, 3.13, 1.56, and 0.78 ml/L ACS. Working solutions of SL were produced by dissolving 2150 µl of Purasal® S in 10.0 ml DI H_2O, followed by dilutions of 2474, 2488, 2493, and 2497 µl of previous solutions into 5.0 ml DI H_2O. ACS solutions were prepared as for previous combinations. For the NIS+LAE combination, all strains were exposed to 6.25,

3.13, 1.56, 0.78, and 0.39 µg/g active NIS in combination with 12.50, 6.25, 3.13, 1.56, and 0.78 µg/g active LAE. A primary working stock of LAE was produced by dissolving 1000 µl CytoGuard LA in 10.0 ml DI H_2O; 974 µl of this primary working stock was then dissolved in 10.0 ml DI H_2O to create a secondary working stock. The first usable solution of LAE was produced by diluting 619 µl of this secondary working stock in 10.0 ml of DI H_2O, with serial dilutions of 2499, 2500, 2500, and 2500 µl of previous solutions in 5.0 ml of DI H_2O. NIS stocks were prepared the same as for NIS+ACS.

Ninety-six well microtiter plates were also used for carrying out the checkerboard assay (Figure 7). Seventy-five µl of antimicrobial A solution (EPL in EPL+ACS, NIS in NIS+ACS and NIS+LAE, and SL in SL+ACS) was added to the test wells in columns along with 50 µl of antimicrobial B solution (ACS in EPL+ACS, NIS+ACS, and SL+ACS, and LAE in NIS+LAE) added in rows. *L. monocytogenes* inoculum in 2x TPB (125 µl) was then added to the 125 µl of combined antimicrobial solutions. Sterile barrier tips were used to load solutions into the wells so as to minimize potential for cross-contamination. Wells containing only antimicrobial-containing solutions and sterile 2x TPB were built in as negative controls for baseline correction adjustment of optical density values. Wells containing only sterile water and inocula in double-strength TPB were again built in as positive controls. Two 96 well plates were used to contain all samples in order that positive and negative controls for all wells would be located on the same plate as test wells. Figure 7 is a schematic of the design and layout of the first plate of each combination used for the checkerboard assay.

After loading plates, OD630 was measured at 0 h and 24 h using the same protocol from the single antimicrobial susceptibility tests (described above). All susceptibility tests for each combination of test antimicrobials were repeated at least twice for each strain. Wells that

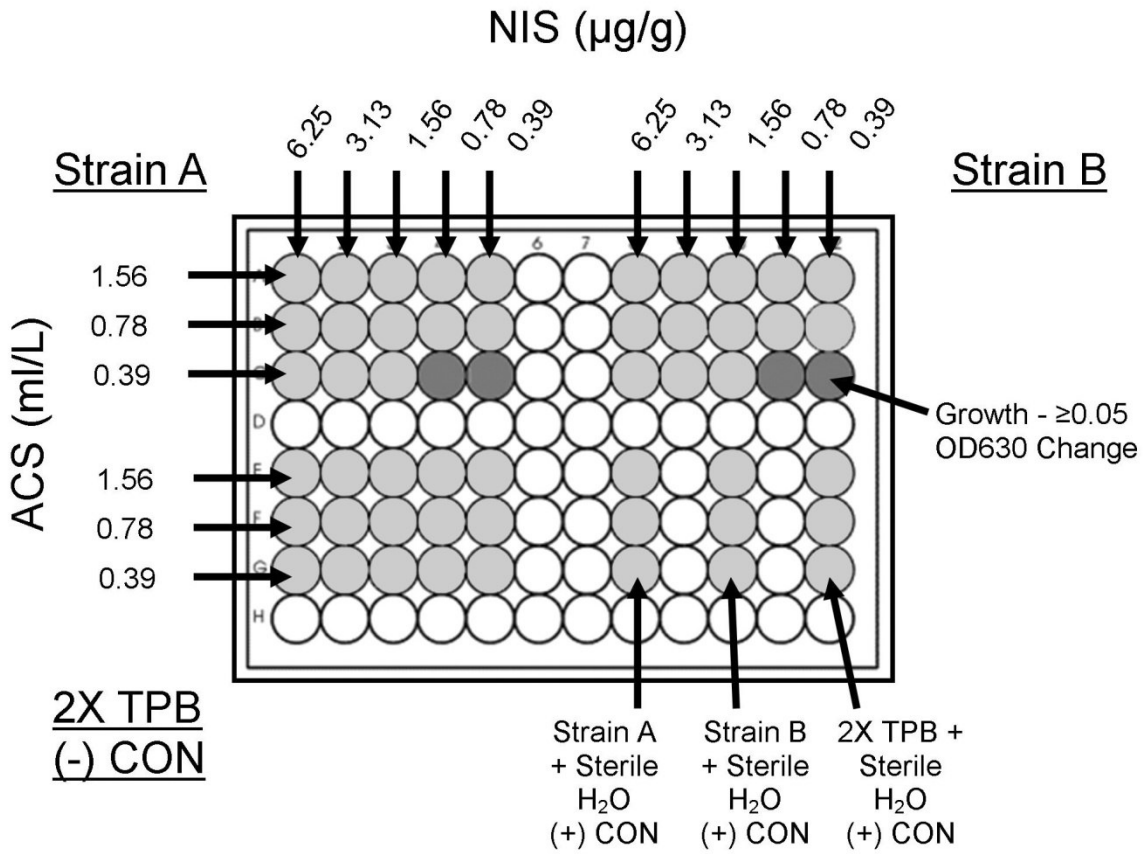

Figure 7-Checkerboard assay microplate setup and interpretation for tests at pH 7.3.

produced a <0.05 ΔOD630 after 24 h incubation over both replications were again classified as being inhibitory. Inhibitory combinations that utilized the least amounts of one compound in combination with the other were then classified as optimal inhibitory combinations (OIC) (Figure 8). An example of differentiation between a normal inhibitory combination and an optimal inhibitory combination would be the case of 3.13 µg/g NIS + 0.78 ml/L ACS and 3.13 µg/g NIS + 1.56 ml/L ACS. Though both are inhibitory, the former would be the OIC because it uses a lower concentration of ACS. The same would hold true for nisin concentrations where 0.39 µg/g NIS + 6.25 ml/L ACS would be an OIC, and 0.78 µg/g NIS + 6.25 ml/L ACS would not be an OIC. Because several combinations may work in this way, multiple OIC points for

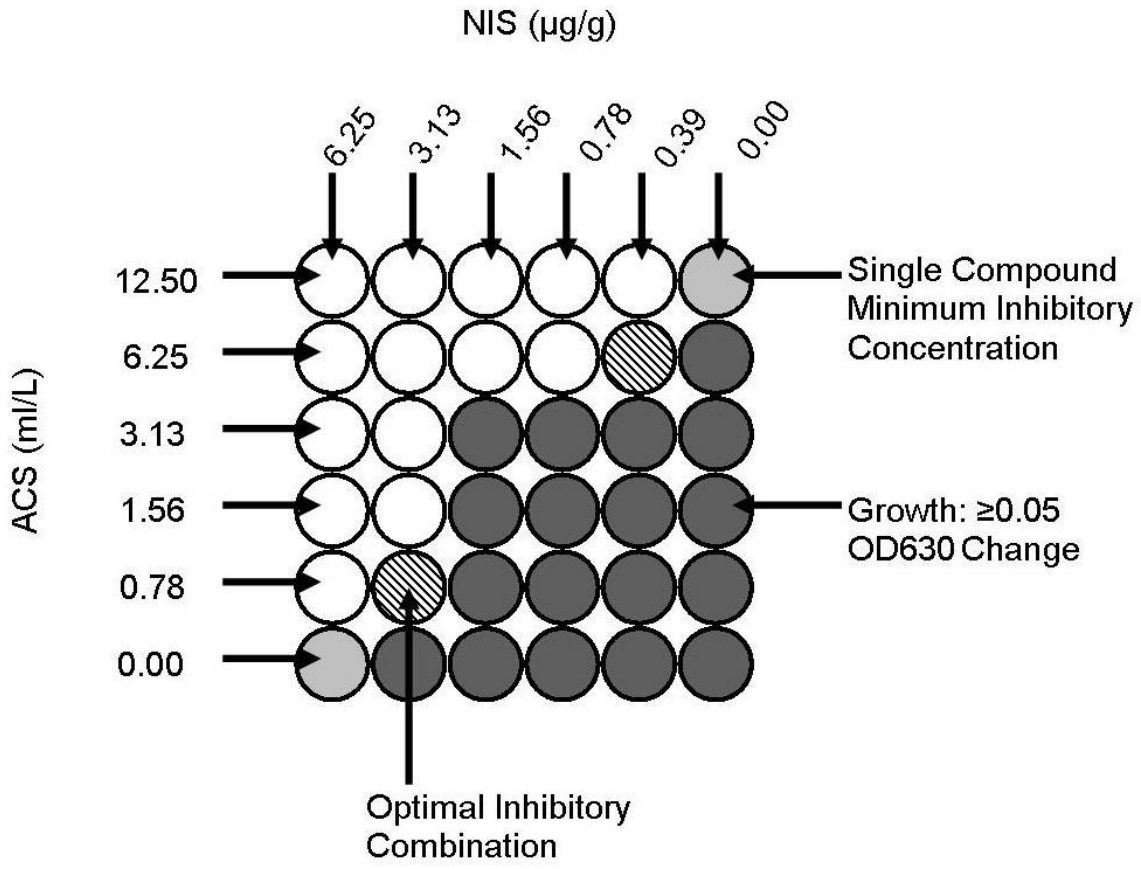

Figure 8-Schematic for determination of combined antimicrobials OICs for antimicrobial interaction characterization and isobologram construction for tests at pH 7.3.
NIS at 3.13 µg/g + 0.78 ml/L ACS is an OIC while 3.13 µg/g NIS + 1.56 ml/L ACS is not since the former uses a lower concentration of ACS. 0.39 µg/g NIS + 6.25 ml/L ACS is also an OIC.

each combination are possible and often do occur. The respective concentrations of the two compounds that were added together in these OICs were the values reported.

Assessment of Antimicrobial Interactions

Fractional inhibitory concentrations were calculated by dividing the concentration of antimicrobial A needed to produce an OIC by the MIC of antimicrobial A from single antimicrobial testing (Figure 4). FIC values for each antimicrobial were plotted to form isobolograms and to calculate factional inhibitory combination indices. Isobologram plots that

displayed a slope =-1 denoted additive interactions, while plots that curved toward or away from the origin defined synergistic or antagonistic interactions, respectively (Parish and Carroll 1988). A schematic for interpretation of isobologram shapes is given in Figure 5. FIC indices were calculated by adding the FIC of antimicrobial A for an OIC point to the FIC of antimicrobial B at the same OIC point (Figure 4). Antimicrobial interactions were defined as antagonistic (FIC_I >1), additive (FIC_I =1.0), or synergistic (FIC_I <1.0) (Branen and Davidson 2004).

Antimicrobial Bactericidal Activity

For both single antimicrobial and combined antimicrobials susceptibility testing, single antimicrobials and antimicrobial combinations were evaluated for bactericidal activity. Bactericidal activity was assessed by spreading 100 µl aliquots from wells that were classified as inhibitory onto TPA. Inoculated plates were aerobically incubated at 35 °C for 24 h and survivors were enumerated. Concentrations of single antimicrobials and antimicrobial combinations that produced at least a 3.0 $\log_{10}$ CFU/ml (99.9%) decrease in viable cells from the starting concentration of the inoculum were classified as bactericidal (Branen and Davidson 2004). The lowest concentration of antimicrobial applied alone or in combination producing bactericidal effects was declared the MBC. Bactericidal combinations that utilized the least amount of one compound in combination with the other were then classified as optimal bactericidal combinations (OBC) using the same principle for determination of optimal points as for the OIC classifications. The respective concentrations of the two compounds that were added together in these OBCs were reported.

Results

Pathogen Inhibition via Single Antimicrobials

The MICs and MBCs of antimicrobials applied to strains of *L. monocytogenes* grown in pH 7.3 TPB at 35 °C are provided in Table 3. The MIC of NIS for the Scott A strain was 3.13

µg/g and was 6.25 µg/g for 310, NADC 2783, and NADC 2045; 6.25 µg/g produced bactericidal activity against all strains except NADC 2045. *L. monocytogenes* Scott A and 310 were inhibited by EPL at a concentration of 6.25 µg/g; for NADC 2783 and NADC 2045, the EPL MIC was 12.50 µg/g. The MBC for EPL against *L. monocytogenes* 310 and NADC 2045 was 12.50 µg/g, but for Scott A and NADC 2783, the MBC was 25.00 µg/g. The MIC of LAE for all *L. monocytogenes* strains was 12.50 µg/g; LAE was also bactericidal to all strains at 12.50 µg/g. Acidic calcium sulfate was consistently inhibitory and bactericidal at 12.50 ml/L against *L. monocytogenes*. Sodium-L-Lactate was not inhibitory at any experimental concentration and no MBC was detected; thus it is excluded from Table 3.

Pathogen Inhibition by Combined Antimicrobials

The sole combination that could be deemed for the SL+ACS combination against all *L. monocytogenes* strains at pH 7.3 was a combination of 3.13 mg/g SL + 6.25 ml/L ACS (Table 4). Though 5.00 mg/g SL combined with 3.13 ml/L ACS also produced inhibitory effects for Scott

Table 3-Experimentally determined MICs and MBCs of antimicrobials against strains of *L. monocytogenes* at pH 7.3.[a]

Antimicrobial[b] (Units)	*L. monocytogenes*							
	Scott A		310		NADC 2783		NADC 2045	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
NIS (µg/g)	3.13	6.25	6.25	6.25	6.25	6.25	6.25	None[c]
EPL (µg/g)	6.25	25.00	6.25	12.50	12.50	25.00	12.50	12.50
ACS (ml/L)	12.50	12.50	12.50	12.50	12.50	12.50	12.50	12.50
LAE (µg/g)	12.50	12.50	12.50	12.50	12.50	12.50	12.50	12.50

a. MIC values are determined as the lowest concentration of antimicrobial for which ΔOD630 is <0.05 after 24 h incubation at 35 °C over two replications. MBC values are determined as the lowest concentration of antimicrobial for which a 3 log cycle reduction is observed.
b. NIS- nisin, EPL- ε-Poly-L-Lysine, ACS- acidic calcium sulfate, LAE- lauric arginate ester.
c. None = No MBC obtained.

A, 310, and NADC 2783, because the point involved a starting concentration (5.00 mg/g SL) it could not be considered an OIC based on definitions. As no MIC had ever been reached by using the maximum concentration allowed by FSIS during single antimicrobials testing, it was not possible to accurately calculate FIC values for the combination, or to construct isobolograms. No combinations of SL and ACS produced bactericidal responses at concentrations below the MBC for ACS, and thus no combination OBCs were determined. For the NIS+ACS combination optimal inhibitory concentrations were 1.56 µg/g NIS + 3.13 ml/L ACS for Scott A; 3.13 µg/g NIS + 0.78 ml/L ACS for 310, NADC 2783, and NADC 2045; and 0.39 µg/g NIS + 6.25 ml/L ACS for all strains (Table 4). Bactericidal effects were observed when 3.13 µg/g NIS was combined with 0.78 ml/L ACS for Scott A and 310; 6.25 µg/g NIS + 0.78 ml/L ACS for NADC 2045; 3.13 µg/g NIS + 1.56 ml/L ACS for NADC 2783 and NADC 2045; 1.56 µg/g NIS + 6.25 ml/L ACS for NADC 2783; 0.78 µg/g NIS + 6.25 ml/L ACS for 310 and NADC 2045; and 0.39 µg/g NIS + 6.25 ml/L ACS for Scott A (Table 5). Combining EPL with ACS resulted in OICs of 0.78 µg/g EPL + 6.25 ml/L ACS for all strains but *L. monocytogenes* NADC 2045. Additional EPL+ACS OICs were 3.13 µg/g EPL + 3.13 ml/L ACS for Scott A; 6.25 µg/g EPL + 1.56 ml/L ACS for 310; and 1.56 µg/g EPL + 6.25 ml/L ACS for NADC 2045 (Table 4). No true OBCs were determined for the EPL+ACS combination since no concentrations of EPL were able to produce consistent bactericidal effects at subsequent concentrations below the MBC of ACS. Thus no combination OBCs are given (Table 5). However, for Scott A, the combination of 12.50 µg/g EPL + 0.78 ml/L ACS and 6.25 µg/g EPL + 1.56 ml/L ACS produced bactericidal activity, while at higher concentrations of ACS, these EPL concentrations were unable to produce bactericidal activity. The same phenomenon occurred for NADC 2783 where 12.50 µg/g EPL + 0.78 ml/L ACS was able to produce bactericidal effects but combinations of 12.50 µg/g EPL with higher concentrations of ACS were unable. Optimal inhibition via LAE+NIS was observed

Table 4-Optimal inhibitory combinations of antimicrobials tested at pH 7.3.[a]

L. monocytogenes Strain	Combination Optimal Inhibitory Combinations Antimicrobial[b] (Units)			
	SL (mg/g) + ACS (ml/L)	EPL (µg/g) + ACS (ml/L)	NIS (µg/g) + ACS (ml/L)	NIS (µg/g) + LAE (µg/g)
Scott A	3.13+6.25	3.13+3.13 0.78+6.25	1.56+3.13 0.39+6.25	0.78+6.25
310	3.13+6.25	6.25+1.56 0.78+6.25	3.13+0.78 0.39+6.25	3.13+1.56 1.56+6.25
NADC 2783	3.13+6.25	0.78+6.25	3.13+0.78 0.39+6.25	3.13+6.25
NADC 2045	3.13+6.25	1.56+6.25	3.13+0.78 0.39+6.25	3.13+3.13 1.56+6.25

a. OICs were the inhibitory (ΔOD630 = <0.05 after 24 h incubation at 35 °C) combinations with the lowest concentrations of antimicrobial A combined with antimicrobial B. For example, though 3.13 µg/g NIS + 0.78 ml/L ACS and 3.13 µg/g NIS + 1.56 ml/L ACS are both inhibitory for NADC 2783, only 3.13 µg/g NIS + 0.78 ml/L ACS would be an OIC since it utilizes a lower concentration of ACS.

b. NIS- nisin, EPL- ε-Poly-L-Lysine, ACS- acidic calcium sulfate, LAE- lauric arginate ester.

at 1.56 µg/g NIS + 6.25 µg/g LAE for 310 and NADC 2045; 0.78 µg/g NIS + 6.25 µg/g LAE for Scott A; 3.13 µg/g NIS + 6.25 µg/g LAE for NADC 2783; 3.13 µg/g NIS + 3.13 µg/g LAE for NADC 2045; and 3.13 µg/g NIS + 1.56 µg/g LAE for 310 (Table 4). LAE+NIS combination OBCs were observed at 3.13 µg/g NIS + 6.25 µg/g LAE for 310 and NADC 2045; 3.13 µg/g NIS + 3.13 µg/g LAE for Scott A; 6.25 µg/g NIS + 0.78 µg/g LAE for NADC 2045; and 1.56 µg/g NIS + 6.25 µg/g LAE for Scott A. No combinations of NIS+LAE produced OBCs for NADC 2783 (Table 5).

Characterization of Antimicrobial Interactions

Combination concentration data in Table 4 were transformed into FIC values for each combination using the operations previously described (Figure 4). FIC values for each antimicrobial combination and strain were plotted onto the isobolograms shown on pages

Table 5-Optimal bactericidal combinations of antimicrobials tested at pH 7.3.[a]

L. monocytogenes Strain	Combination Optimal Bactericidal Combinations Antimicrobial[b] (Units)			
	SL (mg/g) + ACS (ml/L)	EPL (µg/g) + ACS (ml/L)	NIS (µg/g) + ACS (ml/L)	NIS (µg/g) + LAE (µg/g)
Scott A	None[c]	None[c]	3.13+0.78 0.39+6.25	3.13+3.13 1.56+6.25
310	None[c]	None[c]	3.13+0.78 0.78+6.25	3.13+6.25
NADC 2783	None[c]	None[c]	3.13+1.56 1.56+6.25	None[c]
NADC 2045	None[c]	None[c]	6.25+0.78 3.13+1.56 0.78+6.25	6.25+0.78 3.13+6.25

a. OBCs were bactericidal (3 log cycle reduction) combinations with the lowest concentrations of antimicrobial A combined with antimicrobial B. For example, though 3.13 µg/g NIS + 1.56 ml/L ACS and 3.13 µg/g NIS + 3.13 ml/L ACS are both bactericidal for NADC 2783, only 3.13 µg/g NIS + 1.56 ml/L ACS would be an OBC since it uses a lower amount of ACS.

b. NIS- nisin, EPL- ε-Poly-L-Lysine, ACS- acidic calcium sulfate, LAE- lauric arginate ester.

c. No combinations produced bactericidal activity at lower levels in combination than the bactericidal activity of the antimicrobials used singly.

90-92 with antimicrobial A FIC values plotted against antimicrobial B FIC values. Interactions were classified based on the shape of the isobologram (Figure 5). FIC_I values for each combination were obtained by adding the values of the two individual compound FIC values together (Table 6). Determinations of synergism, additivism, and antagonism were deduced using these values and the scale that was previously described (<1, 1, >1). In combination with ACS, NIS functioned synergistically against all strains of *L. monocytogenes* based on the shape of the isobologram (Figure 9). NIS+ACS combination FIC_I values were 0.75 and 0.63 for Scott A, 0.56 and 0.56 for 310, 0.56 and 0.56 for NADC 2783, and 0.56 and 0.56 for NADC 2045 (Table 6). With the exception of *L. monocytogenes* 310, which showed slight antagonism, the interaction of EPL with ACS was mostly additive (indifferent) in nature based on isobologram

Table 6-Fractional inhibitory concentration indices for antimicrobials tested at pH 7.3.[a]

L. monocytogenes Strain	Fractional Inhibitory Concentration Indices Antimicrobial[b] (Units)			
	SL (mg/g) + ACS (ml/L)	EPL (µg/g) + ACS (ml/L)	NIS (µg/g) + ACS (ml/L)	NIS (µg/g) + LAE (µg/g)
Scott A	None[c]	0.75	0.75	0.75
		0.63	0.63	
310	None[c]	1.13	0.56	0.63
		0.63	0.56	0.75
NADC 2783	None[c]	0.56	0.56	1.00
			0.56	
NADC 2045	None[c]	0.63	0.56	0.75
			0.56	0.75

a. Fractional inhibitory concentration index values were determined by dividing concentrations comprising OIC combinations by their respective MICs from single antimicrobial testing and adding FIC values from antimicrobial A and antimicrobial B.
b. NIS- nisin, EPL- ε-Poly-L-Lysine, ACS- acidic calcium sulfate, LAE- lauric arginate ester.
c. None = No FIC values were able to be calculated because no MIC values were obtained from single antimicrobial testing.

interpretation (Figure 10). FIC_I values for Scott A were 0.75 and 0.63, for 310 they were 1.13 and 0.63, for NADC 2783 the sole FIC_I was 0.56, and for NADC 2045 it was 0.63 (Table 6). Combination of Nisin with LAE resulted in additive-type inhibition (indifference) between antimicrobials as evidenced by the isobologram shape (Figure 11). FIC index values were determined to be 0.75 for Scott A, 0.63 and 0.75 for 310, 1.00 for NADC 2783, and 0.75 and 0.75 for NADC 2045 (Table 6). As noted before, because no MIC was determined for SL at pH 7.3 either alone or in combination, or in combination, FIC values, FIC_I values, nor isobolograms were able to be used to determine the antimicrobial interaction type. Thus, no conclusions were able to be made to denote how the two compounds were able to interact with one another when paired together. Possible expectations for the interaction of the compounds are provided below in the discussion section.

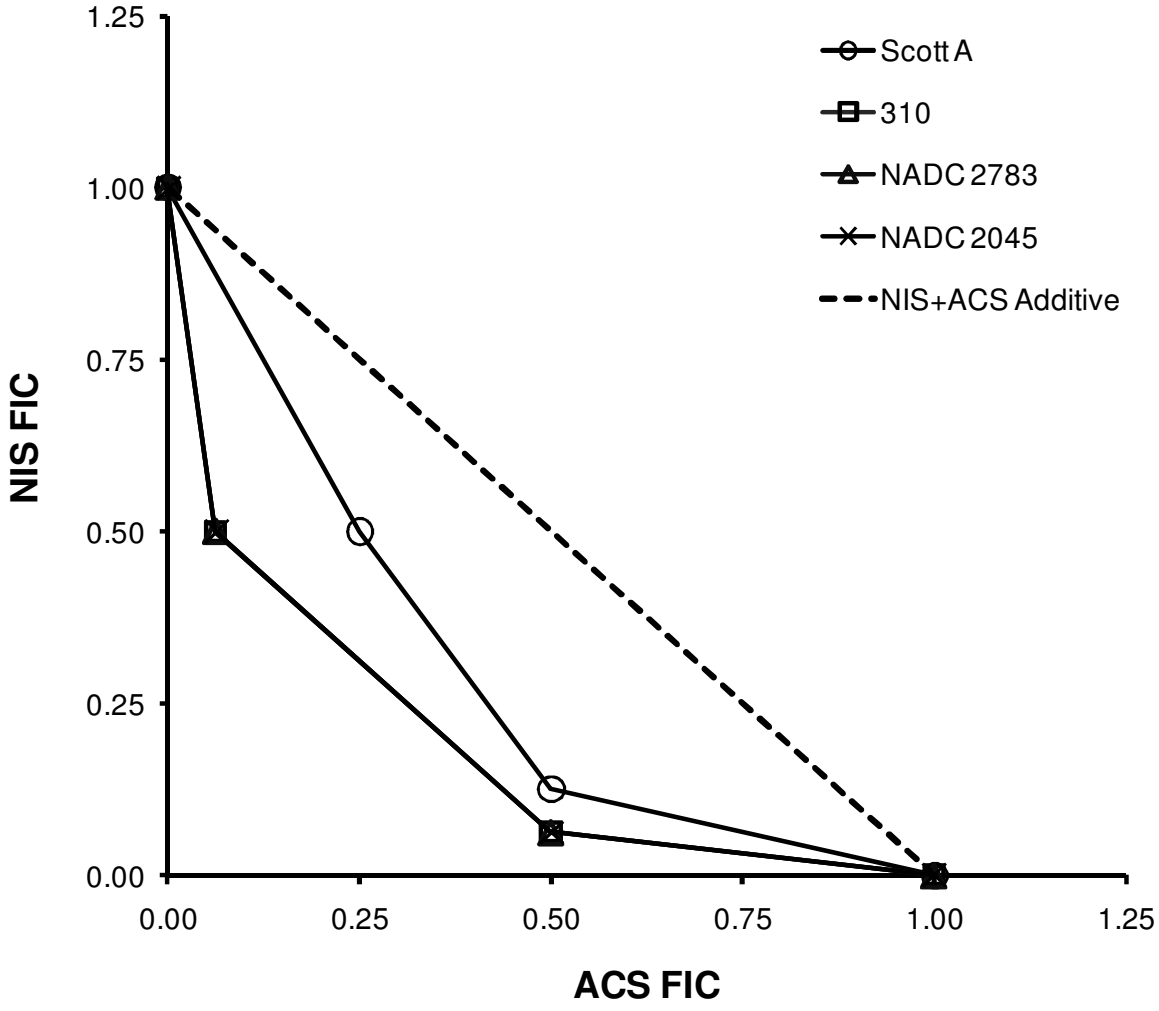

Figure 9-Isobologram of Antimicrobial FIC values for Nisin (NIS) in combination with Acidic Calcium Sulfate (ACS).
An FIC was defined as the OIC concentration of antimicrobial A divided by the MIC of antimicrobial A from single antimicrobial susceptibility testing. FIC values are ratios and are therefore unitless.

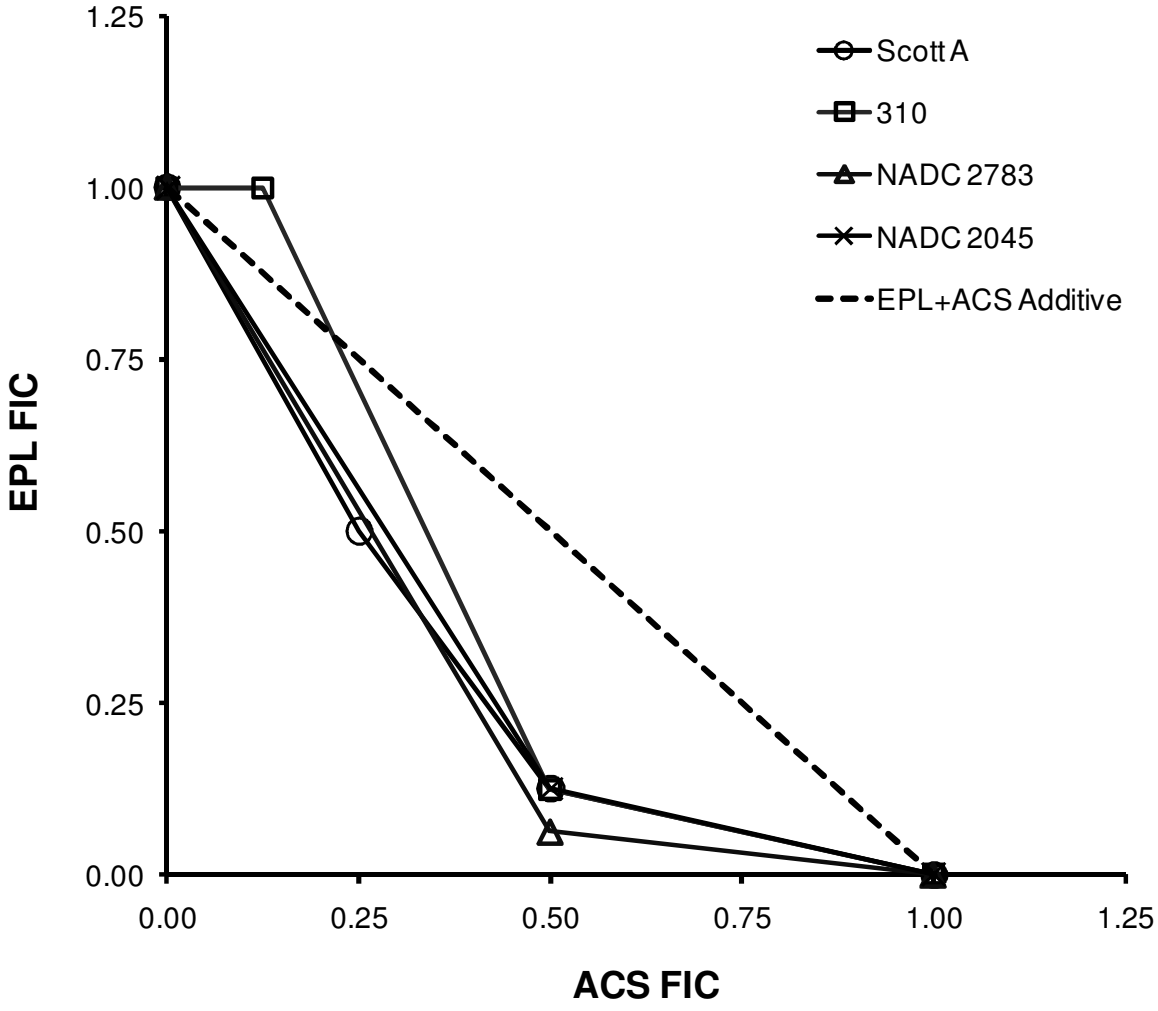

Figure 10-Isobologram of antimicrobial FIC values for ε-Poly-L-lysine (EPL) in combination with Acidic Calcium Sulfate (ACS).
An FIC was defined as the OIC concentration of antimicrobial A divided by the MIC of antimicrobial A from single antimicrobial susceptibility testing. FIC values are ratios and are therefore unitless.

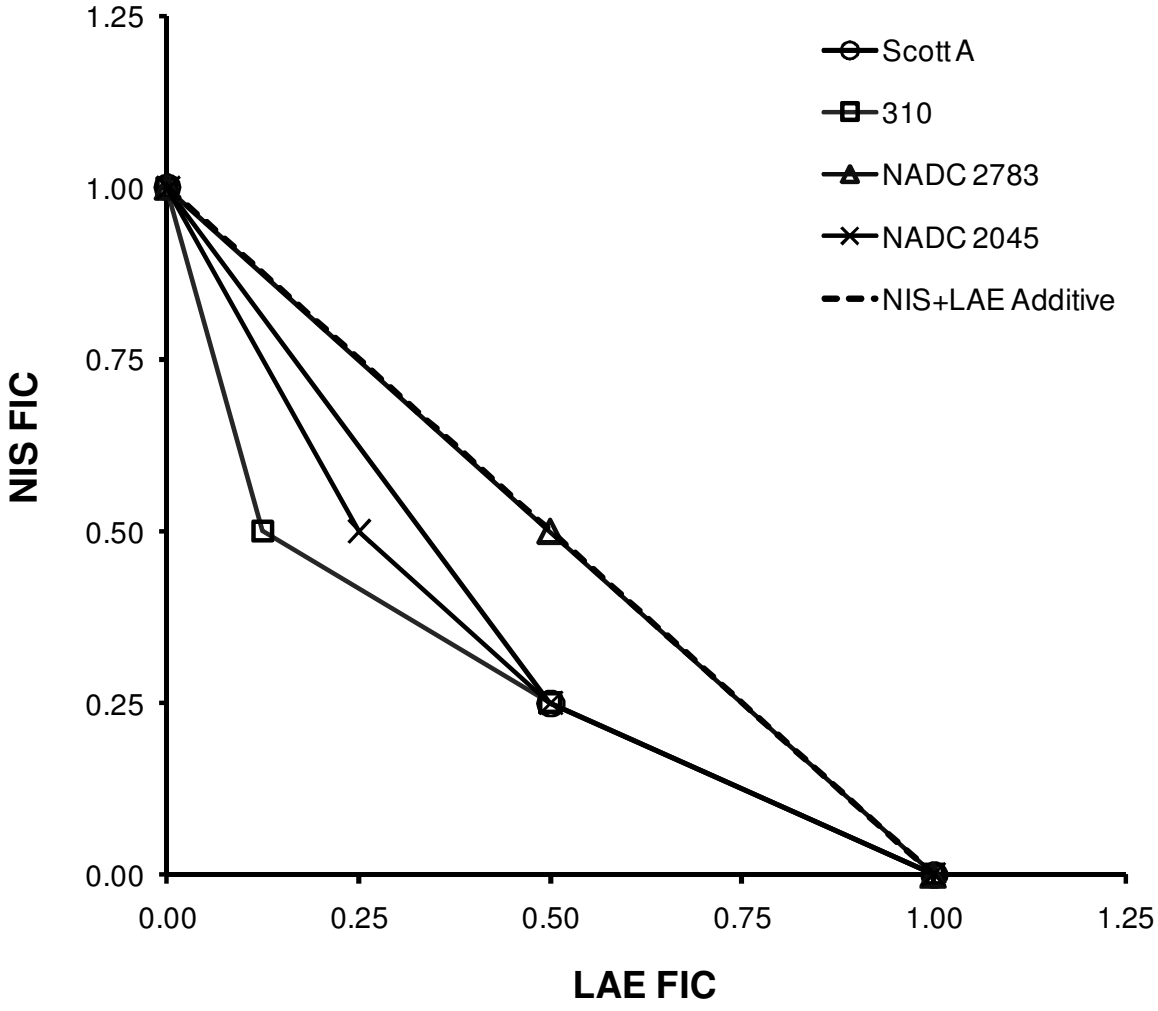

Figure 11-Isobologram of antimicrobial FIC values for Nisin (NIS) in combination with Lauric Arginate Ester (LAE).
An FIC was defined as the OIC concentration of antimicrobial A divided by the MIC of antimicrobial A from single antimicrobial susceptibility testing. FIC values are ratios and are therefore unitless.

Discussion

The experimental results indicate that the *in vitro* inhibition and inactivation of the *L. monocytogenes* strains by individual antimicrobial treatments occurred with intermittent strain dependencies. Indeed, the *L. monocytogenes* Scott A seemed to have a greater sensitivity to nisin as compared to other strains, and NADC 2045 seemed to exhibit a decreased response to the bacteriocin, as evidenced by its ability to survive at concentrations that were cidal to other strains. Though strains were wild-type and had not been exposed to the compound prior to testing, the inherent ability of some strains to readily form nisin-resistant mutants (Harris and others 1991; Davies and Adams 1994) which are then able to counteract the ability of nisin by altering membrane fluidity (Ming and Daeschel 1993; Mazzotta and Montville 1997; Crandall and Montville 1998) may have led to the differences observed in this study. Such variations in sensitivity to nisin between strains are consistent with other studies where almost 8-fold differences in MIC values have been seen before (Mohamed and others 1984). Likewise, differences in EPL sensitivity were noted with 310 being the most sensitive strain with MIC and MBC values of 6.25 and 12.50 µg/g, respectively, and NADC 2783 being the most resistant strain with an MIC and MBC of 12.50 and 25.00 µg/g, respectively. Though instances of EPL resistance have not been previously reported, it is evident from these results that strain variations in MIC and MBC values can occur, and that unknown strain-specific factors may predispose certain strains to increased tolerance or increased sensitivity. The inability of SL to produce an inhibitory response under the test concentrations and growth conditions has been observed previously, where levels of SL at 5.2% have permitted growth of the pathogen at levels of 4.5-5.2 $\log_{10}$ CFU/ml in tryptic soy broth at 35 °C after 24 h (Shelef and Yang 1991); thus inhibition through use of the individual compound was not expected at these experimental conditions and is consistent with previous findings. Though ACS has been shown to induce some acid tolerance in

E. coli O157:H7 (Beuchat and Scouten 2004), and inherent acid resistance could possibly drive variations in sensitivity among strains of *L. monocytogenes* (Dykes and Moorhead 2000; Lianou and others 2006; Barmpalia-Davis and others 2008), MIC and MBC values of ACS were identical for all four *L. monocytogenes* strains. Such uniform acid sensitivity responses may be due to the novel acid regeneration means that the solution possesses, which may counteract normal acid tolerance responses (Kemp and others 2003). Uniformity among strains with respect to LAE was also noted, as the same concentration was both the MIC and MBC for all strains. Because decreased sensitivity to LAE has not been previously reported, the lack of variation in sensitivity observed in the results may further the notion that tolerance to the compound is difficult for *L. monocytogenes* to develop.

Though strain variation in response to combinations of antimicrobials also existed, conclusive interaction types were able to be determined for each pair of compounds, with the exception of SL+ACS. The combination of ε-Poly-L-Lysine with acidic calcium sulfate and the combination of nisin with lauric arginate ester both produced additive-type (indifferent) interactions when paired against *L. monocytogenes in vitro*. The FIC_I values determined for each interaction were all slightly below 1.000, which would indicate moderate synergistic activity, but because at least one FIC_I value of 1.00 or greater (1.13) was obtained for each interaction, the pairings were classified as additive. The conclusion of additivity can be also be drawn from the shape of the isobolograms of all strains for both antimicrobial pairs. As noted previously, a curve toward the origin would be indicative of synergism, while a curve away from the origin would be characteristic of antagonism (Figure 5). Because both sets of isobolograms display neither trend but lie along the line with a slope of -1.0 (Figures 10-11), the isobolograms were further evidence that the interactions of both sets are additive in nature with respect to inhibition of the pathogen. Thus, inhibition that results from the use of the combinations would be expected to

display the indifferent type effect described by Barry (1976) whereas the combined effect is equal to the sum of the effects observed with the two antimicrobials used separately or equal to that of the most active antimicrobial. A numerical analogy to additivism can be made using the assumptions that if Compound A = 1 and Compound B = 1, then ½ Compound A + ½ Compound B = 1; no enhancing effect results from using the antimicrobials together and each just essentially replaces the other in terms of the total inhibitory activity of the combination.

Research conducted on the interaction of EPL with organic acids and their salts with respect to *L. monocytogenes* growth inhibition reinforces the observations of the additive nature of the EPL+ACS interaction observed in this study. Geornaras and Sofos (2005) found similar results when they demonstrated that EPL (0.02%) was able to interact with lactic acid (0.1%), a major component of the acidic calcium sulfate solution, to produce greater than 6.6 $\log_{10}$ CFU/ml differences in growth suppression when compared to controls after 24 h incubation at 24 °C. However, because the effect of using the combination was not significantly different ($p > 0.05$) than the effects of EPL alone, the additive, rather than synergistic, effect seen in this experiment agrees with the observations (Geornaras and Sofos 2005). Yet, because Poly-L-Lysine molecules have been shown to increase membrane permeability to hydrophobic materials (Vaara and Vaara 1983b; Vaara and Vaara 1983a; Vaara 1992), and lactic acid functions primarily due to its ability to cross the cytoplasmic membrane as a hydrophobic molecule, it would seem that the combination might yield enhanced activity as opposed to the additive behavior observed. As a result, other complexities of the interaction must exist, and the biochemistry of the pairing should be further characterized.

At present, no published research is available on the inhibitory effects of the NIS+LAE pairing to substantiate the observed additive behavior of the compounds *in vitro*. However, results obtained from pairing monolaurin (a compound similar to LAE in structure) with nisin

that have shown the combination to be synergistic with respect to inhibition of *E. coli*, *S. aureus*, and *Bacillus subtilis* (Zhang and others 2009) are evidence that such classes of compounds can be used simultaneously without antagonistic effects. Synergism of NIS and LAE may be prevented by the fact that they have the same cellular target: the cytoplasmic membrane. Thus, destabilization of the membrane provided by LAE, may not act to potentiate nisin activity, and inhibition may just result from the N^{α}-acyl amino acid working alongside the bacteriocin using its normal mode of action to produce the additive/indifferent response.

The additive nature of *L. monocytogenes* inhibition that results from EPL+ACS and NIS+LAE is also well characterized by the presence of bactericidal combinations of the two compounds at levels below their individual MBCs. Indeed, for three of the four strains the NIS+LAE combination produced at least one OBC point. As mentioned, the EPL+ACS combination did produce bactericidal activity toward *L. monocytogenes* when the highest concentration of ACS (12.50 ml/L ACS) was paired with all concentrations of EPL. This effect was not seen for any strain at the highest concentration of EPL (12.50 µg/g) in combination with the second-most concentrated ACS level (6.25 ml/L ACS). However, the bactericidal effect resumed as the concentration of ACS decreased (0.78 ml/L) and the concentration of EPL remained constant (12.50 µg/g). The same phenomenon occurred for lower concentrations of EPL (6.25 µg/g), where bactericidal activity was present at 12.50 ml/L ACS, was absent at 6.25 ml/L ACS, and was resumed again at lower concentrations (0.78 ml/L ACS). Though inactivation of a polyamino acid like EPL would be expected to be due to precipitation at an isoelectric point, the isoelectric point of EPL is near pH 9.0 (Yoshida and Nagasawa 2003), which was well above the pH of the test solution. However, deactivation of EPL through the use of sodium lactate (3.0%) in place of lactic acid, was observed by Geornaras and Sofos (2005). Indeed, EPL alone (0.02%) was able to keep *L. monocytogenes* numbers below 1.3 $\log_{10}$ CFU/ml

after 24 h at 35 °C, but at the same concentration combined with 3.0% SL, numbers of the pathogen were near to 5.6 $\log_{10}$ CFU/ml. Thus, conversion of lactic acid from the ACS solution into sodium lactate at a pH provided by the specific concentration of ACS (6.25 ml/L) may cause deactivation of the bactericidal activity of EPL when that threshold is reached. The occurrence of this additional phenomenon is further motive to continue investigations to obtain a better understanding of the EPL+ACS combination.

In contrast to EPL+ACS and NIS+LAE, the pairing of nisin and acidic calcium sulfate yielded synergistic activity in terms of inhibition of the pathogen. With all FIC_I values below 1.00, the interaction between the two compounds was characteristic of synergism. The isobolograms for each strain reiterated this conclusion as the low FIC values for each antimicrobial produced a distinct curvature toward the origin when plotted against each other. As a result, the compounds would be expected to behave in a manner that yields a greater effect than the sum of the effects observed when the two antimicrobials are used separately to inhibit growth of the pathogen (Barry 1976); thus, an enhancement of the individual inhibitory activities would be observed. In contrast to the replacement-type effect of additivism, the numerical analogy of synergism would, with assumptions that Compound A = 1 and Compound B = 1, appear as ½ Compound A + ½ Compound B = 2 (or a greater number). The synergistic effect of the combination was also evident in the bactericidal responses produced by combinations of the two compounds at sub-MBC levels for each strain. Indeed, at least two OBC combinations were generated for each strain, and three were generated for NADC 2045, which had previously been the strain most resistant to nisin in the single compound testing.

Though a number of mechanistic explanations can be generated to justify the synergistic interaction between nisin and ACS, the activity that results is most likely due to the enhanced solubility of the bacteriocin that accompanies acidification and because of simultaneous attack

on cellular proton motive force. Indeed, it has been shown that nisin solubility is enhanced by acidification (Tramer and Fowler 1964), and thus it would be expected that ACS, which contains organic acids, could potentiate solubility and activity of the bacteriocin when the two are used together. Also, nisin is able to form pores (Sahl and others 1987) that can cause leakage of cellular materials (Moll and others 1997) and a disruption of proton motive force (Bruno and others 1992; Okereke and Montville 1992). Organic acid components of ACS are also able to penetrate the membrane, acidify the cytoplasm, and create disruptions in proton motive force (Salmond and others 1984). Thus, simultaneous attack of both compounds may yield a deleterious effect on proton motive force which is too extensive for the cell to handle. Therefore, coupled with increased solubility of the bacteriocin, these disruptions of homeostasis could be the means that are necessary for the combination to produce the synergistic inhibition observed.

As noted, a conclusive interaction type was not able to be determined for the SL+ACS combination, as no MIC was determined for SL at pH 7.3. Though the concentration of SL used as the starting point for the checkerboard assay arrangement was not inhibitory across all concentrations of ACS, as would be typical for results from such an assay, it was still apparent that ACS did function to potentiate the inhibition potential of SL to some degree. Indeed, combinations of lower SL concentrations with sub-MIC concentrations of ACS did produce inhibition, which was indicative of some sort of interaction between the pair, likely via protonation of the lactate. Though one may classify the combination as antagonistic based on the appearance of the inhibition profile with respect to the checkerboard assay, this is likely not the case for the interaction. Likewise, synergistic interaction of the two compounds would not be expected due to similarities in chemical makeup and targets for cellular attack. Thus, even though limitations of procedures disallow correct characterizations of the interaction, it would be assumed that the interaction of the two antimicrobials would most likely be additive in nature.

Further investigations of the activity of the combination through the use of modified methods and at different test conditions are encouraged and might yield different interpretations of the activity of the two compounds in combination.

CHAPTER V

IN VITRO INHIBITION OF *LISTERIA MONOCYTOGENES* BY FOOD ANTIMICROBIAL COMBINATIONS TESTED AT PH 5.0

Introduction

L. monocytogenes persists as a challenge to the safety of processed ready-to-eat (RTE) meat products. Several outbreaks of listeriosis associated with consumption of contaminated RTE meat products have occurred in recent years (CDC 2002b; CDC 2005; Gottlieb and others 2006; Warriner and Namvar 2009). As a result, the United States Food and Drug Administration and the Food Safety and Inspection Service continue to maintain a policy that considers the pathogen an adulterant in such products (Klontz and others 2008). FSIS also mandates the incorporation of certain control alternatives in food processes to further reduce the pathogen's potential to contaminate and grow in RTE foods (FSIS 2003a). Several of these alternatives involve the use of antimicrobials to achieve reductions of the pathogen during processing and throughout storage. Combining antimicrobials with processing procedures or with one another is also an integral part of fulfilling the standards set by the regulatory agency (FSIS 2003a). A number of antimicrobials are approved for use to control the pathogen in meat and poultry products (FSIS 2009), but extensive research on using combinations of these antimicrobials with one another is fairly limited. Thus, investigations of novel combinations of antimicrobials that are able to achieve efficient inhibition of *L. monocytogenes* are readily welcomed by the food industry. In addition, the use of such pairings may also afford greater control without the need for sacrifices in the sensory appeal of products, and can provide greater reductions while still meeting the stipulations of maximum use levels.

The primary objective of this study was to expose four *L. monocytogenes* strains to nisin, octanoic acid, and acidic calcium sulfate *in vitro* at pH 5.0 and to characterize the inhibition that resulted from their use. A broth dilution microassay was used to assess antimicrobial susceptibility and the minimum inhibitory concentration for each antimicrobial was determined. After completion of the incubation period used for inhibitory determinations, the bactericidal activity of each antimicrobial was also assessed.

A second objective was to investigate the efficacy of combinations of food antimicrobials for the *in vitro* inhibition of *L. monocytogenes* growth at pH 5.0. *L. monocytogenes* strains were exposed to octanoic acid combined with acidic calcium sulfate and octanoic acid combined with nisin through use of a checkerboard broth dilution microassay. The inhibition that resulted from each combination was determined and optimal inhibitory combinations of each antimicrobial pairing were established. As with single antimicrobial testing, bactericidal responses were also ascertained for those combinations of concentrations that were inhibitory to growth of the pathogen.

Lastly, the purpose of the study was to characterize the interaction associated with antimicrobial pairing. Fractional inhibitory concentrations were calculated using the optimal inhibitory combinations derived from the combinations assay and the minimum inhibitory combinations from single antimicrobial testing. Using these values, fractional inhibitory concentration index values were determined and isobolograms were plotted for each combination and strain to classify interactions as synergistic, additive, or antagonistic in nature. Through the use of these classifications and suggested modes of action for the antimicrobials, postulations pertaining to possible reasons for increased or decreased activity were made for each pairing.

Materials and Methods

Bacterial Culture Preparation and Maintenance

Listeria monocytogenes Scott A (clinical isolate), 310 (goat cheese-associated outbreak isolate), National Animal Disease Center (NADC) 2783 and NADC 2045 were obtained from the Center for Food Safety culture collection (Department of Animal Science, Texas A&M University, College Station, TX). All strains were biochemically confirmed as *Listeria monocytogenes* using the API® *Listeria* identification system (bioMérieux, Inc., Hazelwood, MO) according to manufacturer instructions. Thereafter, cultures were maintained on Tryptic Soy Agar (TSA; Becton Dickinson and Co., Sparks MD) slants at 5 °C. In order to prevent potential development of antimicrobial resistance via repeated sub-culturing, working cultures were obtained by transferring a loopfull of culture from TSA slants to 10 ml of Fraser Broth (Becton Dickinson and Co.) and incubating aerobically for 24 h without agitation at 35 °C. After confirming a *Listeria* species correct phenotype (esculin hydrolysis) via medium blackening (Fraser and Sperber 1988), a loopfull of culture was transferred to 10 ml Tryptose Phosphate Broth (TPB; Becton Dickinson and Co.), which was incubated aerobically for an additional 24 h at 35 °C without agitation. Cultures from Fraser Broth were streaked for isolation on Tryptose Phosphate Agar (TPA; Becton Dickinson and Co.) plates at the time of TPB inoculation and incubated at 35 °C for 48 h to confirm culture purity.

Inoculum Preparation

Overnight (24 h) cultures of each *L. monocytogenes* strain in TPB were serially diluted in 9.9 ml volumes of double-strength TPB (2x TPB) to achieve a final inoculum concentration of approximately 5.0 $\log_{10}$ CFU/ml. Double-strength TPB was adjusted to pH 5.0 using 6 M HCl prior to use according to antimicrobial manufacturer recommendations. Enumeration of inocula was completed by diluting to approximately 3.0 $\log_{10}$ CFU/ml in sterile 0.1% Peptone (Becton

Dickinson and Co.), and then spread-plating 100 µl on both TPA and PALCAM Agar (Becton Dickinson and Co.). Plates were aerobically incubated at 35 °C for 48 h prior to enumeration. Use of PALCAM Agar served to ensure that cells from the inoculum displayed a correct phenotype for *Listeria* species (medium blackening via esculin hydrolysis) (Van Netten and others 1989), while simultaneous plating on TSA plates confirmed inoculum purity.

Test Antimicrobial Preparation

A 250.0 µg/g stock solution of active nisin was prepared by dissolving a mass of 0.10 g powdered nisin fermentate (NIS; Sigma-Aldrich, St. Louis, MO, 2.5% w/w nisin) in 10.0 ml sterile 0.02 M hydrochloric acid (HCl; Thermo-Fisher Scientific, Waltham, MA) according to previously reported methods (Rogers and Montville 1991). The solution was boiled in water for 4 minutes to aid in dissolution of nisin (Wolf and Gibbons 1996; Taylor and others 2008). Octa-Gone® (OCT; Ecolab, Inc., St. Paul, MN; 3.6% w/w Octanoic Acid/Sodium Octanoate) was diluted in sterile DI H_2O to obtain a working stock of approximately 9000 µg/g active Octanoic Acid/Sodium Octanoate. Safe$_2$O® RTE:01 (ACS; Mionix Corp., Round Rock, TX; saturated Acidic Calcium Sulfate solution) was dissolved in sterile DI H_2O to obtain working solutions. After preparation, 100 µl of stock solutions were spread-plated on TPA and incubated aerobically at 35 °C for 48 h to ensure that solutions were free of microbial contamination. Antimicrobials, active agent, stock solution concentrations, and manufacturers are in Table 7.

Single Antimicrobial Inhibition Assay

A broth dilution microassay (Barry 1976) was used to determine the strain-specific minimum inhibitory concentration for each antimicrobial. Strains were exposed to NIS at 1.56, 0.78, 0.39, 0.20, 0.10, and 0.05 µg/g. Solutions of NIS used for transfer to test wells were generated by dissolving 500 µl of the 250 µg/g stock solution in 10.0 ml DI H_2O to create a

Table 7-Experimental antimicrobials, active agents, and manufacturer for tests at pH 5.0.

Antimicrobial	Active Agent	Stock Concentration	Manufacturer	Headquarters
Nisin	Nisin	2.50% w/w	Sigma-Aldrich, Inc.	St. Louis, MO
Octa-Gone®	Octanoic Acid	3.60% w/w	Ecolab, Inc.	St. Paul, MN
$Safe_2O$® RTE:01	Acidic calcium sulfate	100% v/v	Mionix Corp.	Rocklin, CA

primary working stock solution, transferring 1250 µl of the primary working stock to 10.0 ml DI H_2O to create the solution for delivering 1.56 µg/g, and transfers of 2500 µl of the previous solution into 5.0 ml DI H_2O for all solutions thereafter. Acidic calcium sulfate was tested at 6.25, 3.13, 1.56, 0.78, and 0.39 ml/L. A preliminary working stock of ACS was obtained by dissolving 1250 µl of $Safe_2O$® RTE:01 in 10.0 ml of DI H_2O; 1000 µl of this stock was then transferred to 10.0 ml DI H_2O to create the solution needed to deliver 6.25 ml/L ACS to test wells. All following solutions were created by serially diluting 2500 µl of the previous solutions into 5.0 ml DI H_2O. OCT was tested at 100.00, 50.00, 25.00, 12.50, and 6.25 µg/g. A preliminary working stock of OCT was produced by dissolving 2340 µl of Octa-Gone® in 10.0 ml DI H_2O; a secondary working stock was produced by dissolving 1095 µl of the primary stock in 10.0 ml DI H_2O. A volume of 1997 µl of the secondary stock solution was dissolved in 10.0 ml DI H_2O to obtain the solution needed to deliver 100.00 µg/g OCT to test wells; all subsequent solutions were created by dissolving 2500 µl of the previous solution into 5.0 ml DI H_2O. Concentrations of working solutions were devised so as to deliver the correct concentration of test antimicrobial to the test wells once all additions were made. Sterile barrier tips and serological pipettes were always used in making dilutions so as to minimize potential for cross-contamination.

Microtiter plates (96-wells; 300 µl capacity) (Microtest™, Becton Dickinson and Co.) were used for all broth dilution assays. Equivalent volumes of test antimicrobial solution (125 µl) and serially diluted *L. monocytogenes* inoculum in 2x TPB (125 µl) were aseptically loaded into test wells. Cells were diluted and inoculated in 2x TPB to allow the correct content of nutrients in the final sample well upon delivery. Sterile barrier tips were used to load solutions into the wells so as to minimize potential for cross-contamination. Figure X shows the individual antimicrobial testing setup for the microtiter plate. Appropriate negative controls containing only antimicrobial-containing solutions and sterile 2x TPB were built in for baseline adjustment of experimental wells. Positive controls containing sterile water and inoculum in 2x TPB were also completed. Immediately following plate preparation, optical density of the test wells at 630 nm (OD630) was measured at 0 h using an EL800 absorbance microplate reader (BioTek® Instruments, Inc., Winooski, VT). Optical density at 630 nm was used in order to minimize absorption of light by the growth medium and optimize light scattering by cells (Burton and Kaguni 1997). Microplates were aerobically incubated for 24 h at 35 °C before being retrieved for a 24 h OD630 reading.

Antimicrobial inhibition testing of each antimicrobial at all concentrations was replicated at least twice for each strain. Concentrations of antimicrobial that produced a <0.05 change (Δ) in OD630 from 0 h to 24 h following appropriate baseline adjustment over replication were classified as inhibitory. The MIC for each antimicrobial was defined as the lowest concentration of antimicrobial that produced <0.05 ΔOD630 after 24 h incubation following subtraction of the baseline OD630 values of the negative controls from the treatment well OD630 values (Figure 12).

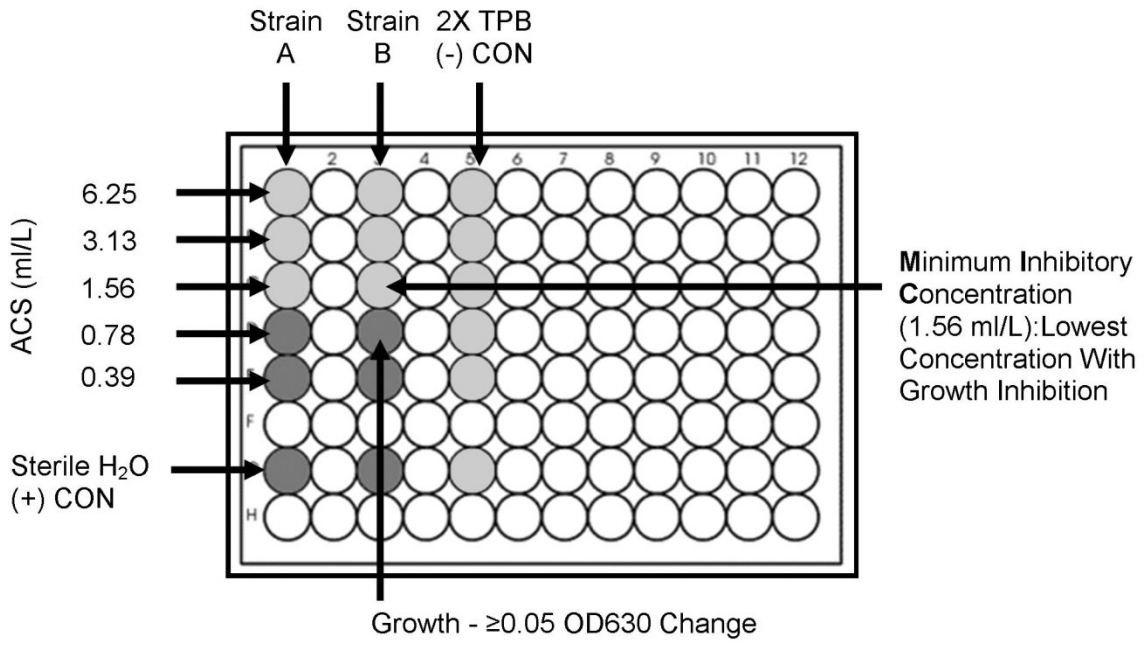

Figure 12-Broth dilution microplate setup and interpretation for tests at pH 5.0.

Antimicrobial Combinations Susceptibility Testing

The response of *L. monocytogenes* to combinations of antimicrobials: OCT combined with ACS (OCT+ACS), and OCT combined with NIS (OCT+NIS), was evaluated using a checkerboard broth dilution microassay. Minimum inhibitory concentrations previously determined from single antimicrobial susceptibility tests were used as starting concentrations for the combination assays. Serial (1:2) dilutions of antimicrobials were made from these starting concentrations so as to produce a 5X5 checkerboard of combinations between the two antimicrobial compounds. Concentrations of working solutions were again devised so as to provide the correct concentration of test antimicrobial when finally delivered to the test wells. Sterile barrier tips and sterile pipettes were used in making dilutions and in plate loading so as to minimize potential for cross-contamination.

For the OCT+ACS combination, *L. monocytogenes* strains were exposed to 25.00, 12.50, 6.25, 3.13, and 1.56 µg/g active OCT in combination with 1.56, 0.78, 0.39, 0.20, and 0.10

ml/L ACS. The first preliminary working solution of OCT was created by dissolving 2340 µl of Octa-Gone® in 10.0 ml DI H_2O; a second working solution of OCT was generated by dissolving 1095 µl of the first solution in 10.0 ml DI H_2O. After diluting 832 µl of the second working solution in 10.0 ml DI H_2O to create the solution needed to deliver 25.00 µg/g OCT, serial dilutions of 2500 µl into 5.0 ml DI H_2O were used to make the remainder of the solutions. A primary working stock of ACS was generated by dissolving 999 µl of $Safe_2O$® RTE:01 in 10.0 ml DI H_2O; the first solution of ACS used for transfer to wells was created by dissolving 782 µl of the primary solution into 10.0 ml of DI H_2O. All subsequent solutions were generated by dissolving 2500 µl of the previous solution into 5.0 ml DI H_2O.

In carrying out the OCT+NIS combination, different concentrations of nisin were utilized for different strains. This was a result of variable sensitivity between strains of *L. monocytogenes* when they were exposed to the compound during single antimicrobial testing. Strains Scott A and 310 were exposed to 25.00, 12.50, 6.25, 3.13, and 1.56 µg/g active OCT in combination with 0.20, 0.10, 0.05, 0.02, and 0.01µg/g NIS. Strains NADC 2783 and NADC 2045 were exposed to 25.00, 12.50, 6.25, 3.13, and 1.56 µ/g active OCT in combination with 1.56, 0.78, 0.39, 0.20, and 0.10 µg/g active NIS. All OCT solutions were generated as previously described for the OCT+ACS combination. For testing of strains Scott A and 310, a primary working solution of NIS was generated by dissolving 500 µl of the 250.0 µg/g stock solution of NIS in 10.0 ml DI H_2O. An aliquot of 781 µl of this primary working solution was dissolved in 10.0 ml of DI H_2O to create the solution needed to deliver 0.20 µg/g NIS; dilutions of 2500 µl of the previous solution into 5.0 ml of DI H_2O were used for the generation of all following solutions. With respect to testing strains NADC 2783 and NADC 2045, a primary working solution of NIS was created by dissolving 1998 µl of the 250.0 µg/g stock solution of NIS in 10.0 ml DI H_2O; the first usable solution of NIS was generated by diluting 1564 µl of this

primary solution into 10.0 ml DI H_2O. Thereafter, all solutions of NIS were made by dissolving 2500 µl of the previous solution into 5.0 ml DI H_2O.

Ninety-six well microtiter plates were again used for carrying out the checkerboard assay (Figure 13). Seventy-five µl of antimicrobial A solution (OCT in OCT+ACS and OCT+NIS) was added to the test wells in columns along with 50 µl of antimicrobial B solution (ACS in OCT+ACS and NIS in OCT+NIS) added in rows. *L. monocytogenes* inoculum in 2x TPB (125 µl) was then added to the 125 µl of combined antimicrobial solutions. Sterile barrier tips were used to load solutions into the wells so as to minimize potential for cross-contamination. Wells containing only antimicrobial-containing solutions and sterile 2x TPB were built in as negative controls for baseline correction adjustment of optical density values. Wells containing only sterile water and inocula in double-strength TPB were again built in as positive controls. Two 96 well plates were used to contain all samples in order that positive and negative controls for all wells would be located on the same plate as test wells. Figure 13 is a schematic of the design and layout of the first plate of each combination used for the checkerboard assay.

After loading plates, OD630 was measured at 0 h and 24 h using the same protocol from the single antimicrobial susceptibility tests (described above). All susceptibility tests for each combination of test antimicrobials were repeated at least twice for each strain. Wells that produced a <0.05 ΔOD630 after 24 h incubation over both replications were again classified as being inhibitory. Inhibitory combinations that utilized the least amounts of one compound in combination with the other were then classified as optimal inhibitory combinations (OIC) (Figure 14). An example of differentiation between a normal inhibitory combination and an optimal inhibitory combination would be the case of 12.50 µg/g OCT + 0.10 ml/L ACS and 12.50 µg/g OCT + 0.20 ml/L ACS. Though both are inhibitory, the former would be the OIC

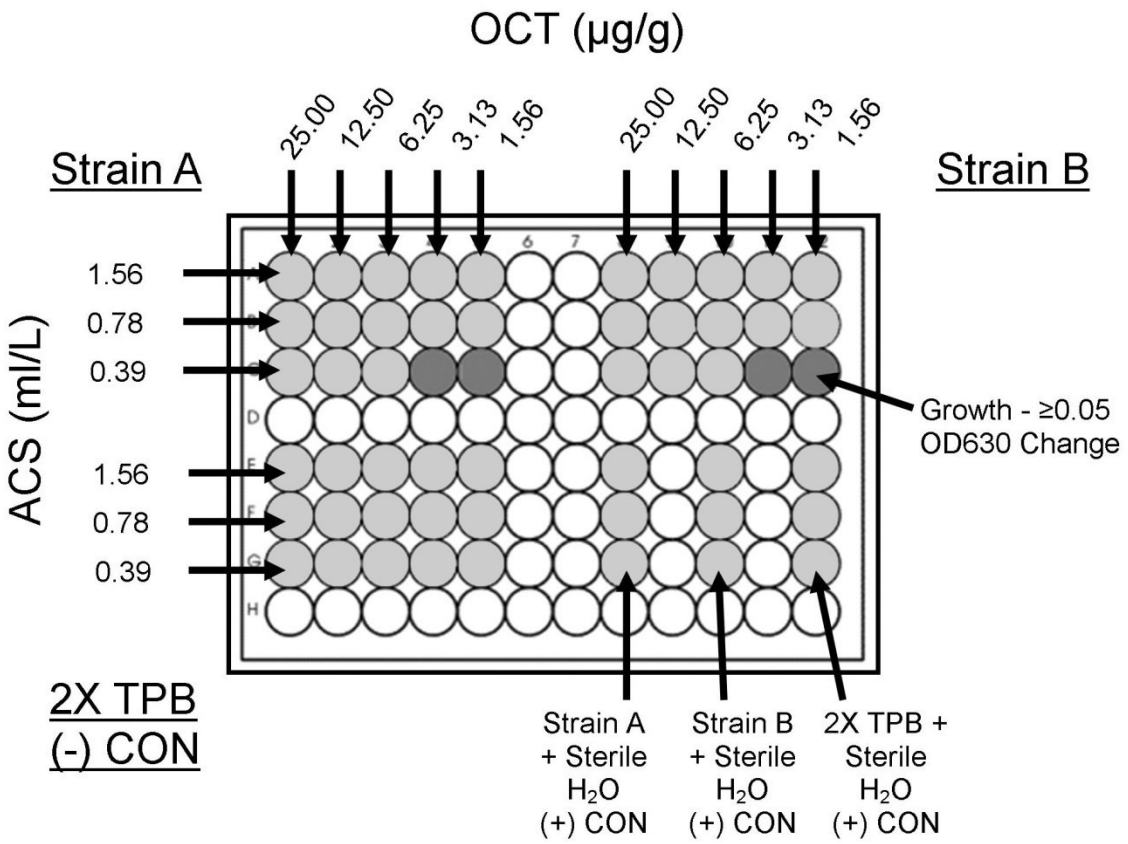

Figure 13-Checkerboard assay microplate setup and interpretation for tests at pH 5.0.

because it uses a lower concentration of ACS. The same would hold true for OCT concentrations where 1.56 µg/g OCT + 0.78 ml/L ACS would be an OIC, and 3.13 µg/g OCT + 0.78 ml/L ACS would not be an OIC. Because several combinations may work in this way, multiple OIC points for each combination are possible and often do occur. The respective concentrations of the two compounds that were added together in these OICs were the values reported.

Assessment of Antimicrobial Interactions

Fractional inhibitory concentrations were calculated by dividing the concentration of antimicrobial A needed to produce an OIC by the MIC of antimicrobial A from single antimicrobial testing (Figure 4). FIC values for each antimicrobial were plotted to form isobolograms (Parish and Carroll 1988). Isobologram plots that displayed a slope =-1 denoted

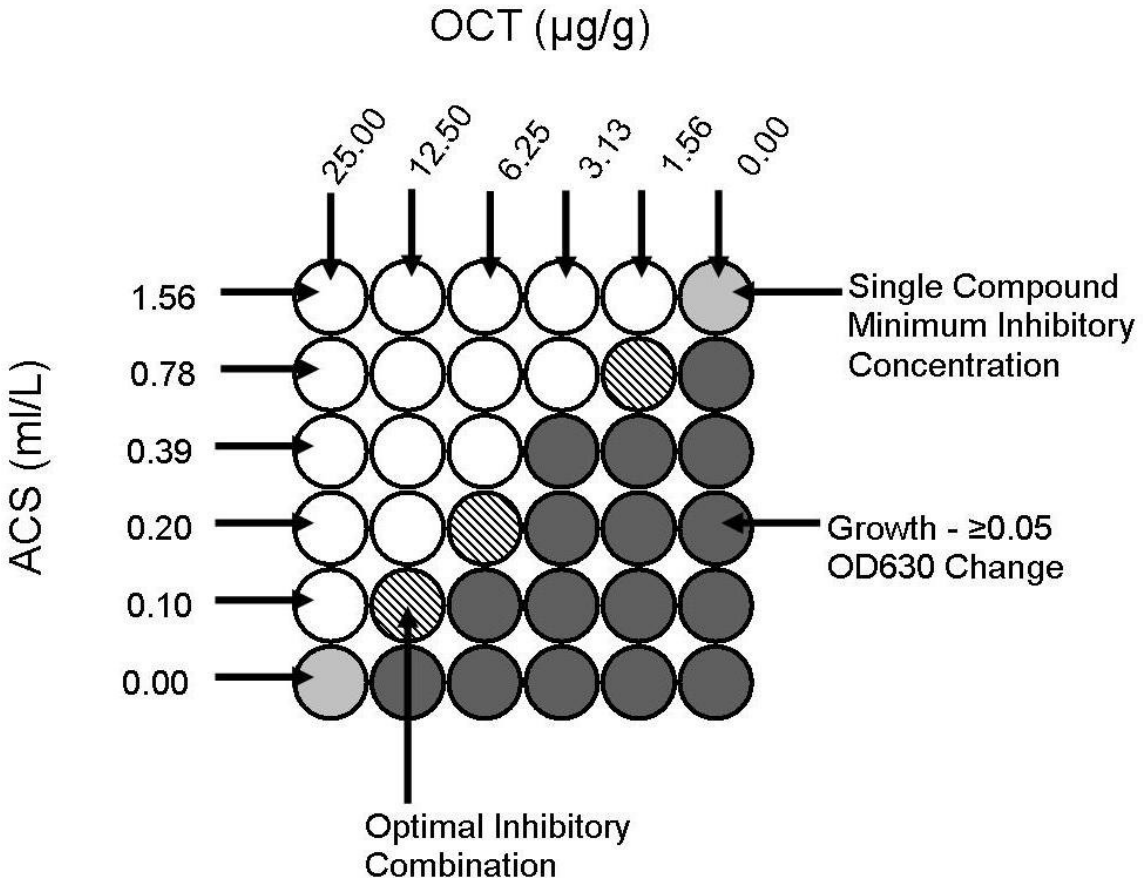

Figure 14-Schematic for determination of combined antimicrobial OICs for antimicrobial interaction characterization and isobologram construction for tests at pH 5.0.

additive interactions, while plots that curved toward or away from the origin defined synergistic or antagonistic interactions, respectively. A schematic for interpretation of isobologram shapes is given in Figure 5. FIC indices were calculated by adding the FIC of antimicrobial A for an OIC point to the FIC of antimicrobial B at the same OIC point (Figure 4). Antimicrobial interactions were defined as antagonistic (FIC_I >1), additive (FIC_I =1.0), or synergistic (FIC_I <1.0) (Branen and Davidson 2004).

Antimicrobial Bactericidal Activity

For both single antimicrobial and combined antimicrobials susceptibility testing, single antimicrobials and antimicrobial combinations were evaluated for bactericidal activity. Bactericidal activity was assessed by spreading 100 µl aliquots from wells that were classified as inhibitory onto TPA. Inoculated plates were aerobically incubated at 35 °C for 24 h and survivors were enumerated. Concentrations of single antimicrobials and antimicrobial combinations that produced at least a 3.0 $\log_{10}$ CFU/ml (99.9%) decrease in viable cells from the starting concentration of the inoculum were classified as bactericidal (Branen and Davidson 2004). The lowest concentration of antimicrobial applied alone or in combination producing bactericidal effects was used to declare the MBC. Bactericidal combinations that utilized the least amounts of one compound in combination with the other were then classified as optimal bactericidal combinations (OBC), which used the same principle for determination of optimal points as the OIC classifications. The respective concentrations of the two compounds that were added together in these OBCs were reported as the combination MBCs in the table on page 114.

Results

Pathogen Inhibition via Single Antimicrobials

The minimum inhibitory and bactericidal concentrations of experimental antimicrobials tested at pH 5.0 are given in Table 8. Significant strain-dependent sensitivity was observed with NIS-driven inhibition of experimental strains. *L. monocytogenes* Scott A and 310 were inhibited at 0.20 µg/g NIS with bactericidal effects being observed at 0.39 µg/g NIS. Conversely, *L. monocytogenes* NADC 2783 and NADC 2045 were inhibited at 1.56 µg/g NIS and 0.78 µg/g NIS, respectively; the observed MBC was 1.56 µg/g NIS for both NADC 2783 and NADC 2045 (Table 8). However, it must be noted that these values for NADC 2783 and NADC 2045 fluctuated between several repetitions, and that only consistently inhibitory and bactericidal

Table 8-Experimentally determined MICs and MBCs of antimicrobials against strains of *L. monocytogenes* at pH 5.0.[a]

Antimicrobial[b] (Units)	*L. monocytogenes*							
	Scott A		310		NADC 2783		NADC 2045	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
NIS (µg/g)	0.20	0.39	0.20	0.39	1.56	1.56	0.78	1.56
ACS (ml/L)	1.56	6.25	1.56	6.25	1.56	6.25	1.56	6.25
OCT (µg/g)	25.00	None[c]	25.00	None[c]	25.00	None[c]	25.00	None[c]

a. MIC values are determined as the lowest concentration of antimicrobial for which ΔOD630 is <0.05 after 24 h incubation at 35 °C over two replications. MBC values are determined as the lowest concentration of antimicrobial for which a 3 log cycle reduction is observed.
b. NIS- nisin, ACS- acidic calcium sulfate, OCT- octanoic acid.
c. None = No MBC obtained.

values are reported. In the case of ACS, MIC and MBC values of the antimicrobial for all *L. monocytogenes* strains were 1.56 ml/L and 6.25 ml/L, respectively. The MIC for octanoic acid against all strains of *L. monocytogenes* was 25.00 µg/g, and no experimental concentration of the antimicrobial exhibited bactericidal capacity against target pathogen strains.

Pathogen Inhibition by Combined Antimicrobials

When octanoic acid was combined with acidic calcium sulfate, no experimental combination exhibited bactericidal activity, resulting in no OBC identifications (Table 9). However, optimal OCT + ACS inhibition was seen at 12.50 µg/g OCT + 0.39 ml/L ACS for 310 and NADC 2045; 12.50 µg/g OCT + 0.20 ml/L ACS for Scott A; 12.50 µg/g OCT + 0.10 ml/L ACS for NADC 2783; 6.25 µg/g OCT + 0.20 ml/L ACS for NADC 2783; 3.13 µg/g OCT + 0.78 ml/L ACS for 310; and 1.56 µg/g OCT + 0.78 ml/L ACS for all strains except 310 (Table 9). With respect to Scott A and 310, no combinations of OCT and NIS produced optimal inhibition (lower than that expected for the compounds by themselves), and amounts greater than the MIC for NIS were required for inhibition in some cases. The combination of 12.50 µg/g OCT + 0.20

µg/g NIS was an example of a combination that produced inhibition at levels below the MIC for OCT, but since 0.20 µg/g NIS was the original MIC for NIS, the combination was not considered to be an OIC (Table 9). The pairing of 12.50 µg/g OCT + 0.20 µg/g NIS was the combination that consistently produced inhibition of strain NADC 2783 with the lowest concentrations of both antimicrobials combined. The same was the case for 3.13 µg/g OCT + 0.39 µg/g NIS against NADC 2045. However, because variations in individual NIS concentrations needed for inhibition occurred across repetitions, and at times were lower than the OIC concentrations, such values were not considered to be OIC values (Table 9). In addition, no combinations of OCT+NIS were bactericidal at levels below the MBCs of NIS for Scott A, 310, and NADC 2783. Strain NADC 2045 did show slightly lower bactericidal concentrations for NIS (0.78 µg/g as opposed to 1.56 µg/g), but such bactericidal activity occurred only when lower concentrations of OCT (>25.00 µg/g) were present in the solution. Because this cidal activity could not be maintained at higher concentrations of OCT, no bactericidal pairings of OCT+NIS were considered to be OBCs for NADC 2045 (Table 9).

Characterization of Antimicrobial Interactions

OIC values in Table 9 were transformed into FIC values for each combination using the operations previously described (Figure 4). FIC values for the OCT+ACS combination and each strain were plotted onto the isobologram on page 116 with antimicrobial A FIC values plotted against antimicrobial B FIC values. Interactions were classified based on the shape of the isobologram (Figure 5). FIC index values for each combination were obtained by adding the values of the two individual compound FIC values together. Determinations of synergism, additivism (also known as indifference), and antagonism were deduced using these values and the scale that was previously described (<1, 1, >1). Because no OIC values were able to be determined for the OCT+NIS interaction and because several combinations of concentrations

Table 9-Optimal inhibitory and optimal bactericidal combinations of antimicrobials tested at pH 5.0.[a]

L. monocytogenes Strain	Optimal Inhibitory (OIC) and Bactericidal (OBC) Combinations Antimicrobial[b] (Units)			
	OCT (µg/g) + NIS (µg/g) OIC	OCT (µg/g) + ACS (ml/L) OIC	OCT (µg/g) + NIS (µg/g) OBC	OCT (µg/g) + ACS (ml/L) OIC
Scott A	None[c]	12.50+0.20 1.56+0.78	None[c]	None[c]
310	None[c]	12.50+0.39 3.13+0.78	None[c]	None[c]
NADC 2783	None[c]	12.50+0.10 6.25+0.20 1.56+0.78	None[c]	None[c]
NADC 2045	None[c]	12.50+0.39 1.56+0.78	None[c]	None[c]

a. OICs were the inhibitory (ΔOD630 = <0.05 after 24 h incubation at 35 °C) combinations with the lowest concentrations of antimicrobial A combined with antimicrobial B. For example, though 12.50 µg/g OCT + 0.10 ml/L ACS and 12.50 µg/g OCT + 0.20 ml/L ACS are both inhibitory for NADC 2783, only 12.50 µg/g OCT + 0.10 ml/L ACS would be an OIC since it utilizes a lower concentration of ACS. OBC values were determined using the same sort of criteria but dealt with bactericidal combinations. Thus, OBCs were the bactericidal (3 log cycle reduction is observed) combinations with the lowest concentrations of antimicrobial A combined with antimicrobial B.

b. NIS = nisin, ACS = acidic calcium sulfate, OCT = octanoic acid.

c. No OICs or OBCs were obtained at tested concentrations.

permitted growth at levels above that of the MIC for NIS in Scott A and 310, no FICs, FIC indices, or isobolograms were constructed for the antimicrobial combination. In contrast, OCT in combination with ACS functioned synergistically against all strains of *L. monocytogenes*, as observed by the shape of the isobologram for all strains (Figure 15). Calculated FIC_I values for the OCT+ACS combination were 0.63 and 0.56 for Scott A; 0.75 and 0.63 for 310; 0.56, 0.38, and 0.56 for NADC 2783; and 0.75 and 0.56 for NADC 2045 (Table 10). All values were less than 1 and were indicative of synergistic interactions.

Discussion

Based on the variability of MIC and MBC results gathered for each strain from the single antimicrobials testing, it can be noted that certain predisposing factors must play a significant role in determining the sensitivity of each *L. monocytogenes* strain to nisin at pH 5.0. Strains Scott A and 310 both demonstrated a greater sensitivity to nisin than did the NADC 2783 strain and the NADC 2045 strain. Both the Scott A and 310 strains had MIC and MBC values of 0.20 and 0.39 µg/g, respectively, while NADC 2783 displayed MIC and MBC values of 1.56 and 1.56 µg/g and NADC 2045 demonstrated MIC and MBC values of 0.78 and 1.56 µg/g. Because increased tolerances to nisin have been shown to result from prior exposures to antimicrobials and acid tolerance responses (Bonnet and others 2006), strains not previously exposed to the antimicrobial were used in this study to control this effect. Yet, because varying tolerances were

Table 10-Fractional inhibitory concentration indices for antimicrobials tested at pH 5.0.[a]

L. monocytogenes Strain	Combination Fractional Inhibitory Concentrations Antimicrobial[b] (Units)	
	OCT (µg/g) + NIS (µg/g)	OCT (µg/g) + ACS (ml/L)
Scott A	None[c]	0.63
		0.56
310	None[c]	0.75
		0.63
NADC 2783	None[c]	0.56
		0.38
		0.56
NADC 2045	None[c]	0.75
		0.56

a. FIC values were determined dividing concentrations comprising OICs combinations by their respective MICs from single antimicrobial testing. FIC values were then added to create the FIC_I values shown above.
b. NIS- nisin, ACS- acidic calcium sulfate, OCT- octanoic acid.
c. None = No FIC values were able to be calculated because no MIC values were obtained from single antimicrobial testing.

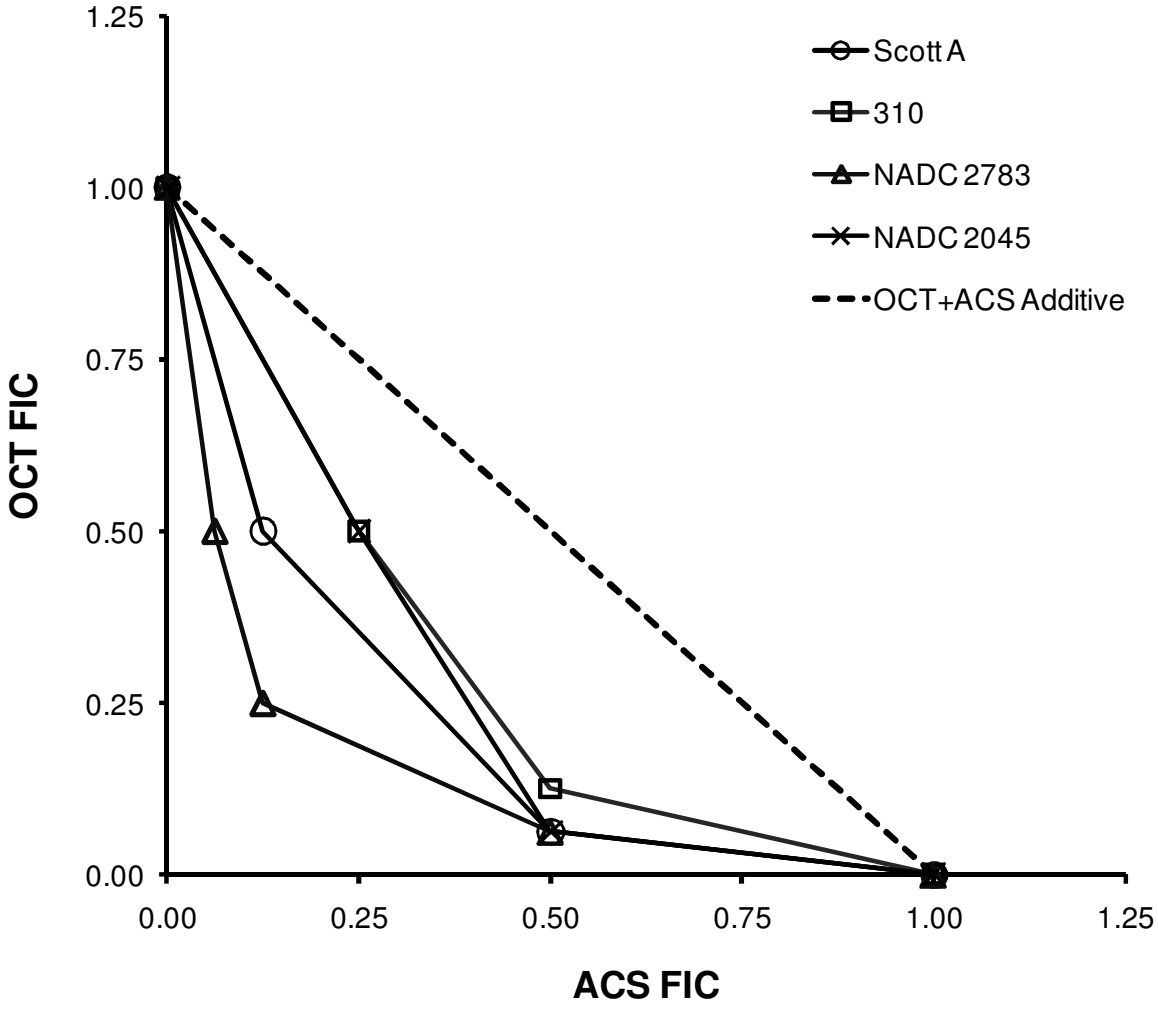

Figure 15-Isobologram of antimicrobial FIC values for Octanoic Acid (OCT) in combination with Acidic Calcium Sulfate (ACS).
An FIC was defined as the OIC concentration of antimicrobial A divided by the MIC of antimicrobial A from single antimicrobial susceptibility testing. FIC values are ratios and are therefore unitless.

observed, the data seems to suggest that strain-specific factors, rather than stress responses, must have led to the divergences in sensitivity. It has been shown that some strains do possess an innate potential to form nisin-resistant mutants over others (Davies and Adams 1994) and to do so with a high degree of frequency (Harris and others 1991). Such decreases in nisin sensitivity may be afforded by the ability of certain strains to rapidly counteract the activity of nisin by creating shifts in membrane fatty acid profiles from more fluid unsaturated fatty acids to less fluid straight-chain fatty acids (Ming and Daeschel 1993; Mazzotta and Montville 1997; Crandall and Montville 1998). Some studies have shown that such disparities among strains can produce nearly 8-fold differences in MIC values for nisin (Mohamed and others 1984). Taking this into account, the variable nisin results seem to be in agreement with previous research.

In contrast to nisin, no differences in MIC and MBC values across strains were observed for ACS or OCT. Variances in sensitivity to such compounds usually stem from some sort of inherent acid resistance (Samelis and Sofos 2003), which leads to production of other stress factor proteins to counteract the damage of other stresses (Farber and Pagotto 1992; Leyer and Johnson 1993). The fact that these variations in acid response across strains were not seen provides further suggestion that strain-specific factors, rather than stress responses, led to the variability with nisin sensitivity. Though the inability of low levels of octanoic acid to produce bactericidal effects at experimental conditions was a concern related to decreased sensitivity toward the fatty acid, previous research with similar experimental parameters has shown that such behavior is not related to an abnormal tolerance. Nair and others (2004) demonstrated that 25 mM octanoic acid was unable to produce bactericidal effects (>3.0 log cycle decrease) on a 5-strain mixture of *L. monocytogenes* grown in milk at 37 °C after 24 h. Thus, even though the compound can produce consistent inhibition, the lack of a complete bactericidal effect is in agreement with previous research and is likely not due to a stress response.

In the analysis of antimicrobial interactions from combinations testing, the OCT+NIS combination presented unique challenges in the interpretation of its interaction type. As previously noted, growth of strains Scott A and 310 occurred at the 0.20 µg/g MIC of nisin for both Scott A and 310 when the bacteriocin was used in combination with several sub-MIC concentrations of OCT. This data seemed to suggest antagonistic activity exists between the two antimicrobials. Additional inconsistencies in the MIC of nisin for the NADC 2783 and NADC 2045 strains when paired with OCT demonstrated the activity of the bacteriocin might be made inconsistent when paired with the organic acid solution. Though irregularities in each strain's sensitivity to nisin could have caused the interaction to appear antagonistic (MICs of nisin alone varied over repetitions for each strain), the disturbance of nisin activity in the presence of fatty acids has been observed before (Henning and others 1986b). Likewise, deactivation of nisin activity by the other minor components of the OCT solution, such as the emulsifiers polysorbate 80 and polysorbate 20, must also be taken into account. Indeed, the emulsifiers Lamegin DW 8000 Schuppe, DK Ester F160, Lamegin NSL, and Lamegin ZE 30 Schuppe and Monomuls 90-25 have all been shown to have an antagonistic effect on the activity of nisin (Henning and others 1986a). Though addition of polysorbate 80 has been shown to counteract loss in activity of nisin in broth and fluid milk (Jung and others 1992; Bhatti and others 2004), the effect of polysorbate 20 on nisin varies between potentiation (Li and others 2002) and deactivation at certain concentrations (Castro and others 2009). Thus, since it is unknown whether actual interactions between octanoic acid itself and nisin produced the antagonistic effect observed, or whether such responses were due to interference by secondary components of the OCT solution, a conclusive interaction type cannot be assigned to the OCT+NIS pairing. Rather, modified procedures should be used to better characterize the interaction and to understand the true effect obtained by pairing the two antimicrobials.

In contrast to OCT+NIS, the pairing of octanoic acid and acidic calcium sulfate yielded synergistic activity in terms of inhibition of the pathogen. Since all FIC_I values were significantly lower than 1, and as low as 0.38 for some strains, the interaction between the two compounds was highly characteristic of synergism. As a result, the compounds would be expected to behave in a manner that yields a greater effect than the sum of the effects observed when the two antimicrobials are used separately to inhibit growth of the pathogen (Barry 1976); thus, an enhancement of the individual inhibitory activities would be observed. However, the synergistic effect of the combination was not evident in the bactericidal responses produced by the two compounds at sub-MBC levels for each strain, as no combinations OBCs were reached. Because no concentration of OCT had been bactericidal during single antimicrobial testing, this was not unexpected.

Though drawing decisive conclusions on the specific mode of action of the two compounds in combination is beyond the scope of this study, suggestions for the synergistic activity observed for the OCT and ACS pairing can be raised. Fatty acids, like octanoic acid, have been shown to pass through the bacterial membrane more easily in their protonated forms and thus be more active (Cramer and Prestegard 1977; Eklund 1983). The ACS solution possesses a unique mechanism to reprotonate its constituent lactic acid and continually challenge cells by producing protonated organic acids (Kemp and others 2003). Thus, one suggestion for the observed synergism is that the same component that is used by the ACS solution to regenerate the protonated form of lactic acid may also act on the octanoic acid to reprotonate it, allow it to work at an even greater extent, and yield an enhanced effect. Also, because the OCT+ACS combination entails the use of two organic acids, which inhibit cell growth by creating disturbances in proton motive force by acidifying the cellular cytoplasm (Salmond and others 1984), a second suggestion is that the simultaneous attack of the two agents may have

produced a level of stress on proton motive force that was unable to be overcome by the microorganism. Though such a mechanism would not entail the targeting of multiple cellular sites to produce starkly different stresses upon the cell, it is possible that the cumulative effect on this one means of cellular energy balance may be enough to cause severe homeostasis disruption. Postulations such as these must be tested in future research to correctly elucidate the inhibitory mechanism of the OCT and ACS combination.

CHAPTER VI

CONCLUSIONS

Conclusions from Assays at pH 7.3

This study investigated the MIC and MBC of five food-approved antimicrobials (nisin, sodium lactate, ε-Poly-L-Lysine, lauric arginate ester, and acidic calcium sulfate) that resulted when *L. monocytogenes* strains were exposed to them *in vitro* at pH 7.3. Strain-dependent sensitivities were noted in the MICs and MBCs of nisin and ε-Poly-L-Lysine, while acidic calcium sulfate and lauric arginate ester showed no signs of strain-dependent tolerances, and sodium lactate was unable to produce inhibition under the test conditions utilized.

Results from antimicrobial testing indicate that nisin and acidic calcium sulfate interact synergistically to inhibit growth of *L. monocytogenes in vitro.* Such synergism is likely due to protonation of the nisin from the organic acid component of ACS, and from simultaneous distress imposed on the proton motive force of the cells. Thus, using lower concentrations of the two compounds in combination is able to produce the same or greater effect than using the compounds individually. Results from combinations testing also indicate that pairings of EPL with ACS and nisin with LAE interact in an additive manner. Therefore, replacing a particular amount of one antimicrobial with a proportional amount of the other antimicrobial will result in the same effect as using either compound individually. The interference in EPL bactericidal activity as a result of combinations with certain concentrations of ACS should also be investigated in further detail to determine the cause for the decreased activity of the pair. The combination of SL+ACS was unable to conform to the limitations of the procedure to produce a conclusive classification, but because interactions were noted between the two compounds, the combination might be considered additive/indifferent in nature if modified testing is performed.

Conclusions from Assays at pH 5.0

This study investigated the minimum inhibitory concentrations and minimum bactericidal concentrations that resulted when *L. monocytogenes* strains were exposed to three food-approved antimicrobials (nisin, octanoic acid, and acidic calcium sulfate) *in vitro* at pH 5.0. Strain-dependent sensitivities were noted in the MICs and MBCs of nisin, while acidic calcium sulfate and octanoic acid showed no signs of strain-dependent tolerances under the test conditions utilized. No bactericidal effect was achieved using the OCT solution alone.

Results from antimicrobial combinations testing indicate that octanoic acid and acidic calcium sulfate are able to synergistically interact to inhibit growth of *L. monocytogenes in vitro.* Thus, using lower concentrations of the two compounds in combination is able to produce the same or greater effect than using the compounds individually. The combination of OCT+NIS appeared to be antagonistic in nature, but because of procedural limitations and uncertainty about interactions with secondary components of the antimicrobial solution, a conclusive classification was not deduced for the combination. Therefore, further examinations and modifications of test procedures are necessary to correctly identify the type of interaction that is observed when octanoic acid and nisin are paired.

Application of Findings

Overall, this research provides valid suggestions for antimicrobial usage conditions for the inhibition of *L. monocytogenes* on RTE meats. However, because these results only demonstrate the *in vitro* activity of the compounds against the pathogen and potential interactions between antimicrobials and food matrices can occur, *in vivo* applications of the antimicrobial pairings must be completed to validate their effectiveness on the surfaces of RTE meats. Also, because differences due to pH and temperature were not used as variables in this experimental design, studies that take these factors into account as variables would also be

avenues for future research. Thus, future research should focus on the application of synergistic pairings like NIS+ACS and OCT+ACS on the surfaces of RTE meat products to help improve their safety, and should investigate the effect that temperature and pH have on the interactions of the combinations

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Education
B.S. (Food Science and Technology), Texas A&M University, May 2008
M.S. (Food Science and Technology), Texas A&M University, Dec 2009

Publications and Reports
Anding JD, Dittmar RS, **Brandt AL**. 2009. Food safety: it's our business. College Station, TX: Texas Agrilife Extension Service. 74 p.

Brandt AL, Harris KB, Castillo A, Hardin MD, Keeton JT, Taylor TM. 2009. Efficacy of novel food antimicrobial combinations for control of *Listeria monocytogenes* for preservation of ready-to-eat (RTE) products. Des Moines, IA: National Pork Board. 17 p.

Recent Scientific Presentations
Brandt AL, Hardin MD, Castillo A, Harris KB, Keeton JT, Taylor TM. 2009. *In vitro* inhibition of *Listeria monocytogenes* with acidic calcium sulfate combined with nisin or ε-polylysine. Poster Presentation. P3-57. International Association for Food Protection Annual Meeting, Grapevine, TX: July 12-15.

Brandt AL, Hardin MD, Castillo A, Harris KB, Keeton JT, Taylor TM. 2009. *In vitro* inhibition of *Listeria monocytogenes* exposed to octanoic acid and acidic calcium sulfate alone and in combination. Oral Presentation. T2-03. International Association for Food Protection Annual Meeting, Grapevine, TX: July 12-15.

Professional Affiliations
Institute of Food Technologists
International Association for Food Protection
Phi Tau Sigma Food Science Honorary Society

Academic and Professional Honors
First Place, Microbiology Poster, Twelfth Annual Texas A&M University Student Research Week, College Station, TX, March 27, 2009

Recipient, 2008 Texas Extension Association of Family and Consumer Sciences Food Safety Award

Recipient, 2007 Texas A&M University Outstanding Senior in Food Science and Technology Award