12-14-2016

Determining the Efficacy of Antimicrobials for the Inhibition and Inactivation of Listeria monocytogenes in Broth, Whole Milk, and Fresh Cheese.

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B.S., Cornell University, 2015

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

At the

University of Connecticut

2016

APPROVAL PAGE

Masters of Science Thesis

Determining the Efficacy of Antimicrobials for the Inhibition and Inactivation of *Listeria monocytogenes* in Broth, Whole Milk, and Fresh Cheese.

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2016

Dedication

This thesis is hereby dedicated to my beautiful little babies, especially Mr. Meatball, may he rest in peace. Of all the things in life that are precious and good, Meaty reigned superior. Blessed to have the honor of being his mama, I can say with my whole heart that his hairlessness and wrinkles are just a few of his unique qualities that made him the most beautiful creature in the world. Kind, caring, and loving, Meaty never failed to comfort me throughout the best and worst of times. His snuggles were the warmest, his purrs the loudest, and his naps the longest. After a long day in the lab, being able to count on the sound of his tiny feet running to the door was what I looked forward to most. If I asked him to nap with me, he never said no, and did not get up from the nap for food until I was also ready for food. This work would not have been possible without his unconditional love and support, and for that I am forever grateful. His brother (Doom) and sisters (Tinky and Shadow Girl) have assisted with snuggles and always know how to put a smile on my face, especially in the time since Meatball's passing. An infinite number of thank-yous to these four impeccable creatures, my babies.

Acknowledgements

First and foremost, I would like to thank my major advisor Dr. Dennis D'Amico and our lab group. As a whole, the D'Amico lab group has been instrumental in my success. My fellow graduate student, Stephanie Barnes, has provided me with every form of support a person could need. Whether it be helping me study, providing helpful tips from her own Master's education experience, watching my cats, or relaxing after a hard week, Stephanie's shoulder is always there to lean on and I would not have been able to complete my education without her continuous support. Our lab group has been blessed with some wonderful undergraduate assistants, all of whom I would like to thank, especially Yustyna Bobak and Emily Forauer, two bright young scientists who will certainly be lifelong friends.

I would like to thank the Department of Animal Science for having me and supporting my education, especially my committee members Dr. Kumar Venkitanarayanan and Dr. Mary Anne Amalaradjou, not only for advising me on my research and thesis but for adding a diverse range of students and research to the department from which I have learned an immeasurable amount. Furthermore, to Dr. Steven Zinn, Tina Burnham, and the rest of the main office members for providing technical support, friendly faces, and financial assistance. A very special thank you to Bill Sciturro and the UConn Creamery for the production of the cheese used in experiments.

Aside from faculty and students at UConn, my success is largely attributed to the support of my friends and family. My family has always supported and encouraged my endeavors, especially my mom, dad, and brothers Alex and Kyle; graduate school was no exception. Voicemails, long phone calls, and care packages from my Grammie became my constant connection to home and my solace in times of stress. A material scientist, physicist, and cheerleader, Alexis Ernst has been an absolute inspiration, and conveniently located across the

hall. Always putting her well-being first, she exemplifies the most important kind of love, self-love, and for allowing me to learn from her I am forever grateful and hope that I will continue to keep learning after my time at UConn has ended. My many friends from Rochester, Irondequoit, and Cornell, I do not have the space to list, but you know who you are (especially you, Katy Zuchowski) and you are so very important to me. To Dr. Anne-Lise Smith, thank you for seeing me, hearing me, and allowing me to grow in ways that have forever changed me; I will carry the lessons you have taught me throughout my entire life.

Lastly, I would like to thank my husband, Ryan Kozak-Weaver as well as Lucy, Thomas, and Julia for being the most open-hearted, loving, and awesome children. Words cannot begin to express how important Ryan been throughout this process. Encouraging, patient, and caring, he is my most valuable person, and I hope he knows how much I love him from the bottom of my heart (of course he does).

List of Tables and Figures

Chapter V

List of Abbreviations

ACSL	Acidified Calcium Sulfate with Lactic Acid
ACSP	Acidified Calcium Sulfate with Propionic Acid
BR	Beta-Resorcylic Acid
BHI	Brain Heart Infusion
BLEB	Buffered *Listeria* Enrichment Broth
BPB	Butterfield's Phosphate Buffer
CA	Caprylic Acid
CDC	Centers for Disease Control and Prevention
CFR	Code of Federal Regulations
CFU	Colony Forming Units
CCP	Critical Control Point
DE	Dey-Engley Broth
DMSO	Dimethyl Sulfoxide
EAFUS	Everything Added to Food in the United States
EPL	ε-Polylysine
FDA	Food and Drug Administration
FBC	Fractional Bactericidal Concentration
FBC_I	Fractional Bactericidal Concentration Index
FIC	Fractional Inhibitory Concentration
FIC_I	Fractional Inhibitory Concentration Index
GRAS	Generally Recognized as Safe
GMP	Good Manufacturing Practices
HP	Hydrogen Peroxide
LAE	Lauric Arginate Ethyl Ester
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
MOX	Modified Oxford Agar
NIFA	National Institute of Food and Agriculture
PDA	Plant-derived Antimicrobials
QF	Queso Fresco
RTE	Ready-to-Eat
SC	Sodium caprylate
SDW	Sterile Deionized Water
TSB	Tryptic Soy Broth
TPB	Tryptose Phosphate Broth
UHT	Ultra-high-temperature Pasteurized
USDA	United States Department of Agriculture

Abstract

Listeria monocytogenes is a bacterial pathogen that causes listeriosis, an illness that is the third-leading cause of death related to foodborne illness. Antimicrobial compounds are currently being investigated as a method for inactivating and/or inhibiting the growth of *L. monocytogenes* in foods, with the ultimate goal of reducing recalls, illnesses, and outbreaks. Based on promising results in other ready-to-eat foods, the efficacy of acidified calcium sulfate with lactic acid (ACSL), beta-resorcylic acid (BR), caprylic acid (CA), ε-polylysine (EPL), hydrogen peroxide (HP), lauric arginate ethyl ester (LAE), and sodium caprylate (SC) was determined in broth, milk, and fresh cheese. Antimicrobials were tested alone and in binary combination in Brain Heart Infusion (BHI) broth over 24 hours, in ultra-high-temperature pasteurized (UHT) milk over 21 days, and/or through application of aqueous dips to fresh cheese [Queso Fresco (QF)] that was then stored for 35 days. Binary combinations were tested to identify antimicrobial interactions, especially synergism, which can reduce usage rates, cost, and potential sensorial changes. Overall, HP was the most efficacious single treatment with concentrations of 40-50 ppm inhibiting *L. monocytogenes* in broth, 200 ppm resulting in undetectable pathogen counts in milk, and a 5% dip treatment resulting in <1 log CFU/g of *L. monocytogenes* recovered from QF. Interactions in broth were characterized using Fractional Inhibitory and Bactericidal Concentration indices (FIC_I and FBC_I, respectively), with an index of <0.5 denoting synergy. A total of thirteen synergistic combinations were identified in broth initially adjusted to either pH 7.4 or 5.5. Sodium caprylate was the most common antimicrobial identified among effective combinations. The combination of EPL + LAE was the most synergistic combination for the inactivation of *L. monocytogenes* in broth at pH 7.4, with an FBC_I of 0.292. Effective concentrations of EPL and LAE increased approximately ten-fold in milk with inhibitory concentrations of 200 ppm and 800 ppm,

respectively, whereas CA and SC produced inhibition at similar concentrations in both media. The most effective antimicrobial pairings for controlling *L. monocytogenes* in QF were ACSL+SC, LAE+SC, and EPL+SC. These combinations resulted in significantly lower counts of *L. monocytogenes* after 35 days of storage compared to the control and their respective individual treatments. These results together identify antimicrobial treatments effective at reducing and/or inhibiting the growth of *L. monocytogenes* in varying conditions and matrices and serve as a basis for additional applications in food.

Chapter I

Introduction

According to the Centers for Disease and Control Prevention (CDC), an estimated 47.8 million cases of foodborne illness occur each year. Among the 31 specified foodborne pathogens, *Listeria monocytogenes* infections result in approximately 1600 illnesses and 260 deaths per year (CDC, 2014). The illness caused by *L. monocytogenes* infection is known as listeriosis. Listeriosis can lead to meningitis, septicemia, and pregnancy complications such as still birth and is the third leading cause of death related to foodborne illness. Groups that are at high-risk for infection include pregnant women, children, the elderly, and the immunocompromised (CDC, 2014).

In the past five years, there have been twelve documented outbreaks of listeriosis, most often linked to the consumption of dairy products (cheese, ice cream, raw milk) and produce (cantaloupes, salad, sprouts). The diversity of foods involved in outbreaks shows the ability of *L. monocytogenes* to adapt to different food matrices and their storage environments. *Listeria monocytogenes* is particularly robust; it can survive and grow at temperatures less than 0˚C (Vos et al., 2011), moderately acidic pH (4-5), and high salinity environments (Schirmer et al., 2014). Several *L. monocytogenes* outbreaks have been directly linked to fresh pasteurized soft cheeses. In 2015 a multistate outbreak resulted in 30 recorded cases of listeriosis, in which the *L. monocytogenes* strains isolated in cases matched strains isolated from environmental swabs taken from the processing facility (CDC, 2015). Also, in 2014 a multistate outbreak of *L. monocytogenes* linked to soft cheese resulted in eight cases of reported illness. Strains isolated from patients matched those isolated from the processing facility (CDC, 2014). While raw milk can be contaminated with the pathogen directly (Sanaa et al., 1993), these outbreaks were a result of post-pasteurization contamination due to the presence of *L. monocytogenes* in the processing facility (Almeida et al., 2013).

Within the processing facility *L. monocytogenes* persists in areas such as drains, floors, and worker footwear (D'Amico & Donnelly, 2009). Molecular typing of isolates has shown that the same strains of *L. monocytogenes* can persist in food processing facilities for long periods of time, ranging from 2 years (D'Amico & Donnelly, 2009) to as long as 12 years (Orsi et al, 2008), displaying the resilient nature of the pathogen. Once contaminated, dairy products with certain desirable growth characteristics allow *L. monocytogenes* populations to grow. Mexican-style soft cheeses such as Queso Fresco (QF) have been implicated in several outbreaks and have been shown to support the growth of *L. monocytogenes* due to their high moisture content, pH >6.0, and low salt content (Van Hekken et al., 2012). Studies have demonstrated that *L. monocytogenes* can grow to levels as high as 8 log CFU/g in as quickly as 7 days at 10˚C (Leggett et al., 2012), as well as in QF stored at proper refrigeration temperature (4˚C). Microbial risk assessments suggest that aside from preventing contamination, the best method for reducing illness occurrence is by limiting *L. monocytogenes* growth in contaminated foods (Chen et al., 2003). Thus, there is a need to identify treatment methods that can effectively reduce levels of *L. monocytogenes* and inhibit its growth in foods.

The use of antimicrobial compounds to control the growth of *L. monocytogenes* has been effective in the ready-to-eat (RTE) meat and poultry industries. The application of antimicrobial compounds is a method that can be applied after lethality steps such as pasteurization in an effort to control post-pasteurization contamination. Although other lethality approaches such as high-pressure processing have been shown to initially reduce *L. monocytogenes* levels, regrowth of the pathogen over the length of storage may occur (Koseki et al., 2007; Turgis et al., 2012) further highlighting the need for post-lethality treatments. Several antimicrobials have demonstrated potential for controlling *L. monocytogenes* either alone or in binary combinations including

acidified calcium sulfate (Brandt et al., 2011), beta-resorcylic acid (Upadhyay et al., 2014), caprylic acid (Gadotti et al., 2014), sodium caprylate (Almeida et al., 2013), ε-polylysine (Geornaras et al., 2007), hydrogen peroxide (Upadhyay et al., 2013), and lauric arginate ethyl ester (Soni et al., 2010).

The specific objectives of this research are as follows:

1)
    a. Determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of individual antimicrobials against *Listeria monocytogenes* in a broth system at pH 7.4 or 5.5.
    b. Determine the inhibitory and bactericidal concentrations of antimicrobials against *Listeria monocytogenes* when applied in binary combinations in a broth system at pH levels 7.4 and 5.5.
    c. Characterize antimicrobial interactions as synergistic, additive, or antagonistic.
2) Determine the efficacy of antimicrobials treatments to control *L. monocytogenes* in whole milk during refrigerated shelf life.
3) Determine the efficacy of antimicrobial dip treatments to control *L. monocytogenes* as surface contaminants on fresh cheese.

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Chapter II

Review of the Literature

1. *Listeria monocytogenes*

1.1. Microbiology/Detection

The taxonomy of *Listeria monocytogenes* is as follows: Kingdom Bacteria, Division Firmicutes, Class Bacilli, Order Bacillales, Family Listeriaceae, Genus *Listeria* (Vos et al., 2011). Within the genus of *Listeria*, only two species are known to be pathogenic to animals. *Listeria. ivanovii* is pathogenic to a wide variety of animal species whereas *L. monocytogenes* is the only *Listeria* spp. that is pathogenic to humans and is therefore the microorganism of concern in terms of human food safety. *Listeria monocytogenes* was first classified by E.D.G Murray under the name *Bacterium monocytogenes* (Murray et al., 1926), and was later changed to the name "*Listeria"* by J.H. Pirie (Pirie, 1940). *Listeria monocytogenes* strains are further classified into serotypes based on somatic (O) and flagellar (H) antigens (Weis & Seeliger, 1975). Currently there are two major phylogenetic divisions of serotypes, with a third consisting of less common serotypes (Borucki & Call, 2003). Of the identified serotypes, studies have found that >90% of clinical cases of listeriosis involve serotypes 1/2a, 1/2b, and 4b (Tappero et al., 1995), with the majority of genetic differences being attributed to phage insertions, transposable elements, and single nucleotide polymorphisms (Nelson et al., 2004). The genus exists as short rods, usually 0.4-0.5µm x 1-2 µm. When stained, *L. monocytogenes* is Gram-positive and not acid fast. Filaments may be formed, but only in cultures that are grown at temperatures <30˚C (Vos et al., 2011). *Listeria monocytogenes* is a facultative anaerobe with an optimal growth temperature of 30-37˚C. However, it is capable of growing from the range of <0 to 45˚C (Vos et al., 2011). This is particularly important in regards to foodborne illness because it facilitates the growth and survival of *L. monocytogenes* in foods at refrigeration temperatures (4-7˚C), which is a common method of food preservation. The optimal pH level for growth is between 6 and 9, although survival has been

demonstrated at pH levels of 4.5-5.0 (Schirmer et al., 2014). *Listeria monocytogenes* also has the ability to grow in environments with salt concentrations as high as 10% (Vos et al., 2011) and survive at 20% and higher (Schirmer et al., 2014).

1.2. Pathogenicity

The virulence factors that result in pathogenesis are controlled by the transcriptional regulator PrfA, (Mandin et al., 2005). When *L. monocytogenes* colonizes the gut through cell adhesion involving the Ami protein (Milohanic et al., 2004), it is able to enter cells using two internalization proteins known as internalin A (InlA) and B (InlB). While both are involved in entry into epithelial cells, InlA in its non-truncated form has been found in approximately 96% of *L. monocytogenes* strains isolated from foodborne outbreaks (Jacquet et al., 2004), suggesting its importance in virulence and human listeriosis. Once inside the cell, *Listeria* are able to grow intracellularly using hemolysin (Portnoy, 1988) and evade vacuoles through the secretion of listeriolysin O (LLO) (Cossart et al., 1989) and PlcA (Camilli et al., 1993). Once it has escaped, *L. monocytogenes* uses actin protein to move from cell to cell, triggering an immune response that is the basis for many of the initial symptoms of illness.

Listeriosis is the illness that occurs when *L. monocytogenes* infects both humans and animals. As soon as hours after consumption, flu-like symptoms are the first indication of listeriosis. Without proper treatment listeriosis may manifest itself in a number of ways, including but not limited to meningitis, encephalitis, septicemia, intrauterine infection, and death (Batt, 2014). Approximately 1600 cases are recorded in the United States each year, with approximately 260 of those cases resulting in mortality (CDC, 2014). The most commonly affected groups include children, the elderly, the immunocompromised, and pregnant women and their fetuses.

Listeria monocytogenes is notable amongst foodborne pathogens in that it has one of the highest mortality rates, third only to *Salmonella* and *Toxoplasma gondii* (FDA, 2014). While the infectious dose of *L. monocytogenes* is not completely known, it is believed to vary based on strain type and susceptibility of the host. However, for severely immunocompromised individuals, less than 1000 cells are needed to cause an infection (FDA, 2016). Infectious dose has also been speculated to be dependent upon the food matrix upon which it grows. For example, *L. monocytogenes* grown in foods with high salt concentrations at ambient temperatures results in a high rate of intracellular invasion by the pathogen (Garner et al., 2006). Environmental stressors such as salt and pH can influence how virulent the specific bacteria become. For example, adaptation to a low pH before ingestion increases the ability of *L. monocytogenes* to survive the stomach acid (Conte et al., 2002).

1.3. Listeria in the Environment

There are several ways that *L. monocytogenes* enters the food system, starting in the natural environment and continuing into the processing facility itself. Within the food industry, so-called "critical control points" (CCP) are designated to identify points within the processing protocol where foods are at high-risk for contamination by biological, physical, and/or chemical agents. At these CCPs, action is taken to prevent or eliminate contamination. In raw milk, pasteurization is not performed and as a result any existing *L. monocytogenes* are not destroyed. Even though pasteurization eliminates pre-existing *L. monocytogenes* in pasteurized products, post-processing contamination is not uncommon due to the presence of *L. monocytogenes* in processing facilities.

In nature, *L. monocytogenes* is ubiquitous, present in soil, water, feeding grounds, and feces (Weis & Seeliger, 1975). The presence of *L. monocytogenes* in a food processing environment begins with transmission of the pathogen from an outside source, such as from a farm environment.

When a food-processing facility is directly adjacent to a farm (such as with production of farmstead cheeses), *L. monocytogenes* that is present on the farm can easily be transmitted to the production facility. If the facility is not near a farm, the pathogen can be brought into the facility via fomites such as worker boots. Researchers studying the presence of *L. monocytogenes* in dairy facilities have shown that the pathogen may be harbored in drains, doorways, and footwear (D'Amico & Donnelly, 2009). Through the use of molecular typing, strains of *Listeria* have been found to persist in dairy processing facilities for as long as 12 years (Orsi et al., 2008). Without proper good manufacturing practices (GMPs) in place, the pathogen can subsequently contaminate food products. One way in which *Listeria* can persist in food processing facilities is through biofilms. A biofilm is a community of bacteria that has attached itself to a surface (O'Toole et al., 2000). In a food processing facility, biofilms can form on a number of different surfaces including polyester floor sealant, nylon, and stainless steel (Alessandria et al., 2010; Blackman & Frank, 1996). With inadequate sanitation practices, biofilms pose a substantial risk for post-processing contamination.

1.4. Listeria in Dairy Foods

According to the Centers for Disease Control and Prevention (CDC), there have been seven recorded listeriosis outbreaks in dairy foods since 2011 (CDC, 2016). In addition, there have been at least 10 recalls associated with *L. monocytogenes* contamination in dairy foods in 2016 alone (FDA, 2016). Soft cheese including the Mexican-style varieties such as Queso Fresco are disproportionately implicated. "Queso Fresco" translates to "fresh cheese", and is considered a high-risk food for *L. monocytogenes* contamination and outbreaks. Studies have shown that *L. monocytogenes* can grow to levels of 8-9 log CFU/ml in milk (Ma et al., 2013) and Queso Fresco (QF) (Gadotti et al., 2014), even when stored at refrigeration temperature. As described by Van Hekken et al. (2012), QF is one of the most popular fresh cheeses in the United States. It is bright

white, crumbles easily, has a mild "fresh-milk" flavor, and does not melt well. The composition of this cheese varies, but ranges from 46-57% moisture, 18-29% fat, 1-3% salt, and has a pH >6.1 (Van Hekken & Farkye, 2003). In the US, Queso Fresco must be made with pasteurized milk in order to be sold across state lines. However, raw milk regulations vary by state, so although the use of pasteurized milk reduces risk of pathogenic outbreaks, it is not necessarily required (Gould et al., 2014). One of the first and most notable outbreaks linked to QF was in 1985 in Los Angeles County, California. In total, 142 cases of listeriosis were reported, 93 of which were pregnant women or their fetuses. Forty-eight resulted in death (Linley et al., 2012). The company responsible for this outbreak had been using non-pasteurized milk in addition to their pasteurized milk. Upon investigation of the dairy plant, it was uncovered that unlicensed employees had been pasteurizing the milk. This investigation also confirmed presence of *Listeria* in the processing environment. This historical case brought attention to *L. monocytogenes* in dairy products and began a national effort to prevent future outbreaks. In 1990, a study was conducted in California to look at Mexican-style soft cheeses across the state, tracking the presence of pathogens and the characteristics of the cheeses in which these pathogens were detected (Genigeorgis et al., 1991). Across 100 cheeses sampled, 31 were determined to have been made from improperly pasteurized milk, which is known to harbor *Listeria* (Lovett et al., 1987). The average salt concentration of these samples was 1.87% and the average moisture content was 50.09%. A total of four samples were positive for *Listeria*, and the cheese samples positive for *Listeria* ranged in pH from 6.1-6.5. This study elucidates the risk factors associated with the survival and growth of *L. monocytogenes* in Mexican-style soft cheeses: high-moisture, low salt content, and near-neutral pH.

1.5. Methods for detection in foods

The standard method for the detection of *L. monocytogenes* in foods in the US is described in the FDA's Bacteriological Analytical Manual (BAM) (FDA, 2016). For a given sample, 25g is mixed with 225ml of Buffered Listeria Enrichment Broth (BLEB) with pyruvate. After homogenization by stomaching, the sample is incubated at 30˚C for 4 hours; after 4 hours three selective supplements are added (cycloheximide, acriflavin, and nalidixic acid). The sample is mixed and incubated at 30˚C for the remaining part of 24 to 48 hours. At 24 and 48 hours, samples are streaked onto two media, one esculin-based and one chromogenic. Esculin-based agars allow for the selection of all *Listeria* species. Examples of esculin-based agars are Oxford, PALCAM, and Modified Oxford (MOX), upon which *Listeria* colonies appear black with a back halo and after 48 hours of incubation, a sunken center. To further classify *Listeria* by species, Chromogenic agars (i.e. CHROMagar Listeria ID) further identify the species based on colony color (caused by enzymatic activity, which releases a chromophore) and presence of a halo due to phospholipase activity. *Listeria monocytogenes* is also able to be differentiated based on a number of defining characteristics such as the inability to use mannitol and xylose and the ability to use rhamnose.

2. Antimicrobials

An antimicrobial is any compound that inhibits the growth of or kills microorganisms including bacteria, viruses, parasites, and fungi (FDA, 2016). The use of antimicrobial compounds in food is regulated by the FDA under the Food Drug & Cosmetic Act, with respect to both raw agricultural commodities and processed foods. The status of each compound in regard to its usage in foods is outlined in the Code of Federal Regulations (CFR) Title 21, which is reserved for rules mandated by the FDA. Food additives are classified as either direct or indirect. A direct food additive is added for a specific purpose, whereas an indirect additive makes its way into a food

product indirectly in trace amounts (i.e. through packaging). Before an additive can be used, it must be preapproved by the FDA, which in short occurs when the FDA has received enough scientific evidence that the additive is safe for human consumption. Direct additives may either be approved as prior-sanctioned ingredients (classified as safe before 1958), or Generally Recognized as Safe (GRAS) based on scientific consensus either prior to 1958 or through research (FDA, 2016). Antimicrobials directly added to food with the purpose of inhibiting microorganisms are classified as food additives. Even if approved for usage in foods, an antimicrobial compound may have limitations concerning the levels that may be used and in which foods. For additives that have been reportedly used but full toxicology reports are not yet in place, the additive falls under the category of "Everything Added to Food in the United States" (EAFUS) (FDA, 2014).

2.1. Antimicrobial Susceptibility Testing

There are a number of methods employed in the research community to determine the susceptibility of a microorganism to a specific antimicrobial (Jorgensen & Ferraro, 2009). One common approach is the broth dilution method. Broth dilution assays use serial dilutions (commonly two-fold) of antimicrobials in broth, to which a microorganism is added. Turbidity of the broth after incubation indicates whether or not the antimicrobial at a given concentration inhibits growth. Two important terms that are used to describe antimicrobial susceptibility are the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). These terms refer to the lowest concentration of a compound at which growth inhibition and cell death occur, respectively. Although many definitions exist, for this thesis the MIC is defined as the concentration of a given compound whereby bacterial counts change ≤ 0.5 log CFU/ml or gram compared to initial inoculation. Furthermore, MBC is defined as the concentration of a given

compound that reduces bacterial counts by >3 log CFU/ml or gram compared to initial inoculation (Brandt et al, 2010).

Concentration of an antimicrobial compound that do not produce growth inhibition are known as sub-inhibitory or sub-lethal. Sub-lethal concentrations can pose a concern due to the development of antimicrobial resistance. Research indicates that exposure to sub-lethal concentrations of antimicrobials can lead to mutagenesis, whereby pathogens are not destroyed/inhibited and multidrug antimicrobial resistance occurs (Kohanski et al., 2010). On the other hand, studies have shown that sub-inhibitory concentrations of antimicrobials can down-regulate the expression of virulence genes and ultimately reduce infection (Upadhyay et al., 2012). Therefore, when considering antimicrobial treatments, sub-inhibitory concentrations need to be further examined for potential effects.

Antimicrobial combinations are often tested to identify whether their interactions are synergistic, additive, or antagonistic. These terms describe the effect of a combination of compounds in comparison to the sum of their separate effects. Synergy indicates a greater combined effect, whereas antagonism indicates a lesser effect and additive is indifferent (Brandt et al., 2011). Compound interactions are often classified through the determination of Fractional Inhibitory and Bactericidal Concentrations indices (FIC_I and FBC_I) (Hall et al., 1982) as follows:

$$FIC = \frac{\text{(Concentration of Compound 1 in interaction with Compound 2)}}{\text{(MIC of Compound 1 alone)}}$$

$$FIC_I = FIC_{\text{Compound 1}} + FIC_{\text{Compound 2}}$$

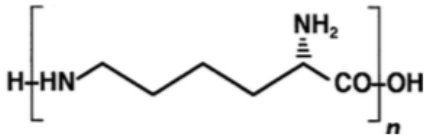
$$FBC = \frac{\text{(Concentration of Compound 1 in interaction with Compound 2)}}{\text{(MBC of Compound 1 alone)}}$$

$$FBC_I = FBC_{\text{Compound 1}} + FBC_{\text{Compound 2}}$$

Although the classifications of interactions based on FIC_I and FBC_I are disputed and vary across research fields and between researchers, an index of >4 is commonly considered antagonistic, and <0.5 as synergistic. The FIC_I and FBC_I take into account the MIC or MBC of an individual compound and its MIC or MBC when used in combination with one or more additional compounds. Combinations of antimicrobials are commonly tested using a checkerboard assay, which tests antimicrobials by diluting each in an opposite direction on a microplate (Hsieh et al., 1993). For example, in a 96-well microplate, the highest concentration of compound 1 would be in the column on the far left, and the antimicrobial is diluted in a horizontal direction to the right. Next, the highest concentration of compound 2 is added to the top row and diluted vertically to the bottom of the plate. In this manner, the interactions of several different combinations of a given antimicrobial pair can be tested.

2.2. ε- Polylysine (EPL)

ε-polylysine (EPL) is a homo-poly-amino acid characterized by a peptide bond connecting the carboxyl and amino groups of L-lysine. It is comprised of approximately 25-35 L-lysine residues, with peptide bond linkages between the carboxyl group and the epsilon amino groups of the residues (Yoshida & Nagasawa, 2003):



Structure of ε-poly-L-lysine (ε-PL)

The number of L-lysine residues is important, as it has been demonstrated that polymers with fewer than 10 residues result in a much lower antimicrobial effect (Shima et al., 1984). ε-polylysine

was originally discovered to be produced by the soil-borne bacteria *Streptomyces albulus* through batch fermentation (Shima & Sakai, 1977), and is currently produced commercially on an industrial scale. ε-polylysine first achieved GRAS status in 2004, but was only initially approved for use in sushi rice/cooked rice at a maximum concentration of 50 ppm (FDA, 2004). In 2011, EPL was then approved for usage up to 250 ppm in a variety of foods, including feta, mozzarella, cream, and Mexican-style cheese (FDA, 2011).

ε-polylysine demonstrates an extensive range of antimicrobial activity across fungi, as well as both Gram-positive and Gram-negative bacteria (Yoshida & Nagasawa, 2003). The antimicrobial activity of EPL has been shown to rely extensively on charge, as EPL is cationic and interacts with negative charges. The negative charges of phospholipid head groups allow EPL to interact with bacterial cell membranes and have an effect of destabilization (Hyldgaard et al., 2014). Antimicrobial resistance to EPL is attributed to a two-component system related to the ViR-regulated operon, which was demonstrated when the inactivation of this operon increased bacterial sensitivity to EPL (Kang et al., 2015). Differences in efficacy between Gram-negative and Gram-positive bacteria occur due to the differences in cell membrane. For example, *L. innocua* (Gram-positive) has a cell membrane that has no net-negative charge, resulting in a decreased interaction of EPL with the cell membrane (Hyldgaard et al., 2014).

The characteristics of EPL as an antimicrobial and the optimal environment in which it may be used has been documented. First, it has been observed that EPL is more effective when used at physiological pH than acidic pH due to reduction of positive charges in a less acidic environment (Najjar et al., 2007). It also has a high resistance to heat, showing no degradation when autoclaved or boiled, implicating that it may be a good candidate for use in foods which are subject to a heat treatment (Yoshida & Nagasawa, 2003). Antimicrobial efficacy against yeasts

was found to decrease in the presence of pectin due to the association of EPL with anionic substances (Chang et al., 2012), which reflects how efficacy may change depending on food components. Similarly, EPL was found to be unstable to aggregation and precipitation in the presence of carrageenan, a common stabilizer used in dairy foods (Lopez-Pena & McClements, 2014). This is important because it reveals how the efficacy of EPL can change based on the presence of other components of a food system, and further highlights the important impact of the food matrix when considering antimicrobial treatments.

The efficacy of EPL against *L. monocytogenes* has been demonstrated extensively, both in broth and food matrices. In a broth system, MIC and MBC levels ranging from 10 to 100 ppm have been reported (Amrouche et al., 2010; Geornaras et al., 2005; Kang et al., 2015; Brandt et al., 2010). The differences in these reported concentrations reflect the effects of different growth media, strain type, and growth conditions on the antimicrobial efficacy of EPL. Geornaras et al. (2007) studied the effect of EPL against *L. monocytogenes* in various food extracts, including fat-free and whole milk, beef, bologna, rice, and vegetables. When EPL was applied at 50 and 200 ppm, there was a significant decrease in pathogen counts compared to the control on the final day of storage for all extracts (Geornaras et al., 2007). Notable in this experiment was that there was a more potent antimicrobial effect in foods that had a lower protein content (rice and vegetables) compared to animal-based foods such as meats, which are high in protein from muscle tissue. This effect is further supported in other studies. For example, the efficacy of EPL to control pathogen growth was diminished in smoked salmon, which was attributed to high levels of protein (Kang et al., 2014; Takahashi et al., 2011). Although EPL has demonstrated a decreased efficacy in foods with higher protein content, it has still been shown to have a significant antimicrobial effect when applied to some meat products. For example, EPL applied at a level of 2% reduced counts of *L.*

monocytogenes by ~6 log CFU/gram on roast beef compared to the control after eight days of refrigerated storage (Chang et al., 2010). In seafood, EPL at 10% was successfully delivered through the use of polymer films on surimi (Muriel-Galet et al., 2014), and in tuna and salmon roe at 0.2% (Takahashi et al., 2011).

When used in combination with other antimicrobial compounds, EPL has been shown to produce a synergistic effect. ε-polylysine has been shown to work synergistically in combination with nisin (Najjar et al., 2007), an antimicrobial derived from *Lactococcus lactis,* and additively with subtilosin (Amrouche et al., 2010), an antimicrobial derived from *Bacillus subtilis*. Also, when combined with either lauric arginate ethyl ester or acidified calcium sulfate with lactic acid, a significant reduction in *Salmonella* on chicken carcasses was observed (Benli et al., 2011). However, at this time data on the use of EPL in combination with other antimicrobials to control pathogens in dairy foods are scant.

2.3. Lauric Arginate Ethyl Ester (LAE; lauric arginate; ethyl-Nα-lauroyl-L-arginine ethyl ester monohydrochloride)

Lauric arginate (LAE) is a cationic surfactant that is derived from lauric acid, arginine, and ethanol (Rodríguez et al., 2004). To date, LAE has GRAS approval for use in a variety of meat and poultry products. These include comminuted and non-comminuted RTE meat products, fresh cuts of meat and poultry, and sausages with a maximum level of 200 ppm of LAE in the finished food product in most cases (FDA 2006). In a study by Romanova et al. (2002), it was observed that alterations occur in the cytoplasmic membrane and in the external membrane in Gram-negative cells in the presence of LAE. However, alterations occur in the cell membrane and cytoplasm in Gram-positive cells, with no cell lysis observed in exposed bacterial cells (Rodríguez

et al., 2004). Cell potential is disrupted due to ion leakage as a result of these structural changes caused by LAE.

The antimicrobial efficacy of LAE has been demonstrated heavily in both broth systems and a variety of food matrices. Although reports of MIC and MBC are variable, studies consistently show that LAE is effective at low concentration in broth systems, with reports of levels as low as ~12 ppm (Ma et al., 2013; Brandt et al., 2010) and 20 ppm (Techathuvanan et al., 2014) producing inhibition. Complete inhibition in broth has also been seen at higher levels of 200 ppm (Soni et al., 2010). The MIC and MBC were found to increase more than ten-fold when soluble starch was added to the broth media, suggesting that the presence of starch or other carbohydrates reduces the antimicrobial efficacy of LAE against *L. monocytogenes* (Ma et al., 2013). Furthermore, effective levels of LAE against *L. monocytogenes* are found to be higher in 2% reduced-fat milk than in broth, with concentrations of 375 and 750 ppm being reported as the MIC and MBC, respectively (Ma et al., 2013). A similar study using skim and whole milk found that 200 ppm of LAE was inhibitory to *L. monocytogenes*, whereas 800 ppm reduced bacteria to undetectable levels with a greater antimicrobial effect in skim milk versus whole milk (Soni et al., 2010). These results show that the efficacy of LAE decreases with the addition of various food components.

The antimicrobial efficacy of LAE can also be affected by components that include a negative charge. Since LAE is cationic, it can form complexes with anionic biopolymers, leading to aggregates. This in turn can decrease its antimicrobial activity as more of the agent becomes bound/unavailable (Bonnaud et al., 2010). When grown on nonfat milk agar, levels of LAE at 0.1 and 0.2% resulted in no visible growth of *L. monocytogenes* over 24 hours of incubation (Taylor & Lathrop, 2015). The same researchers also demonstrated that *Listeria* growth was inhibited by 0.2% LAE only when inoculum level was less than 4 log CFU/ml when grown on cheese agar that

contains fat (Taylor & Lathrop, 2015), further contributing to the body of evidence that fat inhibits the antimicrobial efficacy of LAE.

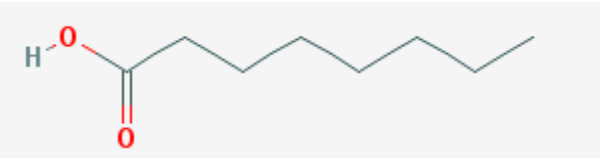
Multiple studies by Soni et al. (2010) have shown that LAE is inhibitory against *L. monocytogenes* inoculated onto QF. Levels of 200 and 800 ppm reduced *L. monocytogenes* levels significantly after 24 hours, but only 800 ppm inhibited growth over 28 days of storage (Soni et al., 2010). A second study showed similar results, with 200 ppm causing significant decreases in the pathogen load over 24 hours, with regrowth leading to levels similar to the control over the course of 28 days of storage. These results indicate that at lower levels, LAE may not be sufficient for inhibiting the growth of *L monocytogenes* in the long-term storage of QF.

Lauric arginate is also effective for an initial decrease in *L. monocytogenes* counts in RTE meat products. In two separate studies, LAE was added to frankfurters and *L. monocytogenes* was enumerated throughout refrigerated storage. The addition of LAE at 43 ppm (Lavieri et al., 2014) and 44 ppm (Porto-fett et al., 2010) caused an initial reduction in *L. monocytogenes*, but counts were >6 log CFU/g at 98 and 120 days of storage, respectively. A five-minute submersion of frankfurters contaminated with *L. monocytogenes* in an antimicrobial dip solution containing 5000 ppm LAE reduced pathogen levels to below detection limit after 48 hours; whether or not growth was inhibited over long-term storage was not investigated (Taormina & Dorsa, 2009). Lastly, LAE applied by adding the aqueous antimicrobial to the food in a bag which was then vacuum-packaged was shown to decrease levels of *L. monocytogenes* in commercially-prepared hams (Luchansky et al., 2005). This method allows for the antimicrobial to be distributed over the entire surface of the food, and will therefore be more likely to make contact with any cells that are present. A similar method was used to apply 200 ppm of LAE to cold-smoked salmon (Soni et al., 2014), which resulted in undetectable levels of *L. monocytogenes* after 24 hours.

When applied in combination with other antimicrobial compounds, the antibacterial effects of LAE can be enhanced by combining mechanisms of action. For example, LAE paired with nisin produces membrane-channel formation, which leads to cell lysis and ion leakage (Pattanayaiying et al., 2014). Lauric arginate has also been found to work additively with the plant-derived antimicrobials cinnamaldehyde, eugenol, and thymol (Ma et al., 2013), as well as white mustard essential oil and flavonoid blends (Techathuvanan et al., 2014). Lauric arginate has also been shown to reduce *L. monocytogenes* counts when applied through the use of polylactic acid and pullulan coatings applied to the surface of ham (Theinsathid et al., 2012; Pattanayaiying et al., 2015), and with alternative treatments such as ultraviolet light (Sommers et al., 2010). Negative organoleptic (sensorial) changes have been documented with the application of this antimicrobial to foods. Although consumers could not detect any sensorial differences in QF that had 200 ppm of LAE applied (Soni et al., 2010), lower levels of LAE applied to frankfurters produced noticeable changes after prolonged storage (Martin et al., 2009).

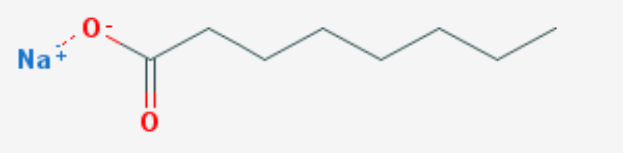
2.4. Caprylic Acid and Sodium Caprylate (CA; octanoic acid / SC; sodium octanoate)

Caprylic acid (CA) ($C_8H_{16}O_2$) is a medium chain saturated fatty acid, consisting of 8 carbons (Marten et al., 2006). It has the chemical structure (Pubchem, 2004):



It is present naturally in a variety of foods including milk and coconut oil, from which it is often commercially produced through fermentation and distillation of volatile fatty acids (21CFR172.860). However, it can also be produced by oxidation of n-octanol (21CFR172.860). Sodium caprylate (SC) is the sodium salt derivative of CA, and is completely soluble in water

whereas CA is less soluble in water and solubilizes completely in alcohol and ether. It has the chemical formula $C_8H_{15}NaO_2$, and structure (Pubchem, 2016):



Caprylic acid is approved for usage in cheese at levels up 400 ppm (21CFR184.1025). Sodium caprylate is a useful alternative to CA because it solubilizes completely in water, making it easier to incorporate into a variety of applications. At the time of writing, there has been no reported limits on the usage of SC and no available toxicology reports. The antimicrobial properties of fatty acids and their derivatives has been tested against a wide variety of microorganisms, including *Salmonella* Enteriditis (Vasudevan et al., 2005), bacterial mastitis pathogens (Nair et al., 2005), bacterial fish pathogens (Kollanoor et al., 2007), *Dermatophilus congolensi* (a pathogen that causes rain rot in animals) (Valipe et al., 2011), and *Listeria* (Wang & Johnson, 1992). Its antimicrobial properties are largely attributed to the free carboxyl group, which aids in the breakage of cellular membranes (Kabara et al., 1972). It has also been shown that fatty acids including CA significantly reduce the ability of *L. monocytogenes* to invade mammalian cells (Wang & Johnson, 1992). Therefore, even if antimicrobial activity does not result in complete inactivation, CA may reduce the ability of surviving cells to infect a host. Also, the efficacy of anionic surface agents (fatty acids) is reduced at physiological pH level (Kabara et al., 1972), suggesting that CA may be a more effective antimicrobial in foods of an acidic nature.

The antimicrobial properties of CA against *L. monocytogenes* have been demonstrated in broth systems, giving indication for its ability to inhibit the pathogen in the absence of confounding

factors. Even at sub-inhibitory levels (<5 mM), CA reduces the lag time and maximum specific growth of *L. monocytogenes* (Nobmann et al., 2009). In tryptose phosphate broth (TPB) adjusted to pH 5.0, the MIC of a commercial mixture of CA and SC (Octa-Gone, Ecolab, St. Paul, MN) has been reported as 25 ppm (Brandt et al., 2011). Petrone et al. (1998) reported growth inhibition at 20 ppm and bactericidal activity at 200 ppm when grown at pH 5.0. In contrast, growth was unaffected at the same antimicrobial concentrations when tested at pH 7.0, suggesting that CA has increased antimicrobial efficacy at more acidic pH levels, which has also been reported by Kinderlerer et al. (1992). However, the reported MIC data vary, with other research groups reporting an MIC as 2360 ppm in unadjusted tryptic soy broth (TSB, pH 7.0) and 2830 ppm in TSB adjusted to pH 5.5 (Nakai & Siebert, 2004), which may be attributed to slightly different pH levels and differences in pathogen strains.

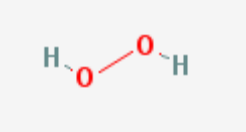
The ability of CA to reduce levels of *L. monocytogenes* in both produce and animal-based products has also been tested. A 2% wash solution of CA reduced levels of *L. monocytogenes* on cantaloupes to undetectable levels after 5 minutes at high temperature (Upadhyay et al., 2014). In milk stored at 8˚C, 50mM of CA reduced *L. monocytogenes* levels approximately 2.5 log CFU/ml after 48 hours (Nair et al., 2004). Despite initial pathogen reductions, studies using CA as an anti-listerial agent in RTE meats have shown that inhibitory properties are limited, with initial reductions followed by outgrowth. When applied to frankfurters, 376.11 ppm of CA resulted in an initial decrease in *L. monocytogenes* counts to undetectable levels over 14 days of refrigerated storage, at which point outgrowth occurred throughout the remaining storage period (Lavieri et al., 2014). A similar effect was seen when CA was applied to RTE hams, with initial inhibition followed by outgrowth by 70 days of storage (Lavieri et al., 2014).

Other antimicrobial compounds have been tested in combination with CA to identify additive, synergistic, and antagonistic effects. When applied to QF, CA at levels 360-720 ppm in combination with nisin were effective at inhibiting the growth of *L. monocytogenes* (Gadotti et al., 2014). When 2% CA was paired with hydrogen peroxide at 2%, no significant difference was seen in levels of *L. monocytogenes* compared to CA 2% alone (Upadhyay et al., 2014), showing that CA works well on its own. However, CA has been shown to exhibit a moderately synergistic effect in combination with oregano essential oil in minced beef (Hulankova et al. 2011, 2013), and with acidified calcium sulfate with lactic acid in broth (Brandt et al., 2011).

To date, data demonstrating the efficacy of SC against *L. monocytogenes* are scant. However, its antimicrobial properties have been demonstrated against a number of other organisms. For example, 120mM of SC completely eliminated *Escherichia coli* in drinking water throughout 20 days (Amalaradjou et al., 2006). The effective anti-listerial concentrations for SC were higher than for CA against four bacterial fish pathogens, with effective concentrations of SC ranging from 25-100mM (Kollanoor et al., 2007). Similarly, when tested against *Dermatophilus congolensis*, a bacterial pathogen causing illness in both animals and humans, the MIC and MBC of SC were higher than CA, with concentrations of SC ranging from 15-75 mM (Valipe et al., 2011). These data suggest that SC has a reduced antimicrobial efficacy compared to CA. However, SC is water soluble and its ability to work in synergy with other antimicrobials has not been investigated.

2.5. Hydrogen Peroxide (HP)

Hydrogen peroxide (HP) (H_2O_2) is a strong oxidizing agent that dissociates into hydrogen and water in an exothermic reaction (Pubchem, 2016). The chemical structure of HP is relatively simple:



Hydrogen peroxide is used in many industries including the food industry as a disinfectant and sanitizer or as an antimicrobial agent added directly to foods. For example, HP has been proposed for use as a post-harvest wash to disinfect apples that may be contaminated with *E. coli* (Sapers & Sites 2003). As of April 2016, HP has GRAS status for use in 13 food categories. It is allowable for use in milk intended to make cheese (up to 500 ppm) and in whey (up to 400 ppm) (21CFR184.1366). Although the antimicrobial mechanism of HP is not completely understood, there is strong evidence of the Fenton reaction, in which HP reacts with metal ions such as iron and produces hydroxyl radicals (Juven & Pierson, 1996). These radicals result in the oxidation of important bacterial biomolecules such as proteins, lipids, and DNA (Linley et al., 2012).

When comparing the antimicrobial susceptibility of *L. monocytogenes* in the form of planktonic cells versus biofilms, biofilms exhibit a stronger resistance to HP (Zameer & Gopal, 2010; Yun et al., 2012). Furthermore, in a study exploring the sensitivity of 19 strains of *L. monocytogenes* to common sanitizers, it was found that the MIC varies depending on strain, but is within the range of 9.4-75 ppm (Romanova et al., 2002). Higher concentrations of HP are needed for bactericidal activity. For example, a 1% solution of HP in Miller's Lysogeny broth produced a 4-log reduction in stationary phase *L. monocytogenes* cells after 15 minutes (Le et al., 2015). Chill brines are used to quickly bring foods to low temperatures, but are a potential source of *L. monocytogenes* contamination (Parikh et al., 2011). When applied at a level of 0.4%, HP reduced pathogen counts to undetectable levels after two hours (Parikh et al., 2011).

The use of HP as a direct food additive to control *L. monocytogenes* has been explored in various food matrices. Hydrogen peroxide has also been shown to have antimicrobial efficacy

when applied as a wash solution. For example, a 2% wash solution of HP reduced *L. monocytogenes* levels on contaminated cantaloupes by at least 5 CFU/cm^2 in 3 minutes and to undetectable levels depending on the temperature of storage (Upadhyay et al., 2014). Hydrogen peroxide can also be applied as a vapor. Application of a 10% concentration of HP in the form of vapor resulted in a 3-log reduction of *L. monocytogenes* when applied to lettuce (Back et al., 2014).

Several groups have demonstrated that HP works in combination with other antimicrobials to produce a synergistic effect. Hydrogen peroxide in combination with plant-derived antimicrobials reduced levels of *L. monocytogenes* on frankfurters (65˚C) to undetectable levels over 70 days of storage (Upadhyay et al., 2013), and on cantaloupes within minutes at temperatures 55˚C and higher (Upadhyay et al., 2014). An increased antimicrobial effect of HP has also been shown when used in combination with alternative treatments such as UV radiation (Parikh et al., 2011). Potential organoleptic changes (smell, taste, odor, etc.) in foods treated with HP present one limitation to broad adoption. Oxidants such as HP can lead to negative organoleptic changes such as rancidity in foods high in fat (Smith et al., 2015; Wambura & Yang, 2011) and thereby reduce shelf-life. Another aspect of using HP as an antimicrobial is that it can inhibit beneficial bacteria. Hydrogen peroxide can inhibit the growth and acid production of lactic starter cultures even at very low levels (Subramanian & Olson, 1968). Residual HP in a product can be neutralized with the addition of the enzyme catalase, which causes HP to dissociate into oxygen and water. In the case of use of HP in cultured products, this can prevent HP from negatively influencing beneficial bacteria. Residual HP can be tested using strips which produce a color reaction, depending on the amount of HP detected.

2.6. Acidified Calcium Sulfate

Acidified calcium sulfate (ACS) products are available commercially from the Mionix company. These include $Safe_2O$ RTE01 (ACS and lactic acid (ACSL)), and RTE03 (ACS and propionic acid (ACSP)). Both products are a unique mixture of sulfuric acid, calcium sulfate, calcium hydroxide, and their respective organic acids. Both are highly acidic with a pH of <2.0. Since *L. monocytogenes* ideally grows in the pH range 6-9, addition of a highly acidic product is thought to disrupt pH homeostasis. Currently, ACSL is approved (GRAS) for use on several animal food products including raw and cooked chicken, beef jerky, comminuted beef, and RTE meats (USDA-FSIS, 2009). Acidified calcium sulfate with propionic acid is approved for use in RTE meat products (i.e. hot dogs) where it may be applied using a spray technique.

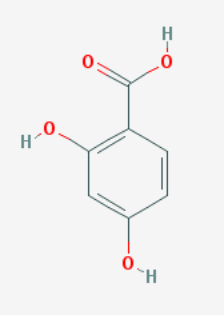
Research using the ACS products listed above show that these compounds are effective in reducing pathogenic foodborne bacteria in both broth and food systems. Brandt et al. (2010) reported that ACSL is inhibitory against *L. monocytogenes* at 1,560 ppm and bactericidal at 6,250 ppm in TPB adjusted to pH 5.0 (Brandt et al., 2011). This same group found in a separate study that inhibition and bactericidal activity both occur at higher levels (12,500 ppm) in unadjusted TPB (pH ~7.0), showing that the antimicrobial efficacy of ACSL is affected by pH. When applied to contaminated ham, a 1:2 solution of ACSL significantly reduced *Listeria* counts. This study did not sample further than 24 hours, so it is not known if bacterial levels remained low or if outgrowth subsequently occurred. When applied to chicken, ACS has been shown to reduce levels of *Salmonella* (Benli et al., 2011) as well as maintain quality by reducing levels of spoilage bacteria (Mehyar et al., 2005).

Acidifed calcium sulfate with lactic acid has been shown to work synergistically in combination with LAE and nisin in broth pH 7.0 (Brandt et al., 2010), and with OctaGone in broth

acidified to pH 5.0 (Brandt et al., 2011). In a study investigating the efficacy of antimicrobials to reduce common foodborne pathogens on pre-rigor beef rounds, ACSL in combination with EPL produced greater bacterial reductions than when used alone (Njongmeta et al., 2011). The ability of ACS to work synergistically with other antimicrobials is important because these combinations allow for a lower usage rate, which could prevent organoleptic changes attributed to the application of ACS, such as changes in aftertaste, texture, and aromatics (Nuñez de Gonzalez et al., 2006). It is currently approved for usage in the meat and poultry industry, and may be used in amounts "sufficient for purpose" (USDA/FSIS 2004).

2.7. Beta-Resorcylic Acid (BR, 2,4-dihydroxybenzoic acid)

Plant-derived antimicrobials have been demonstrated to exert inhibitory effects against pathogenic bacteria, both Gram-positive and Gram-negative (Burt et al., 2004). Beta-resorcylic acid (BR) ($C_7H_6O_4$) is a phytophenolic compound that is commonly found among the angiosperms of plants (Julkunen-Tiitto, 1985), with the given structure (Pubchem, 2016):



Beta-resorcylic acid is listed under EAFUS meaning it has reportedly been used as an additive but toxicology research has not yet been completed. Therefore, direct addition of BR is not yet approved for usage without toxicology studies to determine toxicity and rule out long-term effects. The mechanism of action for BR is not well documented at this time. Beta-resorcylic acid has shown to be effective at inhibiting the growth of both Gram-positive and Gram-negative bacteria,

including *Staphylococcus aureus* and *Escherichia coli* (Alves et al., 2013). The antimicrobial efficacy of BR against *L. monocytogenes* has been tested several times. However, the highest concentration of BR tested in these studies (1000 ppm) was not effective at inhibiting growth on its own (Friedman et al., 2003; Alves et al., 2013). Beta-resorcylic acid applied through a chitosan coating at 2% on cantaloupes reduced *L. monocytogenes* to undetectable levels over the course of 7 days, compared to the control which reached >7 log CFU/cm^2 (Upadhyay et al., 2014). In this same study, *L. monocytogenes* was not detectable when contaminated cantaloupes were washed with a 2% solution of BR, but only at high temperature (65˚C). A study by this same group of researchers looked at BR to inactivate *L. monocytogenes* on frankfurters. A 1.5% solution of BR was used as a dip treatment, and after 50 days of refrigerated storage *Listeria* was still present at levels >3 log CFU/cm^2 (Upadhyay et al., 2013), whereas in the control levels stayed consistently between 4 and 5 log CFU/cm^2 . One potential hindrance to broad application is that BR does not dissolve in water alone. In most studies a 4% dimethyl sulfoxide (DMSO) solution has been reportedly used as a solvent (Capriotti & Capriotti, 2012). DMSO is not currently recognized as GRAS, but falls under EAFUS as well.

3. Hypothesis and Objectives

Based on published research regarding antimicrobials and their ability to inhibit the growth of *L. monocytogenes,* it was hypothesized that the antimicrobials ACSL, BR, CA, EPL, HP, LAE, and SC would exhibit inhibitory and bactericidal activity against *L. monocytogenes* both alone and in binary combinations. It was further hypothesized that the use of antimicrobials combinations would exert synergistic effects, thereby minimizing antimicrobial application while maintaining equivalent inhibition and inactivation levels.

The specific objectives of this research are as follows:

1)

   a. Determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of individual antimicrobials against *Listeria monocytogenes* in a broth system at pH 7.4 or 5.5.
   b. Determine the inhibitory and bactericidal concentrations of antimicrobials against *Listeria monocytogenes* when applied in binary combinations in a broth system at pH levels 7.4 and 5.5.
   c. Characterize antimicrobial interactions as synergistic, additive, or antagonistic.

2) Determine the efficacy of antimicrobials treatments to control *L. monocytogenes* in whole milk during refrigerated shelf life.
3) Determine the efficacy of antimicrobial dip treatments to control *L. monocytogenes* as surface contaminants on fresh cheese.

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Chapter III:

Synergistic antimicrobial combinations inhibit and inactivate *Listeria monocytogenes* in neutral and acidic broth systems

1. Abstract

The use of effective antimicrobials to reduce *Listeria monocytogenes* levels in ready-to-eat (RTE) foods and prevent pathogen growth can be limited by regulation of usage levels, cost, and organoleptic changes. The identification of combinatorial approaches to produce additive, synergistic, and antagonistic effects is desirable to limit usage levels without diminishing inhibitory or bactericidal effects. The present study identified minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of antimicrobials when used alone or in binary combinations against *L. monocytogenes* in growth media adjusted to pH 7.4 and 5.5. Resulting interactions were characterized as synergistic, additive, or antagonistic. When applied alone at pH 7.4, lauric arginate (LAE) and ε-polylysine (EPL) were effective at the lowest concentrations with MICs of 6.25 and 20 ppm, respectively. Lauric arginate was also the most effective antimicrobial at pH 5.5 with an MIC of 10 ppm. Efficacy of caprylic acid (CA) and sodium caprylate (SC) was enhanced in broth at the lower pH with MICs of 50 ppm. With reference to bactericidal concentrations, EPL, HP, and LAE had the lowest MBCs in broth adjusted to both pH 7.4 (60, 50, and 25 ppm, respectively) and pH 5.5 (300, 100, and 20 ppm, respectively). Two inhibitory combinations at pH 7.4 and three at pH 5.5 were classified as synergistic. Five synergistic bactericidal combinations were identified in broth pH 7.4, with three identified at pH 5.5. The most synergistic bactericidal pairing was EPL + LAE. Of all synergistic combinations identified, SC was involved in approximately half. No combinations were characterized as antagonistic. These data serve as a basis for identifying effective antimicrobial approaches at varying pH levels for more effective application for the control of *L. monocytogenes* in foods.

2. Introduction

An estimated 1600 illness and 260 deaths occur annually in the U.S. due to foodborne listeriosis, an illness caused by the bacterial pathogen *Listeria monocytogenes* (Scallan et al., 2011). Pregnant women, children, the elderly, and the immunocompromised (Batt, 2014) are most susceptible to infection with an estimated mortality rate of 30% (CDC, 2014). While less serious cases can mimic flu-like symptoms, more serious symptoms in high-risk groups include convulsions, septicemia, meningitis, as well as premature delivery and stillbirth in pregnancy associated cases (CDC, 2014). This pathogen is able to survive and grow under adverse conditions, including salt concentrations as high as 20% (Schirmer et al., 2014), a wide pH range (5-10) (Batt, 2014), and low temperatures including those used for refrigeration (Coles et al., 1990).

Outbreaks of listeriosis have been attributed to the consumption of a variety of foods, demonstrating the range of conditions in which *L. monocytogenes* can survive and grow. According to the Centers for Disease Control and Prevention (CDC) there have been 12 recorded listeriosis outbreaks in the United States in the past five years. These cases were linked to ready-to-eat (RTE) foods that vary considerably in their levels of acidity including lettuce (pH 5.8-6.0), caramel apples (pH 3.5-4.0), and soft cheese (pH 6.0-7.0) (CDC, 2016). As highlighted by these outbreaks, the ability of *L. monocytogenes* to survive and grow over a wide range of pH values necessitates the need to determine the efficacy of antimicrobials at different pH levels.

Many antimicrobial compounds are Generally Recognized as Safe (GRAS) by the United States Food and Drug Administration (FDA), and have demonstrated efficacy against *L. monocytogenes* and/or other prevalent pathogens in foods. For example, ε-polylysine (EPL) has been shown to effectively reduce *L. monocytogenes* in a variety of food extracts, including milk,

meats, rice, and vegetables (Geornaras et al., 2007). Similarly, there is a growing body of research showing that lauric arginiate ethyl ester (LAE) application is effective in reducing *L. monocytogenes* counts in milk and cheese (Soni et al., 2010) as well as RTE meat products (Luchansky et al., 2005). Acidified calcium sulfate with lactic acid (ACSL) has been shown to effectively reduce levels of *L. monocytogenes* in both a broth system and on various RTE foods including ham, beef, and frankfurters (Nuñez de Gonzalez et al., 2006). Beta-resoryclic acid (BR) is a phytophenolic compound commonly found among the angiosperms of plants (Friedman et al., 2003). Although its antimicrobial mechanism is unknown and research is limited, it has been shown to reduce levels of *L. monocytogenes* on cantaloupes (Upadhyay et al., 2014) and frankfurters (Upadhyay et al., 2013), particularly in combination with hydrogen peroxide. In addition to a long history of use as a disinfectant, (HP) has been shown to inhibit *L. monocytogenes* in a number of food systems including cantaloupes (Upadhyay et al., 2014), frankfurters (Upadhyay et al., 2013), and lettuce (Back et al., 2014).

Fatty acids and their derivatives have also been shown to exert antimicrobial effects against bacteria, yeasts, and fungi (Marten et al., 2006; Kabara et al., 1972) with efficacy varying with chain length, saturation, and bond type (Kabara et al., 1972). Caprylic acid (CA) is a medium chain fatty acid found naturally in foods including milk and has been shown to an effective antimicrobial against *L. monocytogenes* in animal based products, including milk, cheese, and RTE meats (Gadotti et al., 2014; Lavieri et al. 2014). Data demonstrating the antimicrobial efficacy of sodium caprylate (SC)—the soluble sodium salt form of CA—are limited. However, since SC is completely soluble in water it may hold more practical potential in food systems.

Although antimicrobial compounds may be effective when used individually, certain combinations may produce a combined effect greater than the sum of their separate effects referred

to as synergy (Brandt et al., 2011) allowing for similar or enhanced efficacy at lower individual usage levels. This can help to limit potential negative aspects of applying antimicrobials, including organoleptic (sensorial) changes, cost, and changes to characteristics associated with quality such as sensory properties. For example, ACSL applied to frankfurters at high levels can create negative sensorial changes including an increase in bitterness and astringency (Nuñez de Gonzalez et al., 2006). Applying higher concentrations of ACSL could impact the pH of the food product, which could negatively impact sensory attributes. Since food matrices can reduce the efficacy of antimicrobials (i.e. protein and fats) (Ma et al., 2013; Kang et al., 2014), synergistic combinations are desirable for reducing usage rates of individual compounds within the combination treatment while maintaining antimicrobial efficacy.

The objectives of the present study were to determine the minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of ACSL, BR, CA, EPL, HP, LAE, and SC when used alone and in binary combinations against *L. monocytogenes* in growth media adjusted to pH 7.4 and to pH 5.5 and to characterize the interactions as synergistic, additive, or antagonistic.

3. Material and Methods

3.1 Bacterial strains, growth conditions, and inoculum preparation

A total of eight *L. monocytogenes* strains were used to represent a diversity of subtypes and sources. Strains included: F5069/ATCC 51414 (Raw Milk, DUP-1044B; serotype 4b), CWD 675-3 (Raw Milk, DUP-1053A; serotype 1a), CWD 1567 (Cheese, DUP-1038B; serotype 4b), Scott A (DUP-1042B; serotype 4b), 2012L-5323 (Ricotta salata outbreak, 2012; serotype 1/2a), 2014L-6025 (Hispanic-style cheese outbreak, 2014; serotype 1/2b), DJD 1 (washed-rind cheese outbreak, 2013), and CWD 193-10 U5-2 (cheese food contact surface, DUP-1030B). All strains were from the author's culture collections except strain DJD 1, which was kindly provided by

Minnesota Department of Agriculture Laboratory Services Division, and strains 2012L-5323 and 2014L-6025 which were kindly provided by the CDC. Frozen (-80°C) stock cultures were inoculated into 9 ml of Brain Heart Infusion (BHI) broth (Becton Dickinson, Franklin Lakes, NJ) and incubated at 37 ± 1°C for 18 h for two subsequent transfers before use. Equal proportions of cells from each of the eight cultures were combined as a cocktail yielding approximately 9 log CFU/ml. The cocktail was then serially diluted in Butterfield's Phosphate Buffer (BPB), pelleted through centrifugation (15 min, 4200 rpm at 4°C), and re-suspended in BHI broth for use in assays.

3.2 Antimicrobials and preparations

Working stock solutions of the following antimicrobials were prepared in BHI and then serially diluted to achieve target concentrations: CA (Sigma Aldrich, St. Louis, MO), SC (Sigma Aldrich, St. Louis MO), BR (Acros Organic, Pittsburgh, PA), LAE (Cytoguard LA2X, A+B Ingredients, Fairfield, NJ), EPL (25%, JNC Corporation, Tokyo, Japan), HP (30%, Acros Organic, Pittsburgh, PA), ACSL (RTE-01, Mionix Corporation, Scottsdale, AZ). Stock solutions were prepared in sterile distilled deionized water. Powdered BR was first solubilized in Dimethyl Sulfoxide (DMSO) followed by dilution in BHI broth for the working stock where applicable, with DMSO less than 4% of final assay concentration (Upadhyay et al., 2014). Stock solutions of CA were vortexed vigorously prior to overcome low solubility and to ensure homogeneity. All antimicrobial stocks were stored at room temperature, except for HP and EPL, which were stored in the dark at 4˚C. Controls (negative) for each antimicrobial were included in each assay to confirm sterility. Sterility of antimicrobial stocks were routinely verified though direct plating 100 µL of each on BHI agar and incubating at 37˚C for 24 h.

3.3 Single antimicrobial inhibition and inactivation

Inhibitory concentrations of ACSL, BR, CA, EPL, HP, LAE, and SC were determined by exposing *L. monocytogenes* at 6 log CFU/ml to serially diluted concentrations of each antimicrobial in BHI broth using a standard broth dilution assay (Andrews, 2001) in 96-well microtiter plates (Corning, Corning, NY). Assays were repeated in BHI broth adjusted to both pH 7.4 and pH 5.5. pH adjustments were made prior to the addition of antimicrobials using 0.1 N NaOH or 0.1 N HCl. Plates were then incubated for 24 h at 37°C in a computer-and temperature-controlled microplate reader (Biotek Epoch 2; Biotek Instruments, Winooski, VT) with optical density at 630 nm (OD630) measurements taken every 30 minutes following five seconds of orbital shaking. Each assay included a positive control, negative controls of antimicrobial solutions as previously mentioned, negative control of 4% DMSO (where applicable), and sterile growth media for baseline OD correction. pH of individual wells was measured using a microtip electrode (Accumet AB150, Fisher Scientific International Inc., Hampton, NH). Based on pilot studies (data not shown), treatments producing <0.15 change in OD630 after 24h of incubation following appropriate baseline adjustment across duplicate replicates were considered at least inhibitory and selected for enumeration. To further determine inhibition or cell death using culture confirmation, aliquots from wells with suspected inhibition were either plated directly onto BHI agar or serially diluted in BPB before plating onto BHI agar. Plates were incubated for 48 h at 37 °C and enumerated. The MIC was defined as the minimum concentration inhibiting *L. monocytogenes* growth to levels <0.5 log CFU/ml compared to the initial inoculum level (Branen & Davidson, 2004). Concentrations of antimicrobials producing ≥3.0 log CFU/ml (99.9%) reduction in *L. monocytogenes* counts from the starting level of the inoculum were classified as bactericidal (Branen & Davidson, 2004).

3.4 *Antimicrobial interactions.*

A checkerboard assay (Najjar et al., 2007; Brandt et al., 2010) was utilized to examine interactions between binary combinations of antimicrobials at varying concentrations. Assays were conducted in a microplate reader as described above. For each binary combination, the first antimicrobial was serially diluted horizontally over six rows, while the second was diluted vertically over six columns that coincided with the aforementioned rows. Each antimicrobial was also serially diluted in a row on its own to serve as controls. Additional controls were included as described in section 3.3 (page 49). MIC values previously determined from single antimicrobial susceptibility tests were used as starting median concentrations for the combination assays. pH of individual wells was measured using a microtip electrode (Accumet AB150, Fisher Scientific International Inc., Hampton, NH). Wells that produced <0.15 change in optical density reading were considered at least inhibitory, and were serially diluted and plated onto BHI agar. Plates were incubated at 37˚C for 24 hours and enumerated. Combinations that produced an inhibitory or bactericidal effect over three replicates were then characterized. Fractional inhibitory (FIC) and bactericidal (FBC) concentrations for binary combinations of antimicrobial compounds were determined as previously described (Hall et al., 1982). Fractional Inhibitory and Bactericidal Concentration Indices (FIC_I and FBC_I, respectively) were calculated to characterize compound interactions as synergistic, additive, or antagonistic (Hollander et al., 1998). Combinations with FIC_I or $FBC_I \leq 0.50$ were defined as synergistic, >0.50 and <4.0 as additive, and >4.0 as antagonistic (Chin et al., 1997; Domaracki et al., 2000; Meletiadis et al., 2010). The following formulae were used for each combination that resulted in inhibition or inactivation:

$$FIC = \frac{\text{(Concentration of Compound 1 in interaction with Compound 2)}}{\text{(MIC of Compound 1 alone)}}$$

$FIC_I = FIC_{Compound\ 1} + FIC_{Compound\ 2}$

$$FBC = \frac{\text{(Concentration of Compound 1 in interaction with Compound 2)}}{\text{(MBC of Compound 1 alone)}}$$

$FBC_I = FBC_{Compound\ 1} + FBC_{Compound\ 2}$

3.5 Statistical Analysis

All assays were performed in triplicate, utilizing three biological replicates and two internal technical replicates per experiment. pH values were compared using a one-way analysis of variance, with significance defined at $p < 0.05$.

4. Results and Discussion

4.1 pH affects the inhibitory and bactericidal efficacy of antimicrobials.

The antimicrobials with the lowest MICs in BHI at pH 7.4 included EPL, HP, and LAE (20, 40, and 6.25 ppm, respectively) (Table 1). When the pH was lowered to 5.5, the MICs of these compounds increased slightly (100, 50, and 10 ppm, respectively), suggesting that they are more effective in less acidic conditions. Values for EPL and LAE are in agreement with findings by Brandt et al., (2010) who reported similarly low MIC values of 6.24 and 12.5 ppm, respectively, when tested in Tryptose Phosphate Broth (TPB) at pH 7.0. Minor differences in observed MICs could be attributed to the differences in the pH of the broth media used for the assays (7 vs. 7.4) and/or strain differences (Brandt et al., 2010). Results comparable to those observed in the present study have been reported for EPL and LAE in broth at pH 7.3. Inhibitory concentrations under these conditions of have been reported as low as 10-15 ppm for EPL and from 11.8 to 23.5 ppm for LAE (Ma et al., 2013; Techathuvanan et al., 2014; Brandt et al., 2010).

Inhibitory concentrations of HP are also in agreement with previous reports ranging from 7.4 to 75 ppm at pH 7.3, varying by strain (Romanova et al., 2002). In contrast to HP, the inhibitory effect of ACSL, BR, CA, and SC was enhanced in the acidified broth system (Table 1), suggesting

that these compounds are more effective at lower pH levels. As seen in Table 1, effective inhibitory concentrations of ACSL, BR, and CA, resulted in a significant reduction in pH level. Notably, effective inhibitory concentrations of ACSL reduced the pH to below 5.0 (Table 1), suggesting that either higher concentrations are needed for antimicrobial efficacy or that the compound is more effective in an acidic environment and a sufficient concentration of ACSL is needed to reduce the pH to a level necessary for antimicrobial activity. In particular, approximate 98 and 99% decreases in the MICs of CA and SC were observed at the lower pH values, respectively (Table 1). A buffer was considered for use in assays to prevent pH changes, however, with the number of wells used the buffering of each well was deemed inefficient. Further studies are needed to investigate the efficacy of these antimicrobials in a buffered system. Although data on the MIC of CA and SC are limited, a commercial product comprised of both CA and SC has a reported MIC of 25 ppm in TPB adjusted to pH 5.0 (Brandt et al., 2011). This is consistent with previous reports suggesting that fatty acids may be more effective as antimicrobials in more acidic matrices (Kabara et al., 1972). Although the efficacy of ACSL and BR were enhanced when the broth medium was more acidic, evidence does not support that either compound is effective at near neutral pH due to reduced pH following addition of compounds to inhibitory concentrations. Also, inhibitory concentrations were still relatively high compared to the other antimicrobials tested. Data on the antimicrobial effect of BR against *L. monocytogenes* are scant, however, reports suggest that inhibitory and bactericidal applications of BR typically require higher usage levels of approximately 1000 to 6700 ppm (Alves et al., 2013; Friedman et al., 2003), which is in agreement with the findings of this study.

Similar to the inhibition assays, EPL, HP, and LAE had the lowest MBCs at pH 7.4 (60, 50, 25 ppm, respectively) (Table 2). However, when the initial pH of the broth medium was

lowered to 5.5, the bactericidal concentrations of EPL and HP increased to 100 and 300 ppm, respectively. The microbial inhibitory efficacy of EPL has been shown to decrease in acidic systems, which is consistent with these findings (Najjar et al., 2007). Overall, ACSL, BR, and SC were more effective listericides at pH 5.5 than at 7.4, whereas CA was more effective at pH 7.4 (Table 2). Similar to inhibitory concentrations, the bactericidal concentrations of ACSL, BR, and CA significantly reduced the pH of BHI (initially 7.4) significantly compared to the control, suggesting that either high concentrations of these compounds are necessary for bactericidal activity or that they require low pH environments in order to produce a bactericidal effect. When tested in broth at pH 7.4 or 5.5, ACSL reduced the pH of broth to <4.0 (3.67, 3.84, Table 2). This increase in the efficacy of ACSL at lower pH level has been reported previously (Brandt et al. 2011) whereby a reduction in media pH from 7 to 5 reduced the MBC from 12,500 to 25,000 ppm (strain dependent) to 1,560 ppm (Brandt et al., 2011). Minimum inhibitory concentrations of 14,000 ppm at pH 7.4 and 6,000 at pH 5.5 in the present study are in general agreement with these results. In addition to differences between media and pH, inhibitory and bactericidal efficacy of antimicrobials has been shown to vary between strains of *L. monocytogenes* (Brandt et al., 2010; Brandt et al., 2011; Romanova et al., 2002). An eight-stain cocktail was used in the present study to account for this variation and to increase the likelihood of including one or more strains that have a higher tolerance to one or more antimicrobials (Ming & Daeschel, 1993). For example, strain Scott A has demonstrated increased tolerance to several antimicrobials compared with other strains and serotypes (Castellano et al., 2001). Also, Nobmann et al. (2009) demonstrated that CA can have an MIC as low as 5mM, depending on the strain of *L. monocytogenes* that is used.

4.2 Combinatory treatments can reduce inhibitory and bactericidal concentrations.

A total of 26 combinations were found to interact additively or synergistically to inhibit *L. monocytogenes* growth (Table 3) at pH 7.4, with 10 producing inactivation (Table 4). Of the inhibitory combinations, nine involved SC, including the two synergistic combinations (SC with either EPL or ACSL). Five of the bactericidal combinations at pH 7.4 were classified as synergistic. However, ACSL with SC or CA reduced the test media pH to 5.39 and 5.24, respectively. Therefore, these pairings may produce synergy due to increased acidity, so it cannot be concluded that synergy is produced at near-neutral pH. Two synergistic combinations involved ACSL and SC, with one combination containing a higher concentration of ACSL and the other a higher concentration of SC. The combination with a higher concentration of ACSL resulted in a significant reduction in pH (Table 3), which indicates that more research is needed to determine whether or not antimicrobial activity is due to the presence of the compound or a pH effect. Combinations of EPL and LAE demonstrated strong synergism (i.e., FBC_I of 0.292) in agreement with previous work by Benli et al. (2011) when applied to a membrane filter model for controlling *Salmonella*. In addition, usage rates for these compounds are comparably low (<10 ppm) resulting in no significant change in media pH (Table 4).

At pH 5.5, a total of twelve combinations were inhibitory (Table 5), with three characterized as synergistic. Acidifed calcium sulfate with lactic acid was the most common compound among these combinations. When combined with either BR or SC, ACSL demonstrated the highest degree of synergy based on FIC indices. Because the addition of ACSL can significantly decrease pH, the observed inhibition in cases where pH was significantly reduced could be pH driven. Although synergy between ACSL and EPL has been previously reported (Brandt et al., 2010) this interaction was not observed in the present study attributed to the decrease in efficacy of EPL at lower pH levels. A total of seven antimicrobial combinations were

bactericidal at pH 5.5. Three of these produced a synergistic effect (Table 6), notably ACSL and SC with an FBC_I of 0.188. By definition, no inhibitory or bactericidal combinations at either pH level were identified as antagonistic.

It is important to note that synergism only suggests the degree to which a combination enhances efficacy compared to the sum of their individual parts. Fractional inhibitory and bactericidal concentration indices alone should not be used to determine which treatments are most effective overall due to the varying concentrations needed to produce the desired effects. Although a particular combination may be characterized as synergistic, the concentrations may still not be low enough to facilitate effective use. For example, ACSL works synergistically in multiple binary combinations but requires relatively high usage rates which may produce negative organoleptic changes (Nuñez de Gonzalez, 2006) and potential changes in pH. However, the identification of synergistic combinations may be useful for reducing levels of ACSL in foods while still producing similar antimicrobial effects.

5. Conclusions

This study identified antimicrobial compounds effective in inhibiting and inactivating *L. monocytogenes* when used alone or in binary combinations in near neutral and acidic pH environments. When used alone, HP, EPL, and LAE were the most effective inhibitory and bactericidal compounds at pH 7.4. Hydrogen peroxide and lauric arginate were also the most effective at pH 5.5 along with CA and SC. Although more synergistic combinations were identified when tested in broth at pH 7.4, in several combinations with acidic compounds (especially ACSL) significantly reduced the pH of broth. Therefore, further studies are needed to elucidate the mechanism behind the inhibition and inactivation of *L. monocytogenes* in which compounds that result in an acidic environment are used. The combination of EPL+LAE is promising for use in

foods at near neutral pH, as it is the most synergistic listericidal combination at pH 7.4 and with the lowest FBC indices. Sodium caprylate and ACSL are both involved in several synergistic combinations, indicating they are promising candidates for combinatorial approaches in foods. They also serve as a basis for future work in food systems with the ultimate goal of enhancing practical applications to control *L. monocytogenes* in foods under various pH conditions. Future studies are necessary to evaluate these combinations when applied to specific foods in order to determine the effective concentrations in more complex matrices.

Acknowledgements

This work was supported by Dairy Management Inc. and the United States Department of Agriculture (USDA) National Institute of Food and Agriculture (NIFA), Multistate project S1056. Any opinions, findings, conclusions, or recommendations expressed in this manuscript are those of the author(s) and do not necessarily reflect the view of the NIFA, the USDA or any other sponsors.

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7. Tables and Figures

Table 1. Minimum inhibitory concentrations (MIC) of antimicrobial compounds in BHI broth adjusted to pH 7.4 and 5.5.

	pH 5.5		pH 7.4	
Antimicrobial	MIC (ppm)	pH[b] (± SD)	MIC (ppm)	pH[b] (± SD)
ACSL	3000	4.41 ± 0.05[a]	6000	4.83± 0.28 [a]
BR	1000	5.16 ± 0.08 [a]	3000	6.34 ± 0.11 [a]
CA	50	5.63 ± 0.02	3250	6.72 ± 0.40 [a]
EPL	100	5.76 ± 0.07	20	7.33 ± 0.13
HP	50	5.60 ± 0.05	40	7.39 ± 0.11
LAE	10	5.59 ± 0.05	6.25	7.37 ± 0.10
SC	50	5.53 ± 0.03	8000	7.35 ± 0.09

[a] pH levels significantly different from BHI broth without antimicrobial addition ($p<0.05$).
[b] pH of BHI broth following addition of antimicrobial compounds.

Table 2. Minimum bactericidal concentrations (MBC) of antimicrobial compounds in BHI broth adjusted to pH 7.4 and 5.5.

	pH 5.5		pH 7.4	
Antimicrobial	MBC (ppm)	pH[b] (± SD)	MBC (ppm)	pH[b] (± SD)
ACSL	10000	3.67 ± 0.12 [a]	14000	3.84 ± 0.05 [a]
BR	1750	4.83 ± 0.11 [a]	4000	5.99 ± 0.24 [a]
CA	20000	5.05 ± 0.12 [a]	4500	6.52 ± 0.42 [a]
EPL	300	5.99 ± 0.24 [a]	60	7.34 ± 0.15
HP	100	5.63 ± 0.03	50	7.39 ± 0.12
LAE	20	5.57 ± 0.06	25	7.39 ± 0.17
SC	1000	5.58 ± 0.04	13000	7.25 ± 0.27

[a] pH levels significantly different BHI broth without antimicrobial addition ($p<0.05$).
[b] pH of BHI broth following addition of antimicrobial compounds.

Table 3: Synergistic and additive inhibitory antimicrobial combinations in BHI adjusted to pH 7.4.

Antimicrobial		Concentration (ppm)		FIC[c]				
1	2	1	2	1	2	FIC_I[d]	pH[b] (± SD)	Interaction
ACSL	SC	5	1250	0.250	0.156	0.406	7.38 ± 0.09	Synergistic
ACSL	SC	2500	500	0.417	0.063	0.479	6.47± 0.1 [a]	Synergistic
BR	SC	1500	500	0.500	0.063	0.563	6.79 ± 0.07 [a]	Additive
ACSL	CA	625	1500	0.104	0.462	0.566	6.05 ± 0.60 [a]	Additive
ACSL	SC	625	4000	0.104	0.500	0.604	7.07 ± 0.08	Additive
EPL	LAE	10	1	0.500	0.125	0.625	7.39 ± 0.07	Additive
ACSL	BR	973.5	1500	0.156	0.500	0.656	6.53 ± 0.29 [a]	Additive
EPL	SC	1.25	5000	0.063	0.625	0.688	7.38 ± 0.08	Additive
CA	EPL	1500	5	0.462	0.250	0.712	6.78 ± 0.11 [a]	Additive
CA	SC	750	4000	0.231	0.500	0.731	7.15 ± 0.16	Additive
ACSL	SC	4000	600	0.667	0.075	0.742	5.77± 0.11 [a]	Additive
BR	SC	750	4000	0.250	0.500	0.750	7.11 ± 0.04	Additive
ACSL	SC	4500	300	0.750	0.038	0.788	5.53 ± 0.06 [a]	Additive
ACSL	BR	1250	1875	0.208	0.625	0.833	6.17± 0.37 [a]	Additive
ACSL	BR	375	750	0.625	0.250	0.875	7.01 ± 0.19	Additive
CA	LAE	2500	1	0.769	0.160	0.929	6.86 ± 0.33 [a]	Additive
ACSL	CA	5000	500	0.833	0.154	0.987	5.24 ± 0.13 [a]	Additive
ACSL	BR	5000	500	0.833	0.166	1.000	4.87 ± 0.35 [a]	Additive
CA	LAE	1625	3.125	0.500	0.500	1.000	7.23 ± 0.11	Additive
CA	EPL	3000	2.5	0.923	0.125	1.050	6.53 ± 0.29 [a]	Additive
CA	LAE	1500	4	0.462	0.640	1.102	6.76 ± 0.12 [a]	Additive
LAE	SC	3.125	5000	0.500	0.625	1.125	7.39 ± 0.02	Additive
HP	LAE	20	4	0.500	0.640	1.140	7.42 ± 0.02	Additive
EPL	HP	12.5	25	0.625	0.625	1.250	7.40 ± 0.03	Additive
HP	SC	25	5000	0.625	0.625	1.250	7.41 ± 0.01	Additive
ACSL	HP	5000	25	0.833	0.625	1.460	5.55 ± 0.31 [a]	Additive

[a] pH levels significantly different BHI broth without antimicrobial ($p<0.05$)

[b] pH of BHI broth following addition of antimicrobial compounds.

[c] FIC: Fractional Inhibitory Concentration

[d] FIC_I: Fractional Inhibitory Concentration Index; ≤0.5=Synergistic, 0.5-4.0=Additive, >4.0=Antagonistic

Table 4. Synergistic and additive bactericidal antimicrobial combinations in BHI broth adjusted to pH 7.4.

Antimicrobial		Concentration (ppm)		FBC[c]				
1	2	1	2	1	2	FBC_I[d]	pH[b] (± SD)	Interaction
EPL	LAE	10	3.125	0.167	0.125	0.292	7.38 ± 0.13	Synergistic
EPL	LAE	2.5	6.25	0.042	0.250	0.292	7.39 ± 0.10	Synergistic
EPL	SC	2.5	5000	0.042	0.384	0.426	7.37 ± 0.08	Synergistic
ACSL	SC	5000	1250	0.358	0.096	0.453	5.39 ± 0.14 [a]	Synergistic
ACSL	CA	5000	500	0.357	0.111	0.468	5.24 ± 0.13 [a]	Synergistic
LAE	SC	6.25	5000	0.375	0.385	0.635	7.39 ± 0.09	Additive
ACSL	BR	3750	1500	0.268	0.375	0.642	5.45 ± 0.51 [a]	Additive
CA	EPL	3000	5	0.667	0.083	0.750	7.04 ± 0.25	Additive
HP	LAE	30	5	0.600	0.200	0.800	7.39 ± 0.11	Additive

[a] pH levels significantly different BHI broth without antimicrobial ($p<0.05$).
[b] pH of BHI broth following addition of antimicrobial compounds.
[c] FBC: Fractional Bactericidal Concentration
[d] FBC_I: Fractional Bactericidal Concentration Index; ≤0.5=Synergistic, 0.5-4.0=Additive, >4.0=Antagonistic

Table 5. Synergistic and additive inhibitory antimicrobial combinations in BHI adjusted to pH 5.5.

Antimicrobial		Concentration (ppm)		FIC[c]				
1	2	1	2	1	2	FIC_I[d]	pH[b] (± SD)	Interaction
ACSL	SC	625	125	0.208	0.250	0.458	5.22 ± 0.02 [a]	Synergistic
ACSL	BR	312.5	375	0.104	0.375	0.479	5.34 ± 0.03	Synergistic
BR	EPL	187.5	31.25	0.188	0.313	0.500	5.47 ± 0.03	Synergistic
ACSL	CA	625	156	0.208	0.312	0.520	4.89 ± 0.09 [a]	Additive
ACSL	BR	1250	187.5	0.416	0.188	0.604	4.97 ± 0.02 [a]	Additive
ACSL	CA	1250	100	0.417	0.200	0.617	4.89 ± 0.09 [a]	Additive
BR	CA	375	156.25	0.375	0.313	0.688	5.19 ± 0.02 [a]	Additive
ACSL	SC	250	312.5	0.083	0.625	0.708	5.31 ± 0.05	Additive
BR	SC	375	250	0.375	0.500	0.875	5.39 ± 0.04	Additive
ACSL	HP	1250	25	0.417	0.500	0.917	4.93 ± 0.06 [a]	Additive
CA	LAE	312.5	3.125	0.625	0.313	0.938	5.43 ± 0.05	Additive
ACSL	HP	2500	6.25	0.833	0.125	0.958	4.59 ± 0.11 [a]	Additive

[a] pH levels significantly different BHI broth without antimicrobial ($p<0.05$).
[b] pH of BHI broth following addition of antimicrobial compounds.
[c] FIC: Fractional Inhibitory Concentration
[d] FIC_I: Fractional Inhibitory Concentration Index; ≤0.5=Synergistic, 0.5-4.0=Additive, >4.0=Antagonistic

Table 6. Synergistic and additive bactericidal antimicrobial combinations in BHI adjusted to pH 5.5.

Antimicrobial		Concentration (ppm)		FBC[c]				
1	2	1	2	1	2	FBC_I[d]	pH[b] (± SD)	Interaction
ACSL	SC	1250	62.5	0.125	0.063	0.188	5.05 ± 0.11 [a]	Synergistic
EPL	SC	31.25	62.5	0.313	0.063	0.375	5.60 ± 0.10	Synergistic
CA	EPL	78.125	50	0.004	0.500	0.504	5.25 ± 0.12 [a]	Synergistic
ACSL	BR	5000	375	0.500	0.214	0.714	4.14 ± 0.44 [a]	Additive
EPL	HP	100	50	0.333	0.500	0.833	5.61 ± 0.09	Additive
EPL	SC	62.5	250	0.625	0.250	0.875	5.62 ± 0.14	Additive
BR	LAE	375	31.25	0.214	1.560	1.777	5.31 ± 0.02 [a]	Additive

[a] pH levels significantly different BHI broth without antimicrobial ($p<0.05$).
[b] pH of BHI broth following addition of antimicrobial compounds.
[c] FBC: Fractional Bactericidal Concentration
[d] FBC_I: Fractional Bactericidal Concentration Index; ≤0.5=Synergistic, 0.5-4.0=Additive, >4.0=Antagonistic

Chapter IV:
Efficacy of antimicrobials for controlling *Listeria monocytogenes* in milk.

1. Abstract

Dairy-related recalls and illnesses are often attributed to *Listeria monocytogenes*. Although post-lethality interventions have been identified for more acidic foods, control options are limited for products where heating, increasing acidity, controlling water activity, and or other processing hurdles are not practical. The objective of this study was to determine the efficacy of five antimicrobials for controlling *L. monocytogenes* in milk. Stock solutions of caprylic acid (CA), ε-polylysine (EPL), hydrogen peroxide (HP), lauric arginate (LAE), and sodium caprylate (SC) were individually added to ultra-high-temperature pasteurized whole milk inoculated with *L. monocytogenes* at 4 log CFU/ml. Samples were stored at 7°C for 21 days to mimic a mild temperature abuse over the course of shelf life, and counts of *L. monocytogenes* were enumerated weekly. Of the five antimicrobials, EPL and HP applied at concentrations within the limits set by the FDA for use in milk significantly reduced bacterial counts over 21 days compared to the control on day 0 ($p<0.05$). At 200 ppm, EPL inhibited the growth of *L. monocytogenes* in milk throughout storage, while HP at 200 ppm rapidly reduced pathogen counts to undetectable levels through 21 days. Demonstrated efficacy within the acceptable usage limits set by the FDA suggests these antimicrobials are promising approaches to control *L. monocytogenes* in milk and serves as a basis for the identification of post-lethality antimicrobials treatments for use in to control *L. monocytogenes* in additional dairy products.

2. Introduction

Contamination of dairy products with *Listeria monocytogenes* can occur throughout the production chain including contamination of raw ingredients or post-pasteurization from the processing environment (Almeida et al., 2013). Though most dairy products are made from milk that undergoes pasteurization as a lethality step, *L. monocytogenes* remains a substantial threat to the dairy industry due to post-pasteurization contamination, the illegal use of raw milk in making cheeses that require pasteurized milk, and the importation of fresh cheeses from Mexico (Gould et al., 2014). *L. monocytogenes* can lead to an infection known as listeriosis when consumed by children, the elderly, pregnant women, and/or other immunocompromised individuals. Listeriosis initially presents itself with flu-like symptoms, but can escalate into more serious symptoms such as meningitis, and miscarriage in pregnant women. According to the Centers for Disease Control and Prevention (CDC) (CDC, 2016), there have been 19 reported outbreaks of listeriosis linked to dairy products since 2003 with 168 illnesses, 132 hospitalizations, and 24 deaths. Three of these outbreaks were linked directly to milk, and 15 to cheese. As an example, a 2007 outbreak of listeriosis linked to pasteurized milk produced by a small dairy producer in Massachusetts that resulted in four illnesses and two deaths highlights the need for rigorous control of post-pasteurization recontamination (Anonymous, 2008). When present in milk, *L. monocytogenes* has been shown to grow to high levels (6-8 log CFU/ml) during storage at refrigeration temperatures (Donnelly & Briggs, 1986; Rosenow & Marth, 1987; Leggett et al., 2012). According to a Quantitative Assessment of Relative Risk to Public Health from Foodborne *Listeria monocytogenes*, pasteurized milk carries a high relative risk per annum despite a moderate predicted per serving relative risk due to the fact that milk is consumed often by a large percentage

of the population (FDA/USDA, 2003). Therefore, there is a continuous need to identify methods to inhibit and inactivate *L. monocytogenes* in milk, as milk contaminated with *L. monocytogenes* can be consumed directly or used in the manufacture of dairy products. The use of antimicrobial compounds can not only reduce or eliminate *L. monocytogenes* in milk, but may also prevent growth in dairy products manufactured from milk containing the antimicrobial as well.

The use of antimicrobial compounds is a practical approach for controlling pathogen survival and growth. A number of antimicrobial compounds Generally Recognized as Safe (GRAS) by the United States Food and Drug Administration (FDA) including caprylic acid (CA), hydrogen peroxide (HP), lauric arginate (LAE), and ε-polylysine (EPL) have demonstrated efficacy in controlling *L. monocytogenes* under varying experimental conditions (Amrouche et al., 2010; Ma et al., 2000; Romanova et al., 2002; Nair et al., 2004). Caprylic acid is a medium-chain fatty acid that is naturally occurring in foods such as milk and coconut oil that has been shown to be effective at reducing levels of *L. monocytogenes* in milk (Nair et al., 2004). Although no research to date has been conducted to investigate the efficacy of sodium caprylate (SC), the sodium salt derivative of CA, against *L. monocytogenes* in milk, several fatty acid derivatives have demonstrated antimicrobial effects against Gram positive organisms in broth (Kabara et al., 1972). Both LAE and EPL are commercially available antimicrobial compounds that are both cationic, relying on interaction of charge to create changes in bacterial cells. Their efficacy against *L. monocytogenes* has not only been demonstrated in broth (Geornaras & Sofos, 2005; Brandt et al., 2010), but also when applied to foods (Geornaras et al., 2007; Luchansky et al., 2005). Hydrogen peroxide is also commercially available and has been shown to effectively inhibit the growth of *L. monocytogenes* in broth at relatively low concentrations (page 58) and in sterilized milk at 495

ppm (Dominguez et al., 1987). Currently, HP is approved for use in milk intended for cheesemaking at levels up to 500 ppm (21 CFR 582.1366).

Studies evaluating the efficacy of antimicrobials against *L. monocytogenes* in milk to date have not been carried out through the typical shelf life of milk. The shelf-life of milk is increasing as the quality of fluid milk improves with increasing technologies and management training (Martin et al., 2012). In fluid milk samples with low somatic cell counts, shelf-life can extend 21 days or longer, with little to no sensory defect (Ma et al., 2000). Therefore, research investigating the efficacy of antimicrobials over the course of a potentially longer shelf-life is imperative in order to reduce the risk of milk-related listeriosis outbreaks. The objective of this study was to determine the ability of five antimicrobial compounds (CA, EPL, HP, LAE, and SC) to control the growth of *L. monocytogenes* in UHT whole milk over 21 days of storage.

3. Materials and Methods

3.1 Bacterial strains, growth conditions and inoculum preparation

A total of eight *L. monocytogenes* strains were used in this study to represent a genetic diversity of ribotypes and sources. Strains included: F5069/ATCC 51414 (Raw Milk, DUP-1044B; serotype 4b), CWD 675-3 (Raw Milk, DUP-1053A; serotype 1a), CWD 1567 (Cheese, DUP-1038B; serotype 4b), Scott A (DUP-1042B; serotype 4b), 2012L-5323 (Ricotta salata outbreak, 2012; serotype 1/2a), 2014L-6025 (Hispanic-style cheese outbreak, 2014; serotype 1/2b), DJD 1 (washed-rind cheese outbreak, 2013), and CWD 193-10 U5-2 (cheese food contact surface, DUP-1030B). Strains DJD 1 was kindly provided by Minnesota Department of Agriculture Laboratory Services Division, and strains 2012L-5323 and 2014L-6025 were kindly provided by the CDC. *L. monocytogenes* strains Scott A has previously demonstrated increased tolerance to several antimicrobials compared with other strains or serotypes in broth (Castellano et al., 2001). Frozen

(-80°C) stock cultures were inoculated into 9 ml of Brain Heart Infusion (BHI) broth and incubated at 37 ± 1°C for 18 h for two subsequent transfers prior to use. Equal volumes from each culture were combined as a cocktail yielding ~9 log CFU/ml. The cocktail was then serially diluted in Butterfield's Phosphate Buffer (BPB), pelleted through centrifugation (15 min, 4200 rpm at 4°C), and re-suspended in ultra-high-temperature pasteurized (UHT) whole milk to the target concentration of ~5 log CFU/ml for use in the assays below. Viable numbers of *L. monocytogenes* in suspension were determined by aerobic plate counts on BHI agar after serial dilution and incubation at 37 ± 1°C for 24 ± 2 h.

3.2. Antimicrobial compounds and preparation

Antimicrobial stock solutions were prepared in sterile deionized water (SDW). The antimicrobials used included: CA (Sigma Aldrich, St. Louis, MO), EPL (25%, San-Ei Gen FFI (USA), Inc., New York, NY), HP (30%, Acros Organic, Pittsburgh, PA), LAE (Cytoguard LA2X, A+B Ingredients, Fairfield, NJ), and SC (Sigma Aldrich, St. Louis, MO). Caprylic acid, SC, and LAE were stored at room temperature, while EPL and HP were stored in the dark at 4˚C. All stocks were prepared on a volume/volume basis, except for SC which was prepared using a ratio of weight/weight. Antimicrobial stock solutions were then serially diluted in SDW to achieve the desired concentrations for the assays below. Stock solutions were vortexed prior to use, with CA vortexed vigorously in order to ensure homogeneity.

3.3. Inhibition and inactivation of L. monocytogenes in milk

The efficacy of the aforementioned antimicrobial compounds against *L. monocytogenes* was determined in UHT whole milk using a standard broth dilution assay (Andrews, 2001). Ultra-high-temperature pasteurized whole milk was used to eliminate the effect of background flora and to account for the impact of fat, which has been shown to reduce the efficacy of some compounds

such as LAE (Soni et al., 2010). Antimicrobials were added to UHT milk in 100µl aliquots to achieve the desired concentrations while minimizing the dilution of the milk. Three antimicrobial compounds (HP, EPL, LAE) were tested at 100, 200, 400, and 800 ppm. Additional compounds (CA and SC) were tested at 400, 800, 1600, and 3200 ppm. Milk samples were then inoculated with 500µl of the *L. monocytogenes* cocktail to achieve a target count of 4 log CFU/ml. Inoculated UHT milk samples with no antimicrobial were included as a positive control and uninoculated UHT milk served as a negative control. pH of each treatment was measured using a microtip electrode (Accumet AB150, Fisher Scientific International Inc., Hampton, NH). Samples were stored for 21 days at 7˚C to mimic mild temperature abuse along the food supply chain, and plated onto BHI agar on days 1, 7, 14, and 21 following serial dilution in BPB. Plates were enumerated following incubation at 37˚C for 24 hours. The positive control was sampled on day of inoculation to confirm the initial *L. monocytogenes* inoculation levels. At a given time point, inhibitory and bactericidal activity are defined as <0.5 log CFU/ml reduction and ≥3 log CFU/ml reduction in *L. monocytogenes* counts, respectively, compared to the inoculum level (Basri et al., 2014).

3.4. Statistical Analysis

Assays were performed in triplicate, with two internal replicates per sampling time point. Bacterial counts were analyzed using the GLM procedure of SAS 9.4 (SAS Institute, Cary, NC). The model accounts for repeated measures and includes treatment*concentration and time as main effects. pH values were compared using a one-way analysis of variance. Data was adjusted using Tukey's test applied to the least squared means of treatment replicates. Significance was defined as having a p-value of <0.05.

4. Results and Discussion

4.1. Sodium caprylate and caprylic acid have similar efficacy against L. monocytogenes in whole milk.

By day 14 of sampling, *L. monocytogenes* levels exceeded 8 log CFU/ml in milk without addition of an antimicrobial treatment. The two highest concentrations of CA tested in the present study (1600 and 3200 ppm) limited the growth of *L. monocytogenes* to levels significantly lower than control through 14 days of storage ($p<0.001$). However, at 21 days, counts of *L. monocytogenes* only differed significantly from the positive control for the 3200 ppm treatment (Figure 1). At day 21, only 3200 ppm CA was inhibitory with counts that were not significantly different from the starting inoculum level. Similarly, a minimum inhibitory concentration (MIC) of 3250 ppm has been reported for *L. monocytogenes* in broth (page 58) compared to 3200 ppm in whole milk at a similar pH of ~6.7 (Table 1). Nair et al. (2004) investigated the effectiveness of CA in sterile whole milk at various temperatures and found that ~7200 ppm resulted in a significant reduction of *L. monocytogenes* after 48 hours at 8˚C while ~3600 ppm did not. Continued efficacy over the shelf life of milk beyond 48 hours was not determined. In the present study, counts were not significantly lower than control until day 7, suggesting that CA at lower concentrations may gradually reduce *L. monocytogenes* over time. Of the four concentrations of SC tested, two (1600, 3200 ppm) resulted in counts of *L. monocytogenes* significantly lower than the control on day 21 of sampling ($p<0.0117$ and $p<.0001$, respectively; Figure 2). Sodium caprylate at 3200 ppm was the most effective treatment of the four tested, with levels of *L. monocytogenes* in milk with 3200ppm significantly lower than in milk with 1600 ppm of SC. There was no significant difference between counts of *L. monocytogenes* on day 21 in milk with CA or SC applied at 3200 ppm ($p=1.0$), suggesting that the solubility of CA did not inhibit its antimicrobial activity. Both

treatments also inhibited the growth of *L. monocytogenes* throughout storage as counts on day 21 did not differ significantly from inoculum level ($p=1.0$). However, none of the concentrations tested were characterized as bactericidal. Higher concentrations may be necessary to see a bactericidal effect, as observed in broth pH 7.4, in which 4500 ppm of CA and 13000 ppm of SC were bactericidal (pages 58-59).

4.2. LAE reduces viable L. monocytogenes counts in milk at higher concentrations.

Currently, the use of LAE has been approved at levels up to 200 ppm in certain animal-based foods (FDA, 2006). Previous research has demonstrated that LAE is effective in the control of *L. monocytogenes* in milk but is reduced in the presence of increasing fat content (Soni et al., 2010). Of the four concentrations of LAE tested, 800 ppm was the only treatment that inhibited *L. monocytogenes* growth, with levels of *L. monocytogenes* reduced by approximately 2 log CFU/ml on day 21 of sampling compared to the starting inoculum (Figure 3). As seen in Figure 3, treatments of 100, 200, and 400 ppm allowed for growth similar to the control throughout storage. For 800 ppm, counts decreased steadily throughout 14 days, with limited change through the last 7 days of storage. Overall, *L. monocytogenes* counts decreased by ~1.7 log CFU/ml over the 21 days of storage as compared to control on day 0. No significant differences in bacterial counts were observed between 100, 200, and 400 ppm treatments at any time point and none of these treatments tested were inhibitory, with counts on day 21 significantly different from the starting inoculum level ($p<0.0001$). Soni et al. (2010) reported similar growth patterns when 400 ppm was used in skim milk, but found that 800 ppm of LAE lowered *L. monocytogenes* to undetectable levels in both skim and whole milk, which persisted throughout storage. Similarly, Ma et. al (2013) found that 750 ppm of LAE in 2% reduced fat skim milk resulted in a 6.20 log CFU/ml reduction in *L. monocytogenes* strain Scott A. Therefore, results observed in the present study were similar

to those reported in the literature aside from the lower efficacy reported for 800 ppm. These differences may be attributed to differences in fat content (i.e. skim vs. whole milk) and/or differences in strains used. The impact of fat content and other food components on the inhibitory concentration of LAE is also demonstrated by the increase of minimum inhibitory concentration in milk (between 400 and 800 ppm) compared to those reported for the same strains of *L. monocytogenes* in broth at pH 7.4 (6.25 ppm) (page 58).

4.3. EPL treatments are inhibitory and produce gradual reductions in L. monocytogenes counts in milk.

As shown in Figure 4, EPL treatments of 100 and 200 ppm resulted in growth inhibition, with no significant change in counts from the starting inoculum to day 21. Treatments at 400 and 800 ppm resulted in a gradual decrease in pathogen counts from and inoculum of ~4.4 log CFU/ml to 2 and 1 log CFU/ml, respectively, after 21 days. On day 7 of sampling, *L. monocytogenes* counts were significantly lower in all treatments as compared to the control, with no significant difference between treatments. By day 21, the 800 ppm treatment resulted in levels significantly lower than 100 and 200 ppm treatments ($p<0.05$). These results are similar to results reported by Geonaras et al. (2007) investigating the efficacy of EPL in fat-free and whole milk diluted 1:10 in distilled water. At 200 ppm, *L. monocytogenes* levels were lowered to undetectable levels in both extracts after 6 days of storage. Bactericidal activity at a lower concentration of 200 ppm may be attributed to the milk dilution performed in preparation of the milk extracts, effectively reducing the inhibitory effect of fat and protein content within the food matrices. Geonaras et al. (2007) also report an increase in the pH of milk with addition of EPL, which is consistent with treatments of 400 and 800 ppm in this study (Table 1). Currently, EPL is approved for use in cream and various cheeses at a level up to 250 ppm (FDA, 2011). Similar to LAE, the inhibitory concentration of

EPL was approximately ten-fold higher in milk than in broth at pH 7.4, where the MIC of EPL against the same cocktail of *L. monocytogenes* strains was reported at 20 ppm (page 58). Again, reductions in antimicrobial efficacy are most likely attributed to the interaction of EPL with food components in milk such as proteins. Casein molecules carry a net-negative charge and EPL is a surface-active positively-charged molecule. The potential binding of positively charged EPL with negatively charged proteins could in theory reduce antimicrobial efficacy of EPL by not allowing EPL to act on bacterial cells.

4.4. Hydrogen peroxide rapidly inactivates Listeria monocytogenes in milk.

Of the antimicrobials tested, HP was the most effective in terms of effective concentrations used and overall inhibition and inactivation of *L. monocytogenes* throughout 21 days of storage. In previous research, HP has been shown to quickly reduce *L. monocytogenes* counts in BHI broth over a range of concentrations at 37˚C (Liu & Ream, 2008), and when applied at 495 ppm to sterilized milk stored at 4˚C (Dominguez et al., 1987). In this study HP treatments were found to be either inhibitory (100 ppm) or bactericidal (200, 400, 800 ppm) (Figure 1). Treatments at 200, 400, and 800 ppm reduced *L. monocytogenes* counts to undetectable levels (<1 CFU/ml) by day 7. On day 21 of sampling, there was no significant difference in *Listeria* counts between any of the treatments. However, 1-2 log CFU/ml remained after 21 days in the 100 ppm treatment group, suggesting that 100 ppm is not enough to completely inactivate *L. monocytogenes* in milk when present at initial levels of 5 log CFU/ml. Two of the three bactericidal treatments (200 and 400 ppm) and the inhibitory treatment (100 ppm) identified for HP in the present study are within currently approved limits and do not significantly change milk pH levels (Table 1). Therefore, HP treatments may be promising for reducing levels of *L. monocytogenes* in whole milk throughout storage. Although these treatments are effective at reducing levels of *L. monocytogenes*, there is

evidence that HP applied as low as 10 ppm may inhibit acid production by starter cultures even after residual HP is seemingly neutralized using catalase (Subramanian & Olson, 1968) thereby potentially limiting its use in the production of cultured dairy products. Future studies are needed to investigate the rate of pathogen inactivation within the first 24 hours and detection of residual peroxide as use in milk for cheese requires neutralization with catalase prior to processing (Subramanian & Olson, 1968). Oxidative rancidity has been known to occur when adding HP to foods with high fat levels (Wambura & Yang, 2011) due to free radical production, suggesting that it could potentially cause sensorial changes in high-fat dairy products.

5. Conclusions

These data identify treatments that inactivate and inhibit *L. monocytogenes* in whole milk during 21-day storage at 7˚C. Of the five antimicrobials tested, HP at 200 ppm and EPL at 100-200 ppm were the most effective treatments with concentrations that fall within current approved usage limitations. Furthermore, neither of these treatments affected milk pH. Future studies are needed to address potential sensorial changes of effective treatments and the impact of combinatorial antimicrobial treatments in milk in an attempt to further reduce usage levels and increase efficacy.

Acknowledgements

This project was supported by the National Dairy Council and the United States Department of Agriculture (USDA) National Institute of Food and Agriculture (NIFA), Multistate project S1056. Any opinions, findings, conclusions, or recommendations expressed in this manuscript are those of the author(s) and do not necessarily reflect the view of the NIFA, the USDA or any other sponsors.

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7. Figures and Tables

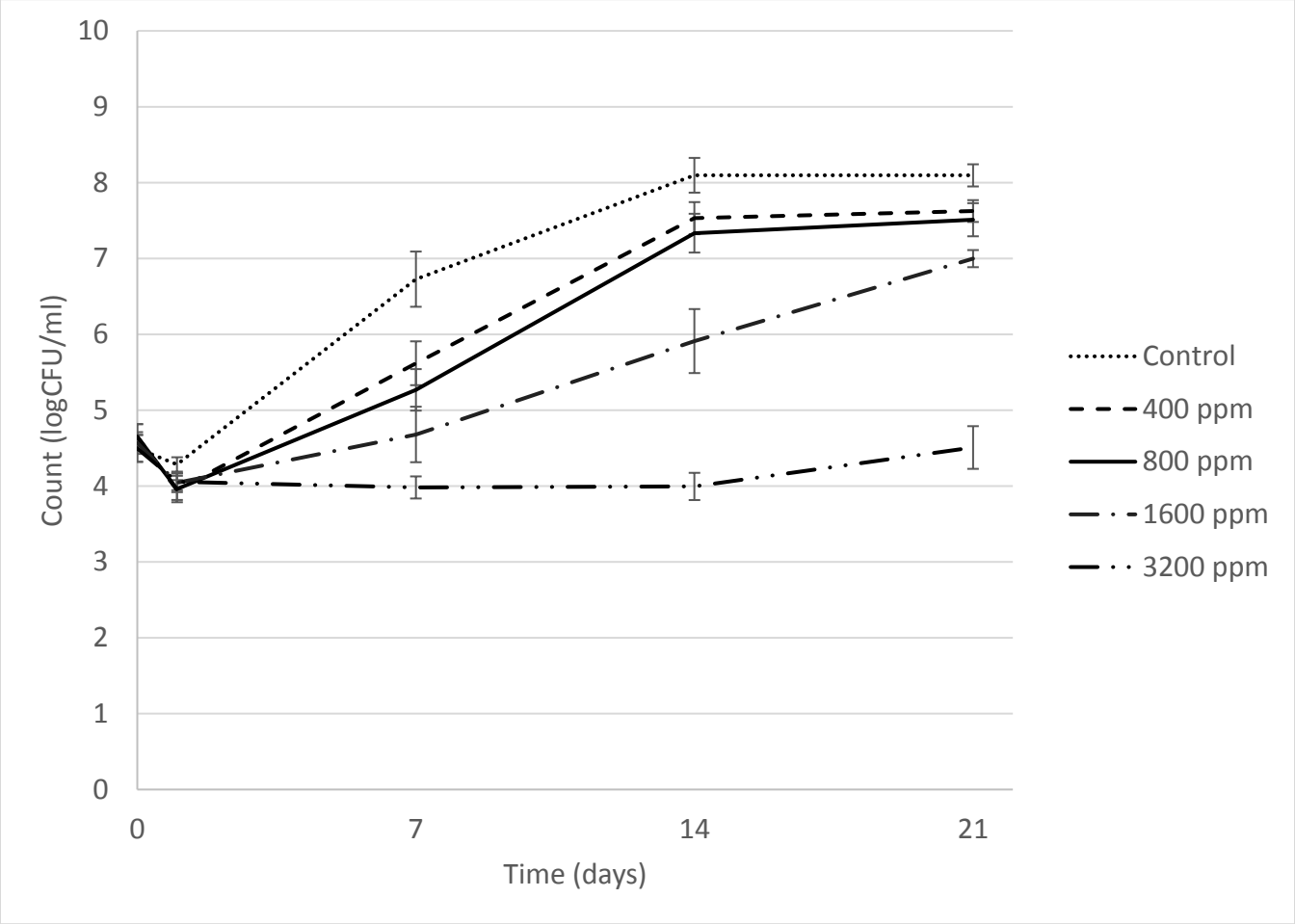

Figure 1. Change in *Listeria monocytogenes* counts in UHT milk with caprylic acid over 21 days of storage at 7˚C.

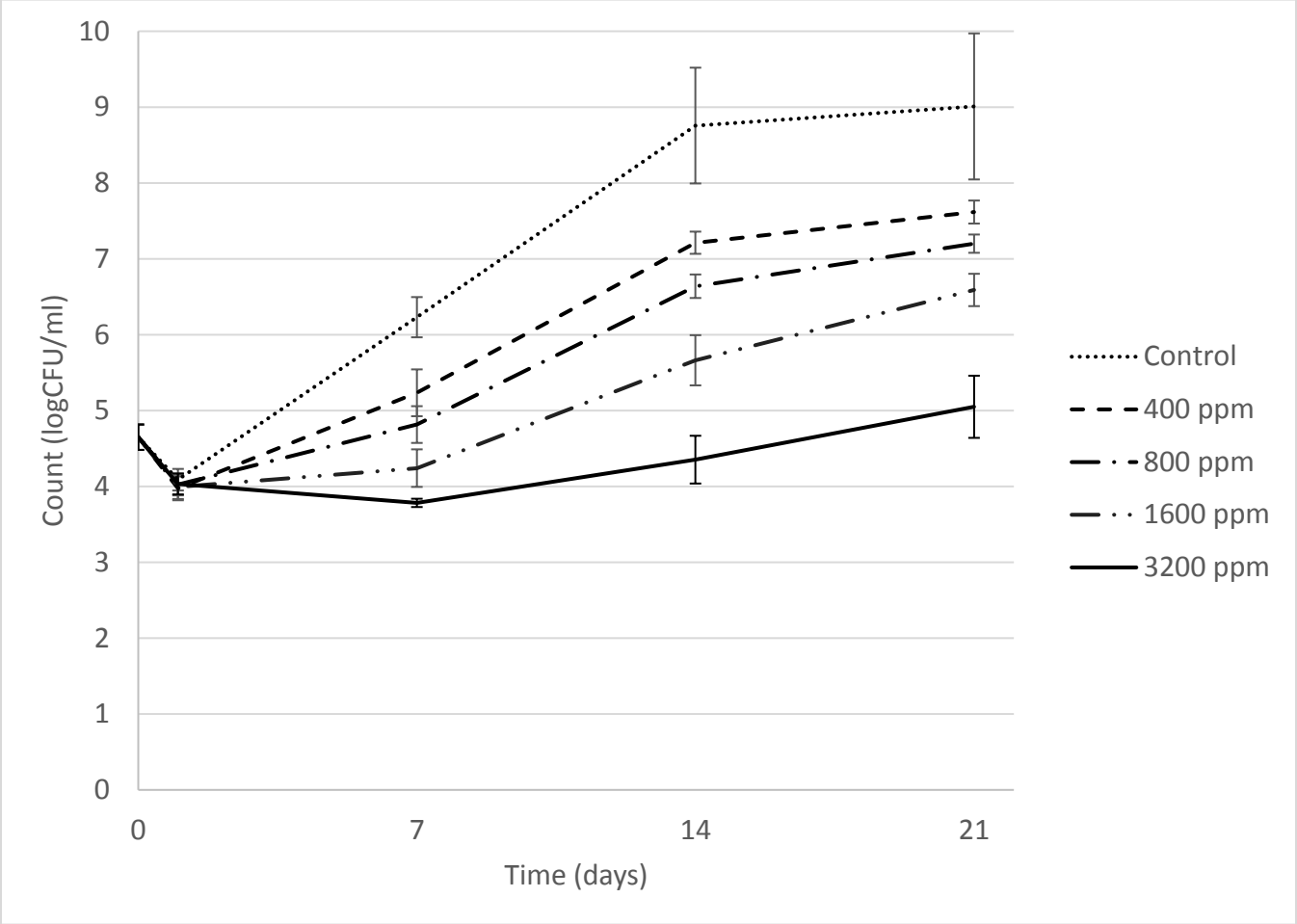

Figure 2. Change in *Listeria monocytogenes* counts in UHT milk with sodium caprylate over 21 days of storage at 7˚C.

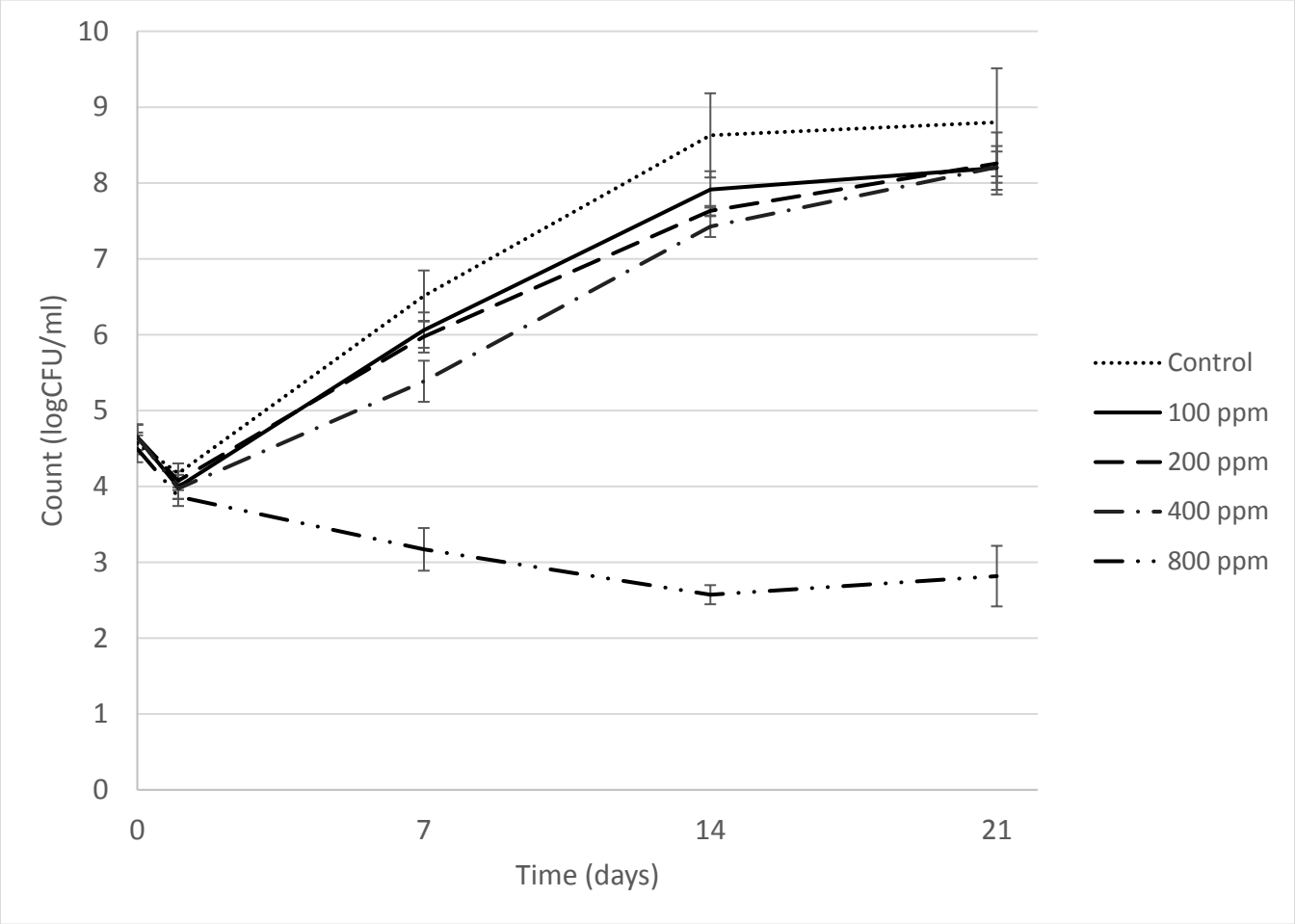

Figure 3. Change in *Listeria monocytogenes* counts in UHT milk with lauric arginate over 21 days of storage at 7˚C.

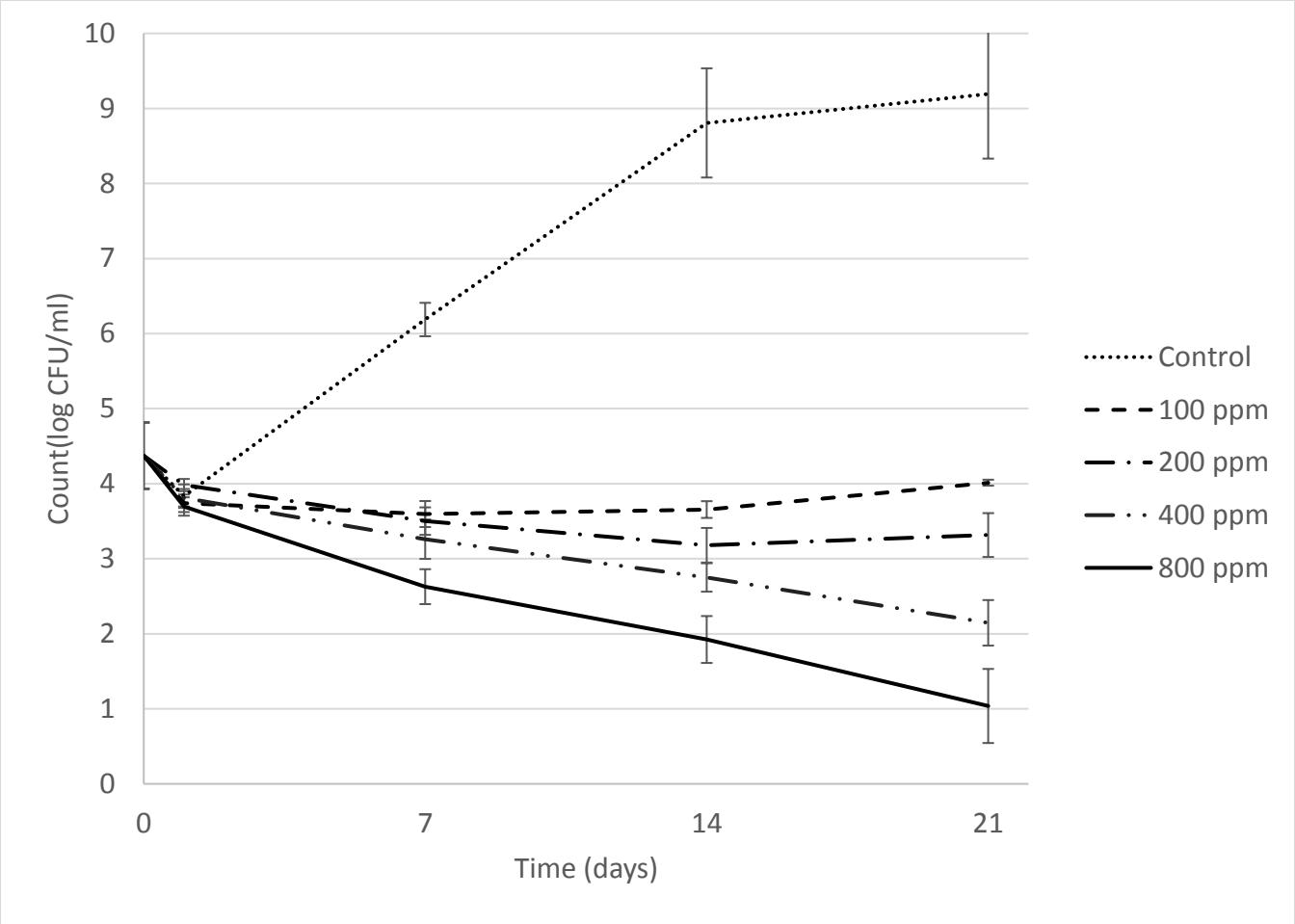

Figure 4. Change in *Listeria monocytogenes* counts in UHT milk with ε-polylysine over 21 days of storage at 7˚C.

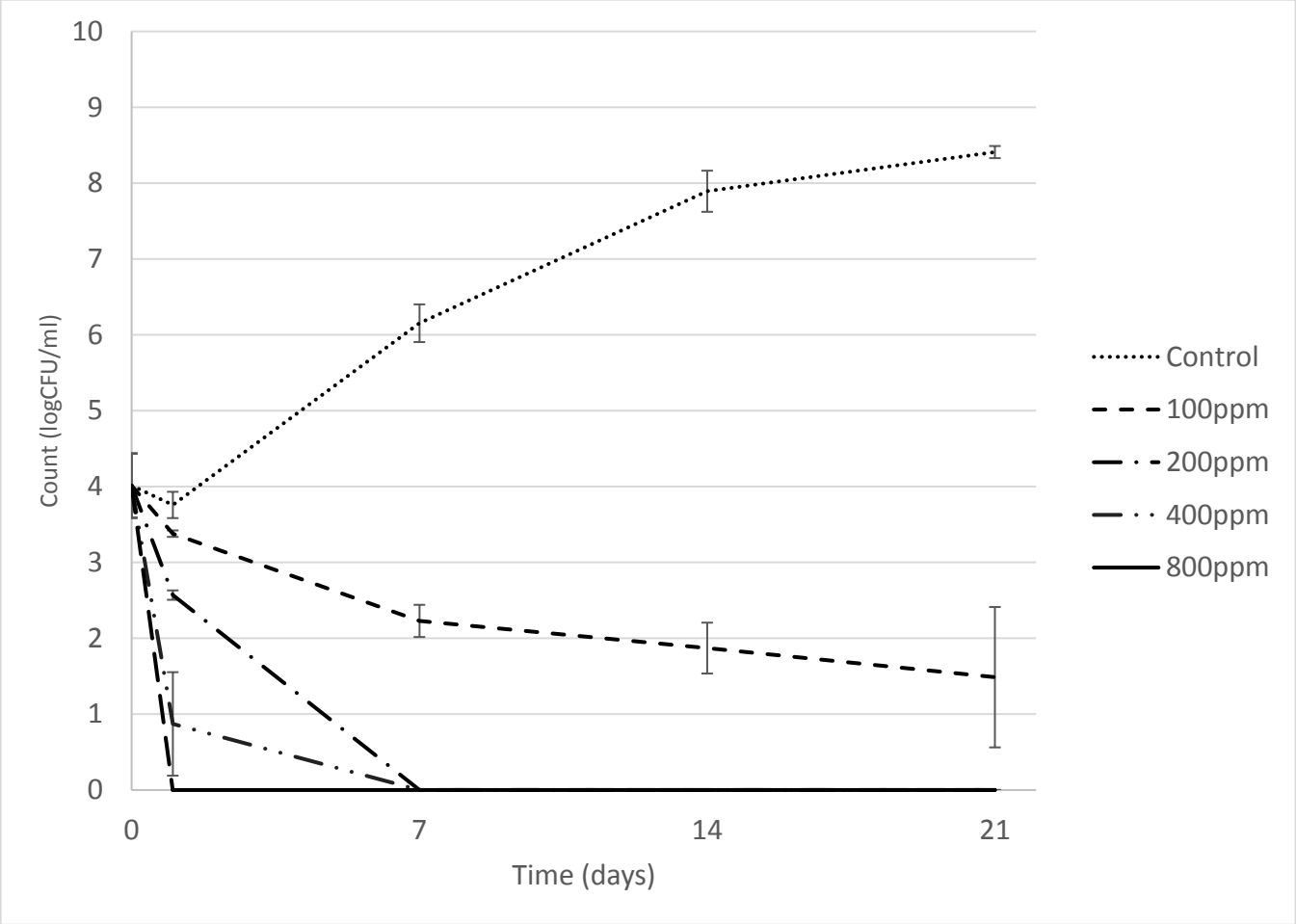

Figure 5. Change in *Listeria monocytogenes* counts in UHT milk with hydrogen peroxide during storage at 7˚C.

Table 1. pH of ultra-high-temperature pasteurized whole milk containing antimicrobials at varying concentrations on day of inoculation.

Treatment	Concentration (ppm)	pH[b] ± SD
Control	-	6.67±0.02
CA	400	6.68±0.03
	800	6.70±0.00
	1600	6.69±0.02
	3200	6.69±0.01
EPL	100	6.67±0.02
	200	6.71±0.01
	400	6.83±0.01[a]
	800	6.78±0.04[a]
HP	100	6.66±0.01
	200	6.68±0.00
	400	6.68±0.01
	800	6.68±0.00
LAE	100	6.57±0.02[a]
	200	6.53±0.02[a]
	400	6.52±0.02[a]
	800	6.53±0.02[a]
SC	400	6.72±0.01[a]
	800	6.75±0.01[a]
	1600	6.74±0.03[a]
	3200	6.73±0.12[a]

[a] pH significantly different from control (UHT milk without antimicrobial added) ($p<0.05$).
[b] pH of milk with added antimicrobials at time of inoculation.

Chapter V

Efficacy of antimicrobials and their combinations

for controlling *Listeria monocytogenes* in Queso Fresco.

1. Abstract

The efficacy of individual and combinatory treatments of acidified calcium sulfate with lactic acid (ACSL), ε-polylysine (EPL), hydrogen peroxide (HP), lauric arginate ethyl ester (LAE), and sodium caprylate (SC) to control *Listeria monocytogenes* as surface contaminants on fresh cheese [Queso Fresco (QF)] was investigated. Antimicrobial treatments applied in the form of aqueous dips included: HP 5%, LAE 2%, LAE 5%, EPL 10%, SC 10%, ACSL 25%, LAE 5%+EPL 10%, EPL 10% + SC 10%, ACSL 25% + SC 10%, and LAE 5% + SC 10%. Combinations were tested to identify potential additive, synergistic, or antagonistic activity. Queso Fresco was inoculated with an eight-strain cocktail of *L. monocytogenes* at ~4 log CFU/g, dipped in antimicrobial solutions, vacuum-packaged, and then stored at 7˚C for 35 days. *Listeria monocytogenes* counts were determined 24h after dip application and weekly throughout storage. Dip treatments in HP at 5% resulted in a decrease in *L. monocytogenes* counts to <1 log CFU/gram by 24 hours, which remained below this level through 35 days of storage. Dip treatments in a 10% solution of SC resulted in significantly lower counts of *L. monocytogenes* compared to the control on day 35. Combinatory treatments of EPL + LAE did not result in levels of *L. monocytogenes* lower than either compound alone. However, the combinations ACSL + SC, EPL + SC, and LAE+ SC produced reductions in *L. monocytogenes* counts that were significantly lower than the control throughout storage and significantly lower than each of the compounds applied individually. These data indicate that HP dip treatments and several combinatory treatments are effective methods for inhibiting and inactivating *L. monocytogenes* on QF throughout 35-day shelf-life.

2. Introduction

Each year, approximately 1 in 6 Americans fall victim to foodborne illness caused by pathogenic foodborne microorganisms (CDC, 2016) with an estimated financial burden of $77.7 billion dollars (Scharff, 2012). Dairy foods are the second most common food category after produce implicated in foodborne illness outbreaks. Of the 31 known biological agents that cause foodborne illness, *Listeria monocytogenes* is the third leading cause of foodborne-illness related deaths, with an estimated 260 deaths per year (CDC, 2016). Furthermore, the presence of *Listeria* or suspected presence in foods can lead to recalls, which causes a financial burden to producers. Therefore, there is a continuous need for the development of methods to prevent listeriosis outbreaks and *Listeria*-related recalls.

Mexican/Hispanic-style soft cheeses, notably Queso Fresco (QF), have been the source of several listeriosis outbreaks and recalls in recent years. Ques Fresco is a fresh, mild cheese, popular amongst the Hispanic community (Van Hekken et al., 2012). In 2015, 30 persons across 10 states were hospitalized due to the consumption of soft cheeses, including QF, which had been contaminated with *L. monocytogenes* (FDA, 2016). This fresh cheese has a short shelf-life of ~ 35 days (Leggett et al., 2012), and is usually consumed before the end of shelf life is reached. In the United States, the Code of Federal Regulations (CFR) Title 21 mandates pasteurization of milk for the production of non-standardized cheeses including fresh soft cheeses like QF (21 CFR 1240.61). Although pasteurization of milk used in the manufacture QF is mandatory in order for it to be sold across state lines, outbreaks have been linked to cheeses made with improper pasteurization or the use of unpasteurized milk mixed in with pasteurized milk (Gould et al., 2014; Genigeorgis et al., 1991). In outbreaks linked to pasteurized cheese, contamination occurs due to presence and persistence of *L. monocytogenes* in the processing facility. Research indicates that *L.*

monocytogenes is commonly found in processing facilities drains, on floors, and on worker boots, and can persist in the same facility for multiple years (D'Amico and Donnelly, 2009). Research has shown that *L. monocytogenes* can grow to levels >7 log CFU/gram in QF over 35 days of storage at 4˚C and more rapidly at 10˚C (Leggett et al., 2012). Queso Fresco supports the survival and growth of *L. monocytogenes* due to its high moisture, low salt, and relatively high pH (>6.0) (Van Hekken et al., 2012).

In an effort to control *L. monocytogenes* outbreaks and recalls, the USDA Food Safety Inspection Service (USDA-FSIS) has issued a final rule for ready-to-eat (RTE) meats and poultry, requiring that producers of these foods employ one of three alternatives to control *L. monocytogenes* as post-lethality contaminants. Several antimicrobial compounds have demonstrated efficacy in the control of *L. monocytogenes* (Koseki et al., 2007; Lavieri et al., 2014; Luchansky et al., 2005; Upadhyay et al., 2013), suggesting they may be promising approaches for the control of *L. monocytogenes* in dairy foods. However, such treatments can produce sensorial changes in the foods which they are applied to (Smith et al., 2015), including changes in color, odor, and taste. In a study conducted on QF in which antimicrobials were applied to curds, Gadotti (2011) found that addition of antimicrobials (caprylic acid, nisin, cinnamaldehyde) produced a significant "off-flavor", and the overall liking of the QF decreased (Gadotti 2011). Therefore, it is desirable to use combinations of antimicrobials to produce a synergistic effect, in which compounds used together produce greater combined effect in comparison to the sum of their separate effects (Brandt et al., 2011). By using synergistic combinations, the usage levels of each antimicrobial in the combination can be reduced, which can limit these sensory effects. Antimicrobials with demonstrated anti-listerial efficacy when used alone or in combinations include acidified calcium sulfate with lactic acid (ACSL), ε-polylysine (EPL), hydrogen peroxide

(HP), lauric arginate ethyl ester (LAE), and sodium caprylate (SC) (Chapter III; Brandt et al., 2011; Brandt et al., 2010; Dominguez et al., 1987). The application of antimicrobials can also reduce the natural microflora of QF (Gadotti, 2011), which could potentially extend the shelf life of the cheese. The purpose of this study was to determine the efficacy of these five antimicrobial compounds on their own and in combination for the control of *L. monocytogenes* as surface contaminants on QF throughout storage at 7˚C for 35 days.

3. Materials and Methods

3.1. Bacterial strains, growth conditions and inoculum preparation

A total of eight *L. monocytogenes* strains were used in this study to represent a genetic diversity of ribotypes and sources. Strains included: F5069/ATCC 51414 (Raw Milk, DUP-1044B; serotype 4b), CWD 675-3 (Raw Milk, DUP-1053A; serotype 1a), CWD 1567 (Cheese, DUP-1038B; serotype 4b), Scott A (DUP-1042B; serotype 4b), 2012L-5323 (Ricotta salata outbreak, 2012; serotype 1/2a), 2014L-6025 (Hispanic-style cheese outbreak, 2014; serotype 1/2b), DJD 1 (washed-rind cheese outbreak, 2013), and CWD 193-10 U5-2 (cheese food contact surface, DUP-1030B). Strains DJD 1 was kindly provided by Minnesota Department of Agriculture Laboratory Services Division, and strains 2012L-5323 and 2014L-6025 were kindly provided by the CDC. Frozen (-80°C) stock cultures were inoculated into 9 ml of Brain Heart Infusion (BHI) broth and incubated at 37 ± 1°C for 18 h for two subsequent transfers prior to use. Equal volumes from each culture were combined as a cocktail yielding ~9 log CFU/ml. Viable numbers of *L. monocytogenes* in suspension were determined by aerobic plate counts on BHI agar after serial dilution, incubated at 37 ± 1°C for 24 ± 2 h. The cocktail was then serially diluted in Butterfield's Phosphate Buffer (BPB), pelleted through centrifugation (15 min, 4200 rpm at 4°C), and re-suspended in BPB to the target concentration of ~5 log CFU/ml for use in the assays below. Cell counts of starting inoculum

were confirmed through serial dilution and enumeration on BHI agar following incubation at 37˚C for 18-24 hours.

3.2. Cheese manufacture.

Queso Fresco was manufactured in the University of Connecticut Creamery in a Kusel double-O style cheese vat according to a standard protocol using 50 gallons of pasteurized cow's milk standardized to 3.5% fat using skim milk and cream (Garelick Farms, Franklin, MA). Briefly, calcium chloride ($CaCl_2$ 0.02% v/v) was added to the milk after filling the vat and thoroughly mixed. Milk temperature was raised to 32°C and pH was adjusted to 6.45 with dilute lactic acid (50% v/v in sterile water) (85% lactic acid, Sigma-Aldrich, St. Louis, MO). Chymosin (DCI Star Double strength microbial rennet, Dairy Connection, Madison, WI) was added at 15.83ml/100 liters and milk was stirred for approximately 45 seconds to mix. Once desired firmness was reached, the coagulum was cut into 1x1 cm curds using sanitized stainless steel curd knives. Cutting time was determined with the following equation: cutting time = time of flocculation + (time of flocculation x 2). Curds were allowed to rest for 5 min and then stirred for 5 min, which was repeated for three cycles (a total of 30 minutes). After an additional 5 min rest, the majority of the whey was removed by draining. The curds were then transferred to standard 20# Wilson hoops lined with disposable cheese cloth and pressed at ~10 PSI in a room maintained between 20-25°C for 20 minutes. Cheeses were then removed from the hoops, broken up and salted with kosher salt (Diamond Crystal, Cargill, Wayzata, MN) to obtain a final NaCl concentration of ~2%. Salted curds were returned to the hoops and pressed ~10 PSI for an additional 75 min. Cheese blocks were then vacuum packaged and stored at 4˚C prior to cutting into experimental units within 48 hours.

3.3. Physicochemical analysis of cheese.

Cheese composition is provided in Table 1. Cheese characteristics were targeted based on previous work identifying cheese compositions associated with *Listeria* presence (Lovett et al., 1987). Physiochemical analysis was conducted on the cheese following manufacture including pH (Accumet AB150 with microtip electrode, Fisher Scientific International Inc., Hampton, NH), dry matter (DM; after drying to constant weight at 102°C), fat (Babcock method), and salt (NaCl as chloride) (Quanttab Chloride, Hach, Loveland, CO). Salt in the aqueous phase, moisture in the non-fat substance (MNFS), and fat in water free substance were determined using the formulae: Salt in the aqueous phase = (salt/ (100-DM))*100; MNFS = (100-DM/ (100-fat))*100; Fat in water free substance = (fat/DM)*100.

3.4. Cheese sample preparation and inoculation

For each trial, samples weighing 25 ± 1g were cut from refrigerated vacuum packaged cheese blocks using sterile knives. For each 25g sample, 100µL of the inoculum was spread over a single surface (~6 cm^2) with a sterile spreader, in order to attain a target contamination level of 4 log CFU/g. Inoculated samples were allowed a 30-minute drying period to enable bacterial attachment prior to dipping application.

3.5. Antimicrobial dip preparation and application

Antimicrobial dip solutions were prepared in sterile deionized water (SDW) in sterile containers. Antimicrobials used are as follows: ACSL (Mionix Corporation, Scottsdale, AZ), EPL (25%, San-Ei Gen FFI (USA), Inc., New York, NY), HP (30%, Acros Organic, Pittsburgh, PA), LAE (Cytoguard LA2X, A+B Ingredients, Fairfield, NJ), and SC (Sigma Aldrich, St. Louis MO). Dip solutions were prepared on a volume/volume basis, except for EPL and SC which were prepared using a ratio of weight/weight. Sample preparation for days 1, 7, 14, 21, 28, and 35

included two inoculated samples dipped in the appropriate aqueous antimicrobial dip solution and two dipped in SDW to serve as controls. Although the volume of antimicrobial dip solution transferred to each individual cheese unit can vary, the approximate transfer of antimicrobial solutions to 25g cheese samples was determined to be ~0.2g. This approximate value was used to estimate antimicrobial concentrations (ppm) in finished product (Table 2). Dip concentrations were selected based on preliminary data to identify potential inhibitory, subinhibitory, and bactericidal concentrations within limits presented by federal regulations and obvious sensorial changes. Single antimicrobial treatments were as follows: 3% HP (250 ppm), 5% HP (400 ppm), 10% SC (800 ppm), 25% ACSL (2000 ppm), 10% EPL (800 ppm), and LAE at both 2% (150 ppm) and 5% (400 ppm). Percentages reflect the antimicrobial concentration in the aqueous dip whereas ppm reflects the approximated final antimicrobial concentration per gram of cheese. Antimicrobial treatments were also tested in combinations through sequential application: 2% LAE + 10% EPL; 5% LAE + 10% EPL; 10%SC + 25% ACSL; 10% EPL + 25% ACSL; and 10% EPL + 10% SC. After inoculation and drying, cheese samples were submerged in respective antimicrobial solutions at room temperature for one minute, then placed in vacuum bags (3 mil, UltraSource LLC, Kansas City, MO), vacuum-sealed, and stored at 7˚C. For combinations, cheese samples were dipped sequentially in two separate antimicrobial solutions, with one minute between the first and second application. The order of antimicrobial application was based on preliminary trials (data not shown).

3.6. Listeria monocytogenes enumeration

At each sampling time point, cheese samples were removed from refrigerated storage and homogenized in 100 ml of Dey-Engley broth (DE; Becton, Dickinson and Company, Sparks, MD) in a Smasher stomacher (Biomerieux, France) for 1 minute at 560 strokes/minute to neutralize

antimicrobials. Bags were then briefly hand mixed to ensure consistent homogenization. Following serial dilutions in Butterfield's Phosphate Buffer (BPB), homogenates were plated onto Modified Oxford Agar (MOX), incubated at 37˚C for 48 hours, and enumerated. On day 0 (day of inoculation), two inoculated samples receiving no dip application were processed to confirm initial inoculation levels. Two uninoculated negative controls were also processed to confirm absence (<5 CFU/g detection limit) of *L. monocytogenes* in uninoculated QF. *Listeria monocytogenes* on MOX plates were enumerated following incubation at 37˚C for 24-48 hours.

3.7. Analysis

For each treatment, inhibitory and bactericidal activity, as well as synergism, were defined based on previous work (Basri et al., 2014). At a given time point, inhibitory and bactericidal activity are defined as <0.5 log CFU/g change and ≥3 log CFU/g reduction in *L. monocytogenes* counts, respectively, compared to the inoculum level. Synergistic interactions of the combined treatments were defined as ≥2 log CFU/g reduction in *L. monocytogenes* counts compared to the most active antimicrobial on its own.

Experiments were performed in triplicate using three independently produced batches of cheese, with two samples per treatment per time point. Bacterial counts were analyzed using the GLIMMIX procedure of SAS 9.4 (SAS Institute, Cary, NC). The model included treatment*concentration and time as major effects. Data was adjusted using Tukey's test applied to the least squared means of treatment replicates. Significance was defined as having a p-value of <0.05.

4. Results and Discussion

4.1. Hydrogen peroxide rapidly inactivates L. monocytogenes on QF.

In samples of cheese without antimicrobial treatment, *L. monocytogenes* reached 8 log CFU/g at day 21 of sampling. Treatments in a 5% solution of HP (400 ppm in final product) for 60 seconds reduced counts of *L. monocytogenes* to <1 log CFU/g by day 1 of sampling, with counts remaining consistently at this level throughout the remainder of storage. With an approximate 3.5 log decrease from the starting inoculum (Figure 1), this treatment was considered bactericidal. Levels of *L. monocytogenes* remained significantly lower than the control and remained below 1 log CFU/g (<0.05) at all sampling days. On the final day of storage (day 35), counts for treated cheese samples were 7.5 log CFU/g lower than the control. These data are in agreement with those observed in milk, as 400 ppm of HP in milk reduced *L. monocytogenes* counts to undetectable levels throughout storage at 7˚C for 21 days (page 82). Results from HP treatments at 3% were inconsistent (data not shown). These inconsistencies were attributed to the use of different lots of HP and the possible degradation of one lot over time. The results from one set reflected results expected of 250 ppm of HP in final product, as 200 ppm in milk resulted in undetectable levels of *Listeria monocytogenes* throughout storage (page 82). If HP behaves similarly in milk and QF as the results of a 400 ppm treatment suggests, it is most likely that the HP used in the less effective trial was in fact degraded. The use of aqueous HP dips has been successful at reducing *L. monocytogenes* levels on other foods as well. When cantaloupes were washed with a 2% aqueous solution at 55˚C, levels of *L. monocytogenes* decreased by 4 log CFU/gram after 10 minutes (Upadhyay et al., 2013).

4.2. EPL and LAE have limited inhibitory effects against L. monocytogenes *on QF when used alone and in combination.*

Based on data from Chapter III showing that LAE and EPL produce inhibition and inactivation at low concentrations, these antimicrobials were first tested individually. Each individual treatment resulted in *L. monocytogenes* counts lower than the control up to 7 days of storage and LAE treatments at 5% remained below control through 14 days of storage. Both treatments of LAE produced 2 log reductions within the first 24 hours of storage and resulted in at least a one log reduction in *L. monocytogenes* counts compared to the control throughout 21 days of storage. By day 35 of sampling there was no significant difference in *L. monocytogenes* counts in samples treated with either LAE at 2% or 5%, compared to the control ($p=0.99$). This is in agreement with previous research, which consistently shows that LAE produces an initial inactivation with no inhibitory effect (Porto-fett et al., 2010; Lavieri et al., 2014). As shown in Table 1, the final concentrations of LAE on cheese was approximated at 150 and 400 ppm for the 2% and 5% dips, respectively. Soni et al. (2010) observed that both 200 and 800 ppm of LAE applied to QF resulted in an initial reduction in *L. monocytogenes* counts, followed by regrowth. In this study counts reached 8 log CFU/g for the 200 ppm treatment and 6 log CFU/g for 800 ppm day 28 (Soni et al., 2010). These results are in agreement with the results of the present study as well, showing that LAE successfully reduces *L. monocytogenes* initially but may not be effective for preventing growth over extended shelf-life on its own. In a study by Taylor (2013), it was observed that lower starting inoculums (1 or 2 log vs. 4 log), delay the onset of *L. monocytogenes* growth on cheese agar. Therefore, further studies are needed to determine if LAE could inhibit *L. monocytogenes* on QF at a lower starting inoculum.

Based on synergistic bactericidal effects of LAE and EPL in combination against *L. monocytogenes* in broth pH 7.4 (page 61), antimicrobial solutions of EPL at 10% were tested with LAE at both 2% and 5%. Cheese samples were dipped in EPL before LAE, based on previous

studies showing that this sequence produced a greater reduction in *Salmonella* on chicken carcasses (Benli et al., 2011). The combined treatment with EPL with either concentration of LAE did not produce counts significantly different than the respective concentration of LAE alone (Figures 2 and 3). By 28 days of storage, neither EPL, LAE, nor any combination resulted in significantly different counts than the control ($p > 0.77$). When applied to milk, the efficacy of both EPL and LAE decreased compared to broth (pages 58 and 59), as evidenced by the ~10-fold increase in inhibitory concentrations. Protein and fat levels are higher in cheese than in milk due to the removal of whey; therefore, it can be speculated that the efficacy of EPL+LAE both alone and in combination against *L. monocytogenes* is diminished in food matrices due to the high levels of fat and protein. Similar observations were reported for the application of EPL+LAE to chicken carcasses (Benli et al., 2011), whereby the combination was more effective against *Salmonella* on filters than chicken carcasses.

4.3. Sodium caprylate produces greater antimicrobial effects when used in combinations.

Of the three individual treatments LAE 5%, EPL 10%, ACSL 25%, no treatment resulted in significantly lower counts of *L. monocytogenes* compared to the control on day 35 of storage ($p <= 1$, $p=1$, $p=.9102$, respectively). On its own, ACSL applied at 25% resulted in a 2 logCFU/g reduction in *L. monocytogenes* counts through 28 days of sampling, and at 35 days of sampling counts were not different from the control. However, SC applied alone at 10% resulted in a 1.5 log CFU/g reduction in *L. monocytogenes* compared to the control on day 35 ($p=0.0001$). When non-inhibitory treatments (LAE 5%, EPL 10%, ACSL 25%) were applied in combination with SC at 10%, *L. monocytogenes* counts were significantly lower than the control at all days of sampling ($p<0.05$). Also, each combinatorial treatment resulted in counts that were significantly lower than either individual treatment alone at 35 days of storage. However, final bacterial counts on day 35

did not differ significantly between any of these treatments. This is important because it then allows producers choices as to which antimicrobials to use, taking into consideration cost and potential sensorial changes. *L. monocytogenes* counts on QF treated with SC in combination with EPL returned to initial inoculation levels by day 21, whereas treatments of ACSL + SC were approximately 1 log lower (Figures 4 and 6, respectively). Combinatory treatments of ACSL + SC were also identified as synergistic (Figure 6).

5. Conclusions

Across all treatments, HP 5% (400 ppm) was the most effective treatment for reducing levels of *L. monocytogenes*, with counts staying below 1 log CFU/g throughout storage, indicating bactericidal activity. In addition to HP, SC 10% was the only single treatment that resulted in delayed growth compared to the control; however, counts still reached >7 log CFU/g by day 35, showing that it was not inhibitory over the entire shelf life. Effective combinatorial treatments included SC 10% combined with either ACSL 25%, LAE 5%, or EPL 10%, which resulted in levels of *L. monocytogenes* 2-4 log CFU/g lower than the control on the final day of storage. The combination of ACSL + SC produced a synergistic effect. Therefore, application of antimicrobial solutions containing either HP 5% or SC in various combinations are promising approaches for controlling *L. monocytogenes* on QF. Future studies are needed to evaluate the survival of L monocytogenes in antimicrobial dip solutions to identify the possibility of cross contamination of cheese through antimicrobial dip applications, as well as potential sensorial changes.

Acknowledgements

This project was supported by the National Dairy Council and the United States Department of Agriculture (USDA) National Institute of Food and Agriculture (NIFA), Multistate project S1056. Any opinions, findings, conclusions, or recommendations expressed in this

manuscript are those of the author(s) and do not necessarily reflect the view of the NIFA, the USDA or any other sponsors.

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7. Tables and Figures

Table 1. Composition of Queso Fresco.

Batch	Dry Matter	Moisture	Fat	Salt	Moisture in the Nonfat Substance	Salt in the Aqueous Phase	Fat in Water-Free Substance
1	47.23	52.77	21	2.15	66.8	4.1	44.5
2	47.36	52.64	21	2.1	66.6	4	44.3
3	45.53	54.47	21.75	2.12	69.6	3.9	47.8
Average	46.71	53.29	21.25	2.12	67.67	4.00	45.53

Table 2. Approximate concentration of antimicrobials in Queso Fresco following antimicrobial dip application. Concentrations are based on a 0.2g transfer of aqueous dip solution to 25±1g samples of cheese.

Antimicrobial	Dip Concentration (%)	Approximate Concentration in Cheese (ppm)
ACSL	25	2000
EPL	10	800
HP	5	400
HP	3	250
LAE	2	150
LAE	5	400
SC	10	800

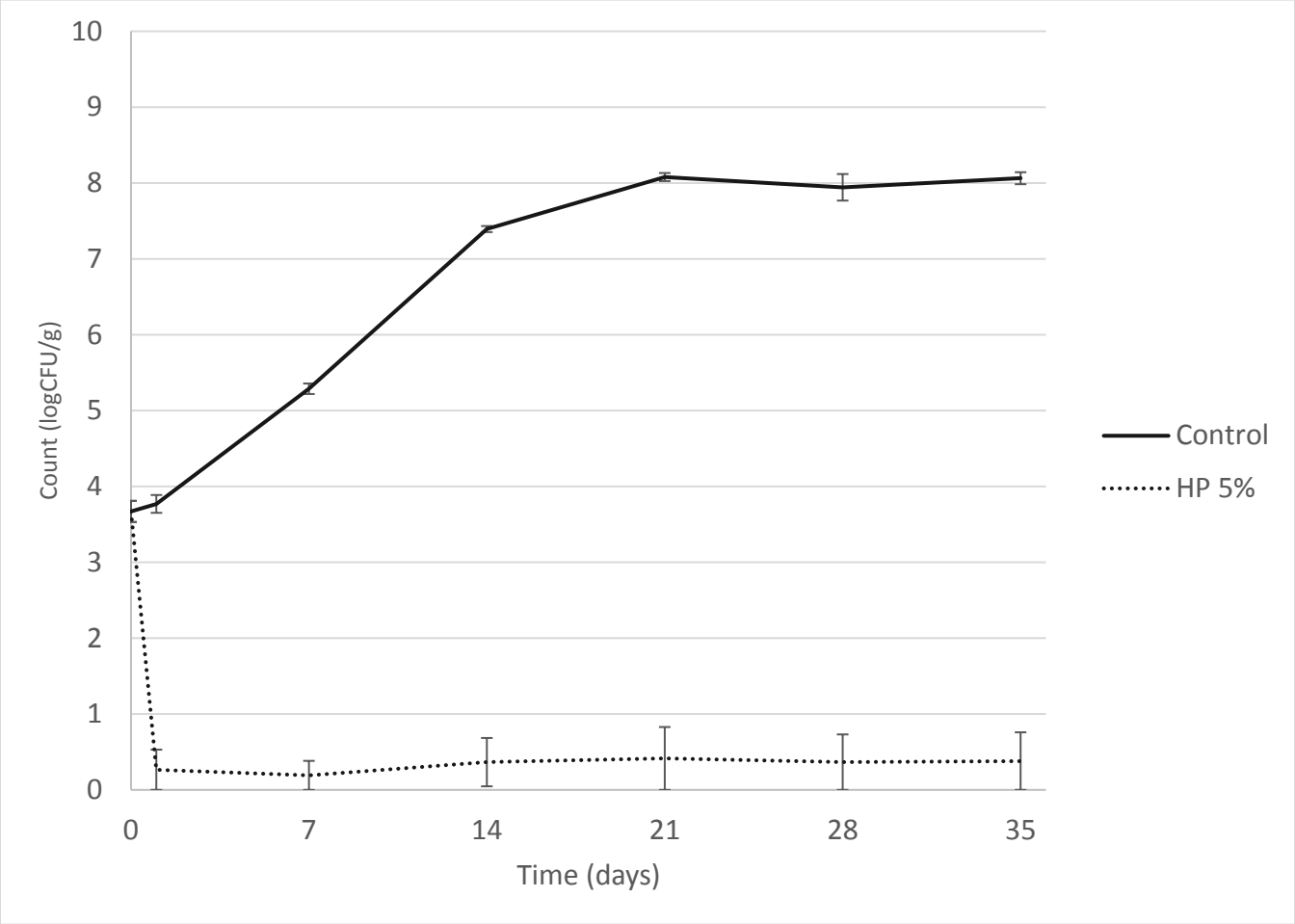

Figure 1. Changes in *Listeria monocytogenes* counts on Queso Fresco during storage at 7˚C following dip application of hydrogen peroxide (HP).

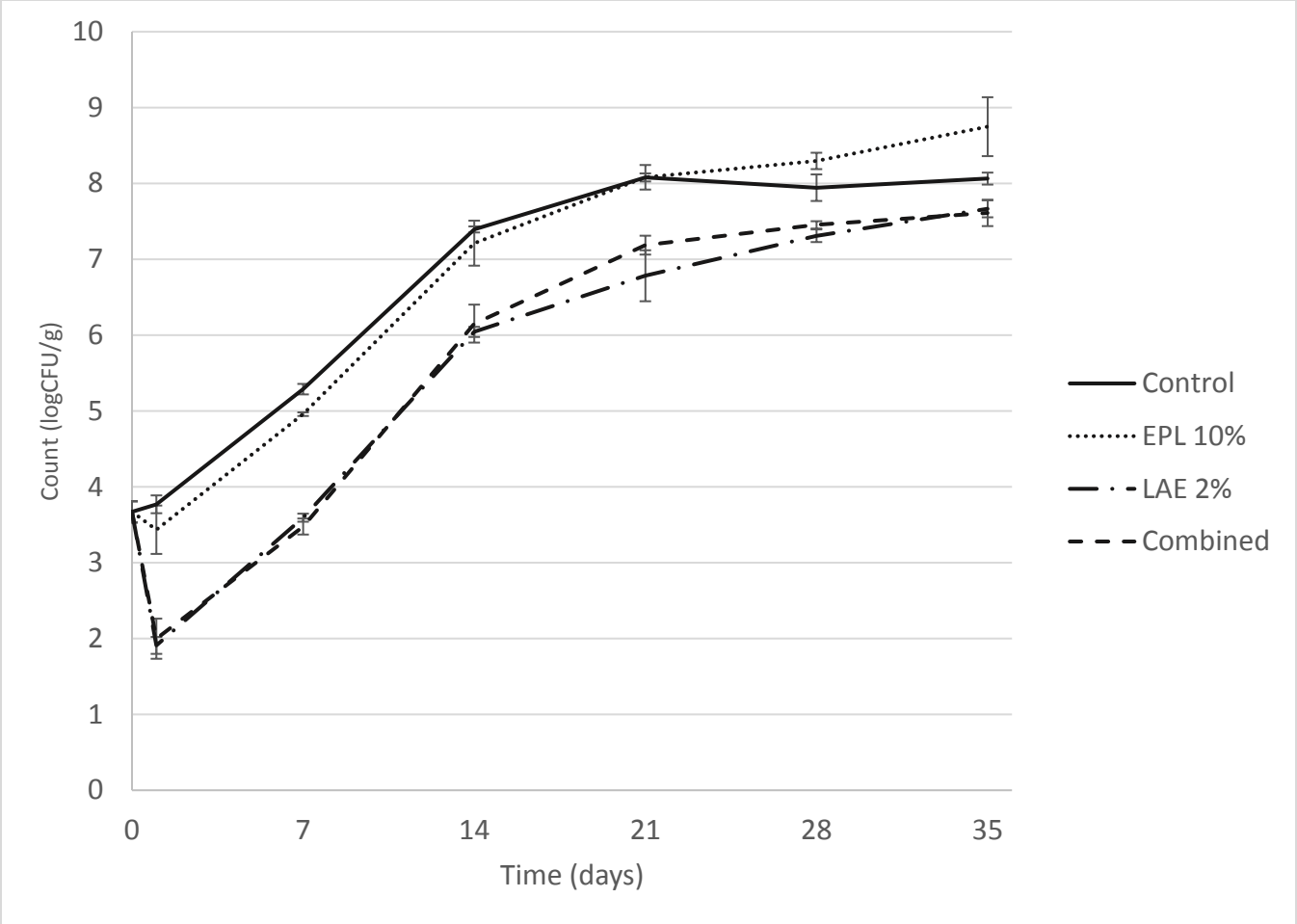

Figure 2. Changes in *Listeria monocytogenes* counts on Queso Fresco during storage at 7˚C following dip application of ε-polylysine (EPL), lauric arginate ethyl ester (LAE), and both antimicrobials sequentially (Combined).

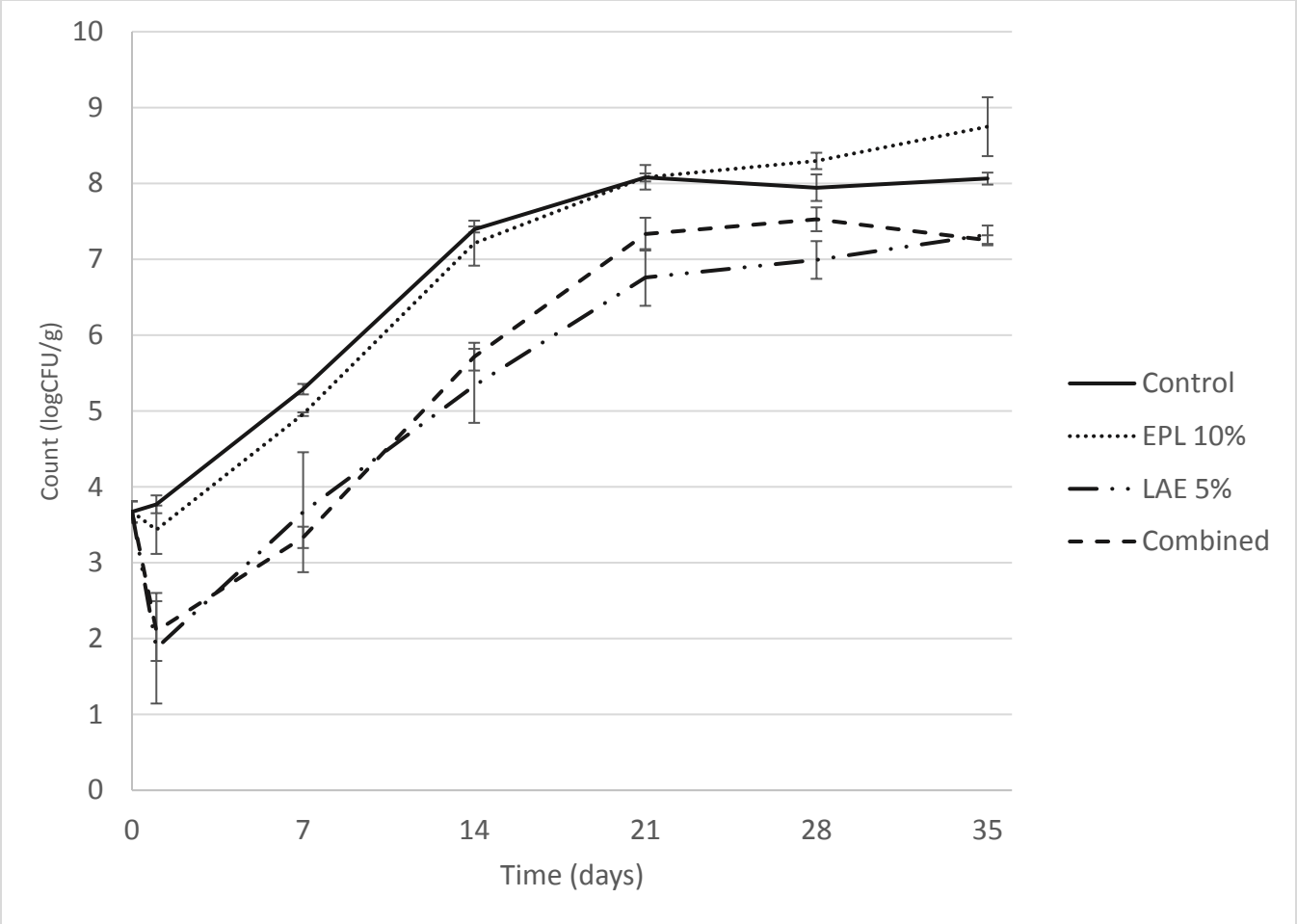

Figure 3. Changes in *Listeria monocytogenes* counts on Queso Fresco during storage at 7˚C following dip application of ε-polylysine (EPL), lauric arginate ethyl ester (LAE), and both antimicrobials sequentially (Combined).

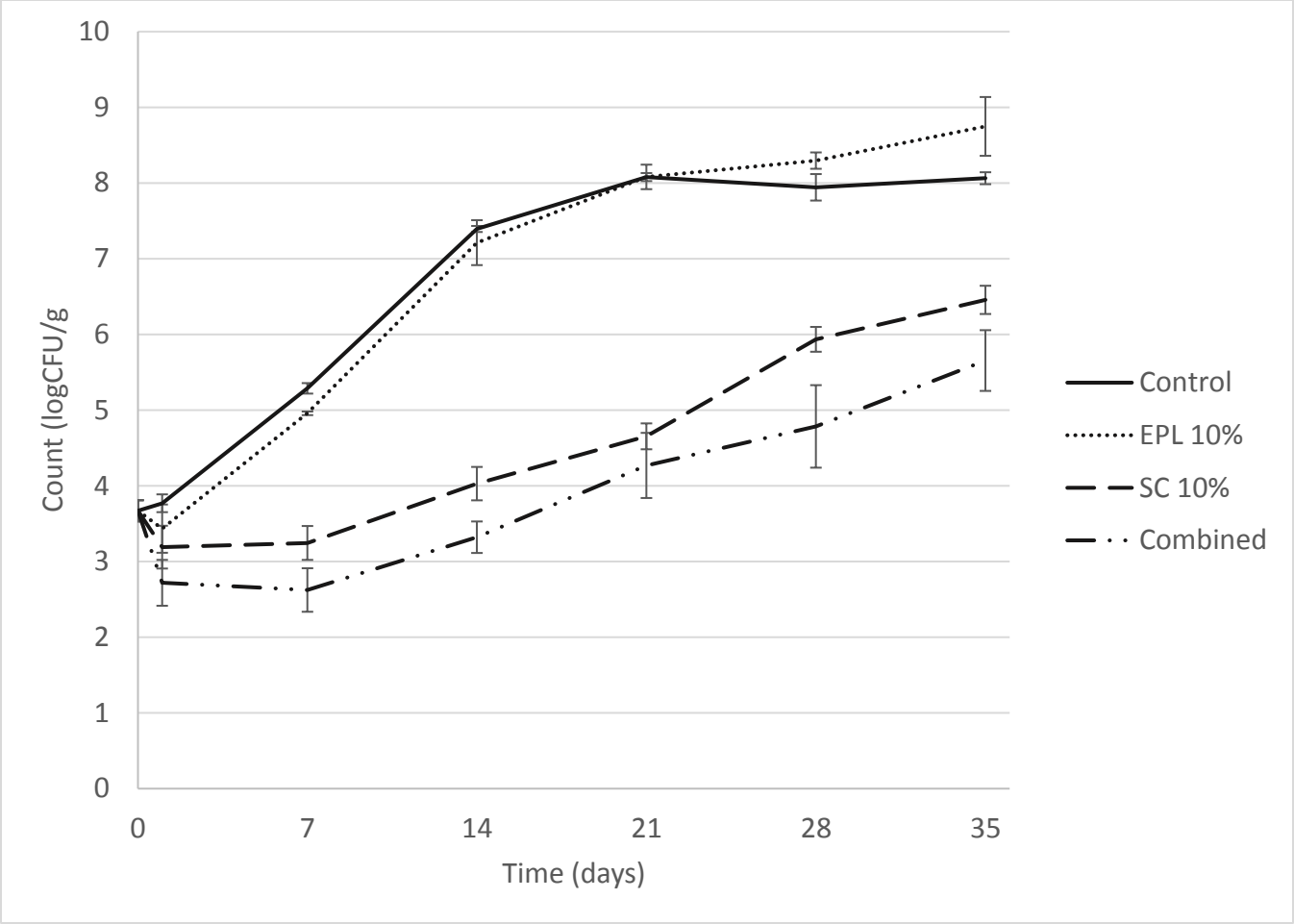

Figure 4. Changes in *Listeria monocytogenes* counts on Queso Fresco during storage at 7˚C following dip application of ε-polylysine (EPL), sodium caprylate (SC), and both antimicrobials sequentially (Combined).

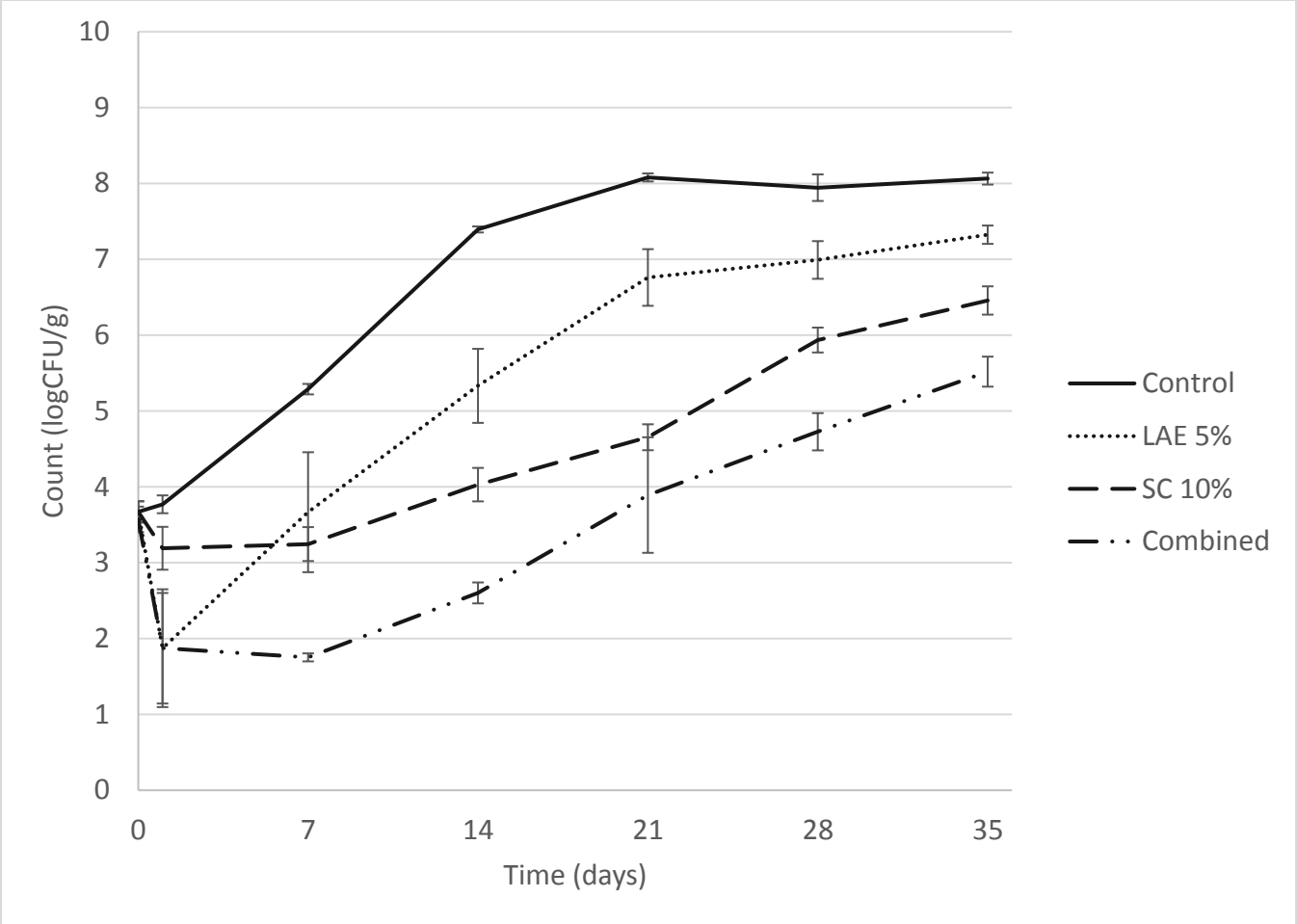

Figure 5. Changes in *Listeria monocytogenes* counts on Queso Fresco during storage at 7˚C following dip application of lauric arginate ethyl ester (LAE), sodium caprylate (SC), and both antimicrobials sequentially (Combined).

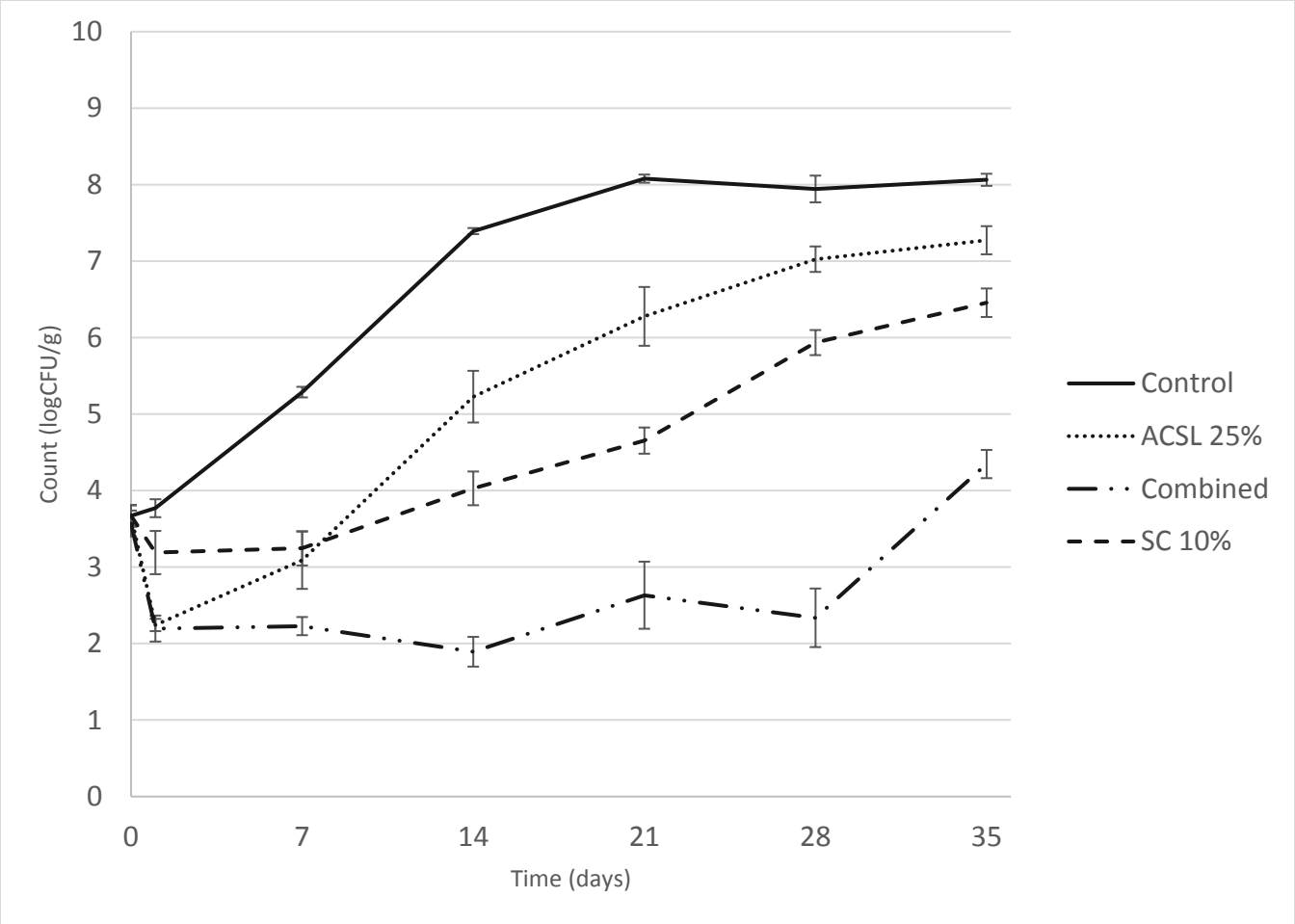

Figure 6. Changes in *Listeria monocytogenes* counts on Queso Fresco during storage at 7˚C following dip application of aqueous solutions of acidified calcium sulfate with lactic acid (ACSL), sodium caprylate (SC), and both antimicrobials sequentially (Combined).

Chapter VI

Summary

Listeria monocytogenes is the major bacterial pathogen associated with foodborne infections linked to dairy foods and is one of the leading causes of death related to foodborne illness. Mexican-style soft cheeses are of particular concern due to their high moisture and pH, and low salt content. Because this pathogen is persistent in food-processing facilities and can survive refrigeration temperatures, there is a continuous need to develop methods to reduce contamination levels and/or inhibit its growth.

This research determined the efficacy of antimicrobials used alone and in combination for the control of *L. monocytogenes* in broth, whole milk, and Queso Fresco (QF). The first objective of this study was to determine the efficacy of antimicrobials against *L. monocytogenes* in a broth system at pH 7.4 and acidified to 5.5. Hydrogen peroxide (HP), ε-polylysine (EPL), and lauric arginate ethyl ester (LAE) were the most effective antimicrobials when used against *L. monocytogenes* in broth at the near neutral pH of 7.4. These antimicrobials maintained efficacy in an acidified broth (pH 5.5). The efficacy of caprylic acid (CA) and sodium caprylate (SC) was enhanced in the acidified system. Overall, the most synergistic antimicrobial pairing in broth at pH 7.4 was EPL+LAE. However, SC was a component of approximately half of all synergistic combinations identified across both pH levels.

The second objective of this study was to determine the efficacy of antimicrobials against *L. monocytogenes* in ultra-high-temperature pasteurized whole milk. In whole milk, the inhibitory concentrations of EPL and LAE both increased by tenfold, while inhibitory concentrations of CA and SC were similar to the inhibitory concentrations identified in broth at pH 7.4. Hydrogen peroxide was the most effective of the antimicrobials, producing a bactericidal effect at 100 ppm and no detectable growth at 200 ppm.

The third objective was to determine the efficacy of antimicrobials in the form of aqueous dips against *L. monocytogenes* on surface-contaminated QF, both alone and in combination. Hydrogen peroxide applied as a 5% aqueous dip solution to QF was the only antimicrobial that was bactericidal when applied alone. Following this treatment, less than 1 log CFU/gram was recovered from QF samples throughout 35 days of storage. The most effective antimicrobial combinations applied to QF as binary combinations included ACSL 25% + SC 10%, LAE 5% + SC 10%, and EPL 10% + SC 10%. Of these three treatments, by definition only ACSL 25% + SC 10% produced a synergistic effect.

These results together suggest that antimicrobial treatments utilizing the aforementioned antimicrobials are effective at reducing and/or inhibiting the growth of *L. monocytogenes.* Antimicrobial efficacy was determined in broth at two pH levels, identifying antimicrobials that may be more effective at different acidity levels. Notably, CA and SC showed a dramatic increase in antimicrobial efficacy in acidified broth. In milk, antimicrobial efficacy of CA and SC was similar as in broth pH 7.4, whereas efficacy of EPL and LAE decreased in milk. Hydrogen peroxide shows the most promise as an antimicrobial treatment in both milk and cheese, with bactericidal activity remaining consistent throughout storage. Applied to QF, LAE +SC, EPL +SC, and ACSL + SC are able to effectively reduce *L. monocytogenes* contamination and therefore should be studied further to determine consumer acceptability.