EVALUATION OF ANTIMICROBIALS AGAINST MULTI-STRAIN COCKTAILS OF *SALMONELLA*, *ESCHERICHIA COLI* O157:H7 AND *LISTERIA MONOCYTOGENES* USING A KINETIC GROWTH INHIBITION ASSAY

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LIST OF COMMON ACRONYNS

µg = micrograms

µl = microliters

CFU = colony forming units

cm = centimeters

g = grams

L = liters

min. = minute

MIC = Minimum Inhibitory Concentration

ml = milliliters

ppm = parts per million

sec = second

CHAPTER I

INTRODUCTION

Salmonella, Escherichia coli O157:H7, and *Listeria monocytogenes* are considered important pathogens responsible for causing food-related human illnesses. In the United States, foodborne illnesses are a significant public health issue. An estimated 76 million cases of illnesses occur annually, resulting in 5,000 deaths and 325,000 hospitalizations (Suo et al., 2010). According to Russell and Gould (2003), the main pathogenic microorganisms responsible for food poisoning and food spoilage are *Salmonella* serovars, *L. monocytogenes*, *E. coli* O157:H7, *Staphylococcus aureus*, *Campylobacter jejuni*, *Clostridium botulinum*, *Clostridium perfringens*, and *Yersinia enterocolitica*. Since the 1990s, the four main foodborne pathogens of concern by government agencies and the food industry have been *Salmonella* spp., *Campylobacter* spp., *E. coli* O157:H7, and *L. monocytogenes* (Newell et al., 2010).

Salmonella is an important foodborne pathogen that causes gastrointestinal infections in humans. It is associated with animals and can be found on raw, animal-related food products such as meat, poultry, and eggs, and has also been found in

contaminated processed foods such as cake mix and peanut butter. The illness, called salmonellosis, may include symptoms such as diarrhea, fever, abdominal pain, nausea, vomiting and loss of appetite (Ray, 2001). The bacteria alter the cells of the villi in the intestinal wall, increasing water secretion into the lumen and decreasing absorption, resulting in diarrhea. *Salmonella* is capable of surviving for long periods in the environment if conditions are favorable (Newell et al., 2010).

Escherichia coli O157:H7 is also a dangerous foodborne pathogen that can cause gastrointestinal illness after consuming contaminated food because the minimum infectious dose is very low. Illness may be acquired by consumption of as little as a few cells and symptoms often show up quickly (1-2 days) and may last for 7 to 12 days, appearing as abdominal cramps, headache, chills, fever, and bloody diarrhea known as Hemorrhagic Colitis Syndrome (Kay et al., 1994). In some cases the infection can cause a more severe syndrome known as Hemolytic Uremic Syndrome (HUS) which may lead to kidney failure or death (Suo et al., 2010).

L. monocytogenes is a Gram-positive, facultative, non-spore forming, rod-shaped intracellular foodborne pathogenic bacterium that may be present on raw meat and sometimes as a contaminant in processed ready-to-eat (RTE) meats. It is been identified since the early 1980s as a microorganism responsible for numerous outbreaks of foodborne disease called listeriosis, which can cause meningitis or sepsis, resulting in high mortality rates (Suo et al., 2010). Newborns and fetuses are the largest group affected, followed by infants, the elderly, immunocompromised people, and pregnant women (Ray, 2001). The microorganism can be found widely distributed in many kinds of foods, such as raw produce, raw poultry, raw dairy products, and is an unwanted

resident in meat processing plants. The pathogen has been a recurring problem in meat processing facilities especially because *L. monocytogenes* is capable of adherence to equipment and/or environmental surfaces in meat processing facilities, resulting in the formation of biofilms (Kushwaha and Muriana, 2010). The microorganism is a psychrotrophic and has the ability to grow in refrigerated food products, such as ready-to-eat (RTE) foods. The increase in consumption of contaminated RTE products that are consumed without heating and which allow an increase in microbial growth from temperature abuse can consequently cause disease (Ray, 2001).

These pathogens have been problematic to the raw and processed meat industry, causing foodborne illnesses and product recalls. The USDA-FSIS has recently developed an incentivized method to promote safety in processed meats by their 'Final Rule for Control of *Listeria* in RTE Meats' which elaborates 3 processing categories: Alternative 3 process (sanitation alone), Alternative 2 (use of either post-process lethality or antimicrobials to prevent growth of *Listeria* during shelf life), or Alternative 1 (post-process lethality and prevention of growth during shelf life). In many instances, the use of antimicrobials can be used in both Alternative 1 and 2 to ensure safe food products

The objective of this study was to evaluate the effect of antimicrobials that are commercially available for the food industry using a kinetic growth assay. The antimicrobials used in our study were tested to evaluate various concentrations in head-to-head trials against multi-strain cocktails of *Salmonella, E. coli* O157:H7*,* and *L. monocytogenes*. We also determined the Minimum Inhibitory Concentration (MIC) which according to Houtsma et al. (1993), was defined as "the lowest concentration at which the absence of growth of a certain microorganism could be observed under the

conditions of testing". Although many of these antimicrobials have been shown to be effective in various peer reviewed publications, they have often been examined on different products, against different challenge strains, and at different concentrations.

CHAPTER II

REVIEW OF LITERATURE

Importance of preservatives

Preservatives, or antimicrobial inhibitors, are important in foods in order to inhibit pathogens and provide a longer shelf life for products by controlling growth of microorganisms that cause food spoilage (Ray, 2001). The purposeful application of methods to preserve foods started around 6000 B.C. and some methods included drying, cooling, fermenting, and heating. Since the early 1900s, chemicals have been introduced into foods to control undesirable microorganisms. Today, the methodology used has changed in comparison to the methods used in the past due to several factors, such as food production and worldwide/global transport, population increase, which all lead to larger food production requirements (Davidson and Branen, 1993). Newer methods used for food preservation as described by Theron and Lues (2007) include the addiction of chemical preservatives, drying, freezing, refrigeration, irradiation, packaging and thermal processing. Consumers are interested in products that are safe for consumption, free of pathogens (Davidson and Branen, 1993), and contain a minimal concentration of preservatives (Ray, 2001). In a processing plant, every food processor has to make the

preservation process a key issue in their business to protect consumers (Theron and Lues, 2007) and the use of a preservative must be approved GRAS substances (Generally Recognized As Safe) before adding into the product (Ray, 2001).

Antimicrobial chemicals are used to either kill microorganisms (i.e., bactericidal) or slow down microbial growth (i.e., bacteriostatic). Some antimicrobials not only serve as a preservative, but also contribute to flavor or color stability (Davidson and Branen, 1993), while others are added to improve functional properties of the product (Ray, 2001). Organic acids are a good example of this, because they not only serve as preservatives inhibiting growth of most naturally found microorganisms in food by pH reduction, but they also improve flavor acceptance qualities of foods (Theron and Lues, 2007). Organic acids used as food preservatives have both bactericidal and bacteriostatic properties. However, they are pH-dependent as their optimal inhibitory activity is expressed at low pH. When applied as decontaminants for raw meats, factors such as tissue type (fat or lean), type of bacteria involved (Gram-negative/Gram-positive), decontamination technique used (spray or dip), and slaughter technology (decontamination of hides) are factors that must be considered to reach optimum activity and minimize resistance (Theron and Lues, 2007).

Benefits that antimicrobial additives provide include reduction of food loss as a result of food spoilage and reduced cases of food poisoning from contaminated food (Davidson and Branen, 1993). Microbiological causes in decreased food quality are important because they may also result in food poisoning. Food poisoning diseases are caused mainly by bacterial and viral agents, and regulatory agencies are responsible for

either identifying those agents, or newly emerging pathogens, and developing efficient methods to combat those agents (Ray, 2001).

Understanding the classification of antimicrobials (traditional or natural) and their sources is of great importance when the selection of antimicrobials is initiated for a particular food product. Identification of the spoilage microorganism and possible preservation methods are the primary steps to start the selection process of the appropriate preservation method. Secondary factors to consider are the spectrum of antimicrobial activity, chemical and physicochemical properties of the food, and composition of the food product in question when selecting antimicrobials (Davidson and Branen, 1993).

The process of meat contamination by microorganisms theoretically starts growth during animal production, but for practical purposes is generally considered to start during processing on the slaughter line. Most contamination of animal-derived meats is carried on the surface of carcasses or smaller cuts of meat. Microbial contamination can be reduced by inhibiting or limiting microbial growth at the surface. Usually the contamination of fresh slaughtered beef carcasses occurs through the transmission of microorganisms via feces, from the hide, from workers, or contact with environmental surfaces. This process may occur by accident when the gastrointestinal tract is damaged when it is removed from the carcass during the slaughtering process (Theron and Lues, 2007). By 1996, the Pathogen Reduction/HACCP (Hazard Analysis Critical Control Point) System was approved by the Food Safety and Inspection Service Agency (FSIS) and U. S. Department of Agriculture (USDA). The objective of this regulation was to

reduce illness risk associated with consumption of meat and poultry products (USDA - FSIS, 1996).

Gill et al., (1999) also suggest that to control and prevent contamination with pathogenic microorganisms, it is mandatory to implement HACCP systems in the plant. Since contamination is a critical meat safety issue, the implementation of HACCP and adherence to GMPs and SOPs are essential mandatory systems required by the USDA - FSIS (Gill et al., 1999).

The increase demand on the food supply normally increases with the population and the use of antimicrobials will become more necessary than it is today in order to supply a larger population (Davidson and Branen, 1993). In compliance with the increase in population, the development of new antimicrobials is essential to face a possible emergence of new pathogenic strains in food products (Theron and Lues, 2007). The constant development of new technologies to process safe food products and reduce contamination is necessary to prevent outbreaks related to the consumption of contaminated foods (Theron and Lues, 2007).

Decontamination methods that provide a safe product without modifying appearance, taste, odor, nutritional value and no more environmental hazards, are ideal substances to be applied, however, it is also important to provide a cost effective method that includes a longer shelf-life for products free of spoilage and pathogenic microorganisms (Dinçer and Baysal, 2004). Decontamination methods that have been applied in slaughter houses in North America have more frequently done so with the aim to reduce microbial loads on meat products (del Río et al., 2007).

Microorganisms of importance

Pathogenic bacteria that are most often the causes of concern are *Salmonella* serovars, *L. monocytogenes*, *E. coli* O157:H7, *Staphylococcus aureus*, *Campylobacter jejuni*, *Clostridium botulinum*, *Clostridium perfringens*, and *Yersinia enterocolitica* (Russell and Gould, 2003; Dinçer and Baysal, 2004). Since the 1960s these microorganisms have been recorded as the major causes of gastrointestinal disease (Newell et al., 2010). Besides foodborne pathogens, food spoilage microorganisms that cause much concern include *Pseudomonas*, *Lactobacillus*, *Acinetobacter*, *Moraxella*, *Aeromonas*, *Alteromonas putrefaciens* and *Brochothrix thermosphacta* (Huffman, 2002).

New foodborne pathogens will likely emerge in the 21st century due to an increase in resistance to antimicrobials by zoonotic foodborne bacteria (Newell et al., 2010), an increase in travelling (especially international travelling that can bring a new pathogen into the country), and changes in food habits by consumers (consumption of raw seafood, undercooked hamburgers, imported foods, raw vegetables, fruits and others) (Ray, 2001).

Salmonella sp.

Salmonella is Gram-negative, rod-shaped, non-spore-forming pathogen and motile enterobacteria. The size of the rods varies from 0.7 μm to 1.5 μm in diameter and from 2 μm to 5 μm in length. These organisms are capable of colonizing livestock species, such as poultry, cattle and pigs, resulting in contaminated meat and other food products. *Salmonella* has been the cause of frequent outbreaks of salmonellosis. There are over 2500 serovars of *Salmonella* that can cause salmonellosis in humans (Ray, 2001). Recently, the association of foodborne outbreaks of salmonellosis has been observed

from vehicles that previously were not common, in which the microorganism evolved to attach and colonize fresh vegetables. This unusual adaptation demonstrates how this pathogen is able to respond and evolve to new niches as a result of environmental changes (Newell et al., 2010).

Escherichia coli O157:H7

E. coli serotype O157:H7 is Gram-negative, rod-shaped enterohemorrhagic strain that can colonize the intestine of various host animals. There are six groups of diarrhoeagenic *E. coli* (DEC) strains, classified as enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC), diffusely adherent *E. coli* (DAEC) and Vero cytotoxin-producing *E. coli* (VTEC) or Shiga toxin-producing *E. coli* (STEC). VTEC and STEC strains are more associated with foodborne outbreaks than EPEC, ETEC and EAggEC strains (Newell et al., 2010). It is estimated that *E. coli* O157:H7 causes 73,000 illnesses and 250 deaths annually (Mead et al., 1999).

Cattle have been often found to be sources and vehicles of contamination. Any processed raw animal product has the potential to carry *E. coli* O157 contamination from animal feces may also spread to fruit and vegetable crops. Contaminated products that have already been involved with outbreaks include vegetables, fruits, juices, sprouts, and drinking water (Powell et al., 2011).

In 1982, *E. coli* O157:H7 was first recognized as a pathogen after an outbreak linked to ground beef consumption that caused an unusual gastrointestinal illness with severe bloody diarrhea in North America. In 1994, a fast food chain in the USA, Jack-in-the-Box, was linked to an outbreak of *E. coli* O157:H7. This outbreak killed four and

sickened hundreds of people who consumed hamburger that was not properly cooked. In 2006, fresh baby spinach was the source of transmission to cause an outbreak of *E. coli* O157:H7, resulting in three deaths and 205 illnesses in 26 U.S. states and in Canada (Powell et al., 2011).

Listeria monocytogenes

L. monocytogenes is rod-shaped, Gram-positive, non-spore forming, facultative, intracellular foodborne pathogenic bacterium that may be found on raw meats and vegetables (Suo et al., 2010). It is ubiquitous in nature by virtue of being shed in the feces of numerous animals, and is an opportunistic foodborne pathogen, affecting humans and animals. This species has been found in soil, leaf litter, vegetation, dust, sewage, water and in the intestines of animals and humans. *L. monocytogenes* has been found in different food products such as coleslaw, raw milk, soft cheese, cabbage, meat and poultry products, and as a contaminant on ready-to-eat products such as jerky, cooked sausages, deli ham and luncheon meats. Due to the seriousness of outbreaks with *L. monocytogenes*, the USDA-FSIS has issued a policy of zero tolerance for this pathogen on RTE products (Fabrizio and Cutter, 2005).

Researchers have observed a pattern change of listeriosis outbreaks in European countries. An increase in testing showing numerous positive results reported in patients 60 years old and over. Clinical changes occurred in the patients presented a bacteremia without involvement of the central nervous system. The cause of this modification is still not known, but a possible relation of the elderly and their changes in food consumption habits may be relevant (Newell et al., 2010).

In 2008, an outbreak of *L. monocytogenes* contaminated deli meats by Maple Leaf Foods, Inc. of Canada, resulted in 22 deaths and 57 illnesses. The cause was due to commercial meat slicers that were not properly cleaned according to the equipment manufacturer's instructions. Failure in the sanitation process was not the only reason, which also included insufficient planning on food safety practices with the company, such as proper implementation of HACCP plans, which today is mandatory in the food industry in the U.S. (Powell et al., 2011).

Improving the safety of foods to reduce foodborne illness

HACCP is an example of a structured approach to be implemented in the food sector to improve food safety and reduce the chance of outbreaks with foodborne diseases. In order to reduce the risk of disease and improve microbiological safety of food products, the implementation of monitoring methods is essential in all sections of the food production system. Farm-to-fork monitoring can aid in avoiding outbreaks (Newell et al., 2010). HACCP is a system revolving around seven basic principles that reduce and prevents contamination on meat products and a safe product (Table 1) (Hulebak and Schlosser, 2002).

According to Powell et al. (2011), it is essential to change the way that people handle food. Public education of consumers and food handlers will be necessary to decrease the occurrence of contamination through cross-contamination or from the cooking or cooling of foods improperly (Ray, 2001). The emergent concept of 'Food Safety Culture' is based on keeping the concepts of food safety in thought and in behavior relevant for the community and for the good and safety of the food system. The concept of Food Safety Culture includes food processing facilities, farmers, restaurants

and domestic kitchens. Food Safety Culture requires the greatest organization and communication systems (Powell et al., 2011). As proposed by Griffith et al. (2010), there are six culture factors to provide improved food safety performance that include leadership, food safety management systems and style, commitment to food safety, food safety environment, risk perception, and communication. Another tool that can provide communication and support to the food safety culture for food handlers are food safety infosheets. Infosheets include details of an outbreak of food illness and the consequences correlated with food handler's behavior, with the objective to change people's behavior (Powell et al., 2011).

When changes in behavior are mentioned, there are multiple factors that correlate to a change tendency in diseases caused by food products. These factors can be explained by fast population growth, increased global market in products with origins in different countries without proper safety procedures, changes of eating habits, higher proportions of immunologically compromised individuals, climate change, and changes in farmer's practices (Newell et al., 2010).

Antimicrobial interventions for raw meats

Table 2 includes safe and suitable antimicrobials used in the production of meat and poultry products from the Food Safety and Inspection Service. Some of the antimicrobials used in this project have been approved as Generally Recognized as Safe (GRAS) substances for use in products and are included in Table 2.

Antimicrobial interventions for raw beef

Lactic acid

In the past, the use of lactic acid was the result of acidification of foods and by microbial cultures accepted sensory qualities. Today, its use is also heavily focused on its antimicrobial decontamination properties mainly in beef, pork and chicken products (Davidson and Branen, 1993). Lactic acid is a weak-organic acid and by tradition is widely used to control pathogens in foods. The inhibitory capacity occurs through diffusion of lactate molecules into microbial cells where they dissociate into toxic anionic forms based on the relationship of the acid's pK_a and the pH of the food (Ibrahim et al., 2008). Organic acids are classified as pure or buffered acids. Lactic acid is a pure acid with a simple and small structure, making it more susceptible to move easily into cells (Theron and Lues, 2007). The use of lactic acid in the food industry is due to its effectiveness when used on a meat surface layer to reduce bacterial counts (Pipek et al., 2005).

An evaluation of the efficacy of surface decontamination of beef carcasses was the objective of a study conducted by Pipek et al. (2005), which was performed using a combination of steam treatment followed by lactic acid solution spray treatment. The lactic acid solution was prepared at 2% concentration for the treatment process. The total count was tested for mesophilic and psychrophilic microorganisms. Results show that mesophilic counts were lower than counts for psychrophilic microorganisms, but the effect of decontamination was similar for both groups. The treatment effectively reduced microbial counts on the beef surface carcass, by 1 to 3 decimal counts of CFU, as well as

slowed down microbial growth during storage, extended shelf life, and increased the safety of the product (Pipek et al., 2005).

Calicioglu et al. (2002) used 2% lactic acid for decontamination of beef carcass quarters and fat-covered sub primal cuts. In this study, the authors inoculated samples with a mixture of manure slurry plus 5 strains of *E. coli* O157:H7 at 4 to 5 $\log_{10}$ CFU/cm^2. They conducted several experiments to identify the effectiveness of lactic acid spray treatment: Experiment A consisted of: (i) control (not treated), (ii) spraying with 2% LA, (iii) tempered at 21^{o}C during 4 h, (iv) tempered followed by 2% LA spray solution. Experiment B involved: (v) water spray, (vi) 2% LA spray, (vii) 2% LA spray plus 0.5% sodium benzoate, (viii) 2% LA spray plus sodium benzoate plus 5% Tween 20 (TW20). For experiment C they used: (ix) water spray (control), (x) pre-spray withTW20 solution followed by 2% LA solution, (xi) pre-sprayed with TW20 followed by 2% LA containing SB. Results for these treatment combinations revealed that lactic acid treatments on beef carcasses reduced the viable number of the pathogen ranged from 1.6 to 2.8 $\log_{10}$ CFU/cm^2, and the pre-spraying with a 5% TW20 solution did increase the inhibitory effect of lactic acid by 2.8 $\log_{10}$ CFU/cm^2 on beef carcass quarters and by 3.2 $\log_{10}$ CFU/cm^2 on fat-covered sub primal cuts. This may be explained by the effect of TW20 on cells, making them more susceptible to the action of lactic acid (Calicioglu et al., 2002).

Decontaminating treatments, such as spraying with 2% lactic acid, vacuum hot water, trimming, washing, and pasteurization with steam or hot water, were placed in four beef packing plants where microbiological properties were examined during the dressing of beef carcasses (Gill and Landers, 2003). All carcass samples were submitted

for bacterial counts to be performed for aerobes, coliforms, and *E. coli*. A non-satisfactory result was obtained from samples that were sprayed with 2% lactic acid, vacuum-hot water and trimming which were not effective, however, the efficacy of washing and pasteurization was satisfactory in reducing bacterial counts on carcasses by 1 and 2.5 log units, respectively (Gill and Landers, 2003).

In another study, beef carcass tissue (BCT) inoculated with *E. coli* O157:H7, *L. innocua*, and *Clostridium sporogenes* were subjected to an industrial spray wash cabinet and submitted for microbial analysis during prolonged refrigerated vacuum-package storage after antimicrobial treatment. The method was used to treat the BCT with: water (W), 1.5 and 3.0% lactic (LA) or acetic (AA) acid, or 12% trisodium phosphate (TSP) washes. Prior to each treatment, fresh bovine feces were inoculated with the following antibiotic-resistant bacteria strains (marked): *E. coli* O157:H7, *L. innocua*, and *Clostridium sporogenes*. After treatments, plates were stored at 5^{o}C and analyzed for up to 21 days. Analyses were made by monitoring levels of mesophilic aerobic bacteria, lactic acid bacteria, pseudomonads, and added antibiotic-resistant bacteria. Mesophilic aerobic bacteria, lactic acid bacteria and pseudomonas were initially 5.6 log CFU/cm^2, after the treatment they were reduced to 1.3 log CFU/cm^2. Labeled antibiotic resistant bacteria had a 1.3 log CFU/cm^2 reduction that remained constant during 21 days of storage. Trisodium phosphate was more effective for mesophilic aerobic bacteria, lactic acid bacteria, and *L. innocua* than for *E. coli* O157:H7 and *Clostridium sporogenes* (Dorsa et al., 1997).

Lactic acid at 4% and 2% concentrations were used by Castillo et al., (2001b) using organic acid spray systems on beef carcass surfaces inoculated with *E. coli*

O157:H7 and *Salmonella* Typhimurium. The use of organic acids applied by spray systems in carcass decontamination have been shown to be effective in reducing pathogens (Anderson and Marshall, 1989). One of the method used in this study consisted of spraying 4% lactic acid at 55^{o}C for 30 seconds applied to the outside beef carcass surfaces already contaminated with the pathogens. The other method was examined on prechilled hot carcasses using only a water wash, or a water wash followed by a 15 second spray with 2% lactic acid at 55^{o}C. The prechill treatment using water wash alone reduced both pathogens by 3.3 to 3.4 log cycles and using water wash and lactic acid the reduction was up to 5.2 log cycles. A postchill treatment was also conducted onto the outside beef carcass surfaces and showed more reduction in both pathogens by 2.0 to 2.4 log cycles for *E. coli* O157:H7 and by 1.6 to 1.9 *Salmonella* Typhimurium, concluding that when using both treatments the counts of pathogens were significantly reduced (Castillo et al., 2001b).

Another decontamination study performed by Castillo et al. (2001a) was the evaluation of chilled beef carcasses using a 4% lactic acid (55^{o}C at source) solution sprayed for 35 s. The objective was to reduce bacterial populations using hot water spray treatment followed by lactic acid spray prior to chilling. Three samples from the brisket, the clod, and the neck, were examined from 40 untreated and 40 treated carcasses, and three bacterial counts were used to examine potential microbial reduction, including: aerobic plate count, total coliform count, and *E. coli* count. The aerobic plate count was reduced by 3.0 to 3.3 log cycles, and for coliforms and *E. coli* the counts were reduced to undetectable levels. According to these results, the conclusion for this study was that hot 4% lactic acid solution spray method was an effective treatment that would considerably

reduce bacterial loads on cold carcass surfaces, which could be implemented in a commercial slaughter environment (Castillo et al., 2001a).

To increase palatability of beef subprimal cuts, two types of mechanical treatments were used, one is blade tenderization (BT) and the other is moisture enhancement (ME). The reduction of *E. coli* O157:H7 was evaluated on the subprimal cuts and the efficacy of five antimicrobial interventions applied before those two mechanical treatments were also evaluated during this study. Since the bacteria can be transferred to the interior of the beef during those two processes, if the product is not cooked thoroughly at 71^{o}C, the product can become a health risk for the consuming population. Antimicrobial interventions used were as the follows: no intervention, surface trimming, hot water (82^{o}C), warm 2.5% lactic acid at 55^{o}C, warm 5.0% lactic acid at 55º C or 2.0% activated lactoferrin followed by warm 5.0% lactic acid at 55^{o}C applied to the surface of subprimal beef cuts using a handheld sprayer and then submitted for mechanical treatments (BT and ME) (Heller et al., 2007). According to Huffman (2002), lactoferrin has the potential to be an antimicrobial classified as antimicrobial blocking agent due to its action on iron proteins, the compound recently received the status of GRAS by US Food and Drug Administration to be applied on fresh beef. The results suggests that the reduction of *E.coli* by 0.93 to 1.10 log CFU/100 cm^2 for all interventions used prior to mechanical tenderization was effective in reducing the pathogen population on the interior of subprimal beef cuts (Heller et al., 2007).

In a recent study using Beefxide antimicrobial solution (a mixture of lactic acid and citric acid) to reduce *Salmonella* and *E. coli* O157:H7 counts on beef trim through spray or immersion treatment intervention showed good results in reducing the pathogen

load. According to the results for spray treatments, the log reduction obtained was 1.4 log CFU/100 cm^2 for *E. coli* and by 1.1 log CFU/100 cm^2 for *Salmonella*, significantly reducing pathogen populations when the initial inoculum of both strains was approximately 10 log CFU/100 cm^2. The use of Beefxide provided a viable treatment to reduce the *Salmonella* and *E. coli* O157:H7 load on raw beef products (Laury et al., 2009).

Acetic acid

Acetic acid is an organic acid also known as ethanoic acid and occurs in the human body by a natural process, playing an important function in metabolic processes such as participating in several biochemical reactions. It has been long used as a natural flavoring and acidulant by the food industry (Lück et al., 1997). This antimicrobial, considered a GRAS substance, has also been used as a sanitizer of red meat carcasses (Anderson and Marshall, 1989). Acetic acid has two main actions to reduce microbial population, by lowering the pH value of the product that will be preserved (Anderson and Marshall, 1989) and by changing the permeability of the membranes of the microbial cells by penetrating inside the cell wall, causing protein denaturation (Lück et al., 1997). Since its approval as a GRAS substance in 1982, many studies have been made with the main pathogens of concern in the food industry such as *E. coli*, *Salmonella*, and *L. monocytogenes* in which its effectiveness has been demonstrated in reducing microbial loads (Bell at al., 1997). Acetic acid is produced naturally by biological oxidation or synthetically (Lück et al., 1997). Natural production occurs when there is spoilage of certain foods by *Acetobacter* microorganisms (genus of acetic acid bacteria), which is also used in the manufacture of vinegar and wine (Theron and Lues, 2007).

A study using spray-washing and rinsing with hot and warm water treatments was tested against *E.coli* inoculated on adipose tissue from beef carcass samples (Delmore et al., 1998). The treatments included: pre-evisceration washing, rinse with 2% acetic acid, final washing and a hot water rinse. Results, based on aerobic and *E. coli* counts, showed that 3 or 4 combinations were more effective than a single treatment. All spray-washing and rinsing treatments were effective in reducing aerobic counts by 1.3 to 2.9 $\log_{10}$ CFU cm^{-2} when the initial inoculum level was 6.5 $\log_{10}$ CFU/cm^2. *E. coli* counts were reduced at least 1 $\log_{10}$ CFU/cm^2 when the initial inoculum level was 5.4 $\log_{10}$ CFU/cm^2.The most effective treatment was a combination of pre-evisceration washing, acetic acid rinsing, final washing, plus acetic acid rinsing (Delmore et al.,1998).

Another study using spray application of acetic acid for decontamination of beef carcass tissues to provide control of possible contamination was by Bell et al. (1997). The method used was spray-wash treatments with the aim to reduce microbial counts of *E. coli*, *L. innocua* and *Salmonella* Wentworth. The following antimicrobial solutions were used alone or in combination: 1% acetic acid, 3% hydrogen peroxide, and 1% sodium bicarbonate. Lean and adipose tissue were inoculated with a solution containing fecal slurry plus the microorganisms listed above at 5 $\log_{10}$ CFU/cm^2. The parameters for the spray washer were 80 psi for 15 seconds at 25°C. After the treatments all samples were analyzed immediately as well as after 1 day for pH, color, bacterial counts, and hydrogen peroxide residue. The most effective result for treating *E. coli* was the combination of 1% acetic acid plus 3% hydrogen peroxide resulting in a reduction of 3.97 $\log_{10}$ CFU/cm^2 on lean tissue and 3.69 $\log_{10}$ CFU/cm^2 on adipose tissue. For *L. innocua*, the reductions were 3.05 $\log_{10}$ CFU/cm^2 and 3.52 $\log_{10}$ CFU/cm^2 on lean tissue and on adipose tissue,

respectively. For *S.* Wentworth, the results showed a reduction of 3.37 $\log_{10}$ CFU/cm^2 and 3.69 $\log_{10}$ CFU/cm^2 on lean and adipose tissue, respectively (Bell et al., 1997).

Suspensions of *E. coli* and *Salmonella* Typhimurium dip-inoculated onto beef semitendinosus muscle (eye of round roasts) where treated using an acetic acid dip method at different concentrations (0, 1, 2, and 3%) for 15 seconds, and at different temperatures (25, 40, 55 and 70°C). Counts were made for aerobic and *Enterobacteriaceae* microorganisms. Results showed that acetic acid solution had no relation with concentration and temperature. Although the treatment that was most effective was 3% acetic acid at 70°C against aerobic counts, it was less effective for *Enterobacteriaceae* followed by *E. coli* and *Salmonella* Typhimurium (Anderson and Marshall, 1989).

Citric acid

Algino et al. (2007) evaluated the effectiveness of intervention treatments applied to carcasses in small slaughter plants against several bacterial indicators of contamination. The treatments were tested against *E. coli* O157:H7, coliforms, *Enterobacteriaceae*, and aerobic plate counts. The interventions examined 5 methods: the first method was dry-aging (refrigeration without water-spray chilling), the second method was 2.5% acetic acid spray (applied to the carcass with a hand-held spray wand before chilling), the third method was low/high pressure hot-water spray (washing the carcass with 150^o F water applied using low-pressure or 120^o F water using pressure washer), and the fourth method was Fresh Bloom spray (mixture of 5% citric acid, ascorbic acid, and erythorbic acid) applied to the carcass with a hand-held spray wand before chilling. After all treatments, the carcass samples were held in a cooler for 24 h

before submitting to fabrication. Results showed that all interventions used were effective in decreasing levels of *E. coli* O157:H7, coliforms, and *Enterobacteriaceae* microorganisms. The reduction average was in a range of 0.6 to 2.0 log CFU/cm^2 for *E. coli*, 0.7 to 2.2 log CFU/cm^2 for coliforms, and 0.4 to 2.2 log CFU/cm^2 for *Enterobacteriaceae*. There were two factors that correlated to bacterial reduction during the experiment, relative humidity and the speed at which samples were brought to refrigeration temperatures. When the low/high pressure, hot-water spray intervention was used, the best factor involved in reducing microbial loads was to increase the spray time. These factors are important considerations for processors to apply these interventions in slaughter facilities to increase the efficacy of treatments (Algino et al., 2007).

Peroxyacetic acid

Peroxyacetic acid (PAA), or peracetic acid, is derived from the peroxide of acetic acid and has a strong function as a disinfectant and an oxidant. It is available commercially as a quaternary mixture, which contains acetic acid, hydrogen peroxide, peroxyacetic acid, and water. This liquid mixture is colorless, clear with a strong pungent acetic acid odor and pH 2.0 or less (Kitis, 2004).

Dairy calves have a high risk to carry *E. coli* O157, especially during weaning time. The transmission of this pathogen can become a potential risk for the meat industry in their facilities (Cobbold and Desmarchelier, 2000). In New Zealand, interventions were implemented to reduce levels of *E. coli* on external carcass surfaces of hot-boned beef (bobby calf flaps – meat younger than 2 weeks of age) and veal (hot-boned beef flaps) using sprays system to remove or minimize the pathogen on the product samples. The culture preparation was added to bovine feces at high dose concentrations (10^6 log

CFU/cm^2) and low dose concentration (10^3 log CFU/cm^2) and inoculated onto the meat samples. Next, treatments were applied for decontamination as a water wash, POAA formulation (marketed as Inspexx 200 – mixture of hydrogen peroxide, acetic acid, octanoic acid, peroxyacetic acid, peroxyoctanoic acid and hydroxyethylidene-1, 1-diphosphoric acid) wash at 180 ppm or water plus POAA wash. The results showed a potential reduction of *E.coli* O157:H7 by 97.4 to 99.9% when POAA treatment was used for carcass decontamination of beef and veal carcasses (Penney et al., 2007).

Acidified sodium chlorite

Several studies about sodium chlorite solutions were shown to be effective in decontaminating fruits and vegetables as a surface disinfectant (Renard et al.,1997; Tanner, 1989). Acidified sodium chlorite (ASC) solution has also been proven to reduce intramammary infections in dairy herds after milking. In a study to compare both sodium chlorite and acidified sodium chlorite, on fish and vegetables, no significant differences were observed between the two solutions. The same author suggests that when sodium chlorite solution is mixed with lactic acid, the potential for antibacterial activity increases (Hasegawa et al., 1990).

ASC has been approved by the USDA - FSIS for the purpose to be applied for carcass decontamination of poultry and beef products. The efficacy of this antimicrobial was studied using spray intervention combined with phosphoric or citric acid against populations of *E. coli* O157:H7 and *Salmonella* Typhimurium on hot beef carcass surfaces. The initial counts were 5.5 and 5.4 logs CFU/cm^2, after inoculation with *E. coli* O157:H7 and *Salmonella* Typhimurium, respectively. After treatment, reductions were 3.8 to 3.9 $\log_{10}$ CFU/cm^2 by acidified sodium chlorite spray intervention combined with

phosphoric acid and 4.5 to 4.6 $\log_{10}$ CFU/cm^2 when using acidified sodium chlorite spray intervention combined with citric acid. According to these results, the efficacy of the acidified sodium chlorite spray intervention combined with citric acid was better than with the other intervention solution, although both showed significantly reduced levels of pathogens. In conclusion, acidified sodium chlorite is a good intervention for beef carcass decontamination without affecting color or odor (Castillo et al., 1998).

Another study to identify which antimicrobial intervention was more effective on beef carcasses was the use of 25% sodium chloride or 0.1% acidified sodium chlorite against *E. coli* O157:H7 and *Staphylococcus aureus* inoculated onto fresh beef briskets. The meat surface samples were inoculated with a mixed strain "cocktail" comprising 5 strains of each microorganism at an initial level of 6.5 $\log_{10}$ CFU/cm^2 followed by spray treatments varying from 10-60 sec of exposure. The water washes were control treatments to determine the effect of liquid displacement on microbial removal. The results showed that all treatments were effective in reducing *E.coli* O157:H7, but only acidified sodium chlorite was more effective with increased exposure time. Water wash treatments were not effective against *S. aureus* and sodium chlorite was only effective after 60 sec of treatment, however, acidified sodium chlorite was the most effective in reducing microbial counts (Hajmeer et al., 2004).

E. coli O157:H7 and *Salmonella* Typhimurium DT 104 were applied to boneless beef strip loins and taken to different antimicrobial interventions. The objective of this recent study was to check the effectiveness of acidified sodium chlorite, lactic acid bacteria, and lactic acid sprays to reduce microbial loads and to prevent their internalization during mechanical blade tenderization (MT) and injection enhancement

(EN) processes in beef loins prior to packaging observed at different locations in the sample, and aging date. Bacterial contamination during MT and EN processes are commonly observed in beef processing plants (Echeverry et al., 2009). Lactic acid bacteria applied as intervention had a significant reduction on *E. coli* O157:H7 in the interior of the meat samples (1.2 to > 2.2 log cycles), while a 0.8 log reduction was observed for acidified acid chlorite and a 3.0 log reduction was obtained with lactic acid. For *Salmonella* Typhimurium DT 104, all the interventions had satisfactory results, varying from 0.9 to 2.2 log reduction in the interior of the samples. The interventions were effective in reducing microbial loads inside the beef strip loins samples for all days analyzed (0, 14, and 21 days) (Echeverry et al., 2009).

Antimicrobial interventions for raw pork

Lactic acid

Castelo et al. (2001), utilized three processes of combination treatments for the decontamination of pork trim. Lean pork trim tissue (LPT) and fat-covered pork trim tissue (FPT) were inoculated with swine feces and treated with 3 combinations processes. Combination 1 (water, hot water [65.5°C, 15 s], hot air [510°C, 60 s], and lactic acid), combination 2 (water, hot water [82.2°C, 15 s], hot air [510°C, 75 s], and lactic acid), and combination 3 (water, hot water [82.2°C, 45 s], hot air [510°C, 90 s], and lactic acid). Lactic acid solution was prepared and applied in all combination treatments at a 2% concentration. The analysis was made before and after treatment by monitoring populations of aerobic bacteria, psychotropic bacteria, coliforms, *E. coli* and lactic acid bacteria during days 2 and 7 at 4^{o}C. Results with lower microbial reductions were

observed with lean pork trim tissue than on fat-covered pork tissue. The reason for this may be explained by the higher buffering capacity of the lean to lactic acid. Treatments that were favorable in maintaining the quality of ground pork was hot water wash and water plus lactic acid, these treatments were most effective in reducing of microbial populations on the sample (Castelo et al., 2001).

Antimicrobial interventions for raw poultry

Trisodium phosphate, acidified sodium chlorite and chlorine dioxide have been introduced as chemical decontaminants commonly used as interventions for the poultry carcass (del Río et al., 2007).

A study was conducted to identify the most effective chemicals for antimicrobial interventions on the natural microbial loads of chicken. In this study, del Rio et al, (2007) used chicken legs and the treatments consisted of dipping samples into solutions of 12% trisodium phosphate, 1200 ppm acidified sodium chlorite, 2% citric acid, 220 ppm peroxyacetic acid, water (control), or no treatment. Following each treatment, samples were drained for 15 minutes and placed into sterilized bags for storage at a temperature ranging from 1 to 3^{o}C. The evaluations consisted of microbiological counts, sensory analyses, and pH values tested at days: 0, 1, 3 and 5. After treatment each sample was homogenized into a stomacher bag containing peptone water for 2 minutes and subjected to microbiological analysis for mesophilic, pseudomonas, psychotropic, coliforms, enterococci, Enterobacteriaceae, Micrococcaceae, lactic acid bacteria, yeasts, and molds. According to the results, all chemical compounds were effective in reducing microbial populations. When analyzed immediately, the most effective antimicrobials were trisodium phosphate, acidified sodium chlorite and citric acid, both during and after the

storage time. In conclusion, those antimicrobials tested above can be valuable in reducing and controlling microbial counts during poultry product storage. A satisfactory sensory result lead to the conclusion that the consumer sensory properties of the samples were not affected by the chemicals used making the samples acceptable for industry and consumer safety (del Río et al., 2007).

Antimicrobial interventions for ready-to-eat meats

Organic acids (Frankfurters)

Organic acid treatments have been shown to improve the shelf-life of red meats. It has been also examined for the control of *L. monocytogenes* in RTE meat processing plants because this microorganism is a common contaminant that is able to survive for long periods of time and conditions in RTE plants are favorable to its survival (Anderson and Marshall, 1989).

In one study to control possible contamination of *L. monocytogenes* on the surface of frankfurters, dip treatments of organic acid was examined prior to packaging, at different concentrations and in combinations. Citric acid combined with acetic acid at 2.5% concentration proved to control microbial growth on samples during 90 day storage at 5^{o}C. Results obtained with organic acid dip treatment of frankfurters controlled growth of *L. monocytogenes* on vacuum-packaged frankfurters. (Palumbo and Williams, 1994). However, dip treatments are difficult to apply in actual commercial production.

Organic acids have shown bactericidal effects when used in combination with ionizing radiation techniques applied to food products. Sommers et al. (2003) examined citric acid, applied on the surfaces of frankfurters in concentrations up to 10% prior

packaging, in combination with ionizing radiation against *L. monocytogenes*. The objective was to use irradiation at a level that would reduce 90% of the viable microorganisms. Results demonstrate that citric acid can improve the lethality of ionizing radiation but it also affected color of raw meat. Citric acid used as antimicrobial has a dual benefit in that it can also act as anti-oxidant in meats, preventing color change on frankfurters, lipid oxidation, and maintaining firmness (Sommers et al., 2003).

In a recent study, Byelashov et al. (2010) evaluated the parameters of concentration, temperature, and time of lactic acid treatment of the surfaces of frankfurters. The frankfurters for this study were inoculated comprising 10 strains of *L. monocytogenes* and the authors tested lactic acid concentration from 0 to 3%, acid solution temperatures ranging from 4, 25, 40, or 55^{o}C, and the treatment times lasting 0-120 seconds. The objective was to evaluate the efficacy of concentration, temperature and time to achieve 1 - 2 log-reductions immediately after treatment. They found it was possible to achieve a 2-log reduction using lactic acid solution against *L. monocytogenes* on frankfurters by 3% lactic acid at 25ºC applied for 120 sec, or by 1% lactic acid at 55ºC for 60 sec (Byelashov et al., 2010).

In another study, the dip method was used prior to packaging, in which organic acids were tested against *L. monocytogenes* on frankfurters, showing that the lactic acid at 5% concentration is also effective against *L. monocytogenes*. Results from this study complement the results in the previous study (Palumbo and Williams, 1994).

Sodium lactate and acetate (Frankfurters)

Sodium lactate and sodium acetate are additives that are considered GRAS and at present the most widely used chemicals to control *L. monocytogenes* in the meat industry

(Bedie et al., 2001). In a study to test these two chemicals against *L. monocytogenes*, Samelis et al. (2002) examined allowable levels for these chemicals and compared their use when applied in frankfurters inoculated with *L. monocytogenes*. The procedure consisted of a preparation of sodium lactate at 1.8% concentration (allowed concentration = 3%) and its use alone and in combination with other antimicrobials such as sodium acetate, sodium diacetate and gluconolactone each one prepared at 0.25%. The antimicrobial preparations were added into the frankfurter formulation, processed (cooked), and then surface inoculated with the pathogen at 10^3 to 10^4 CFU/cm^2. After this step the samples were vacuum-packaged and kept at 4^{o}C for up to 120 days. The use of sodium lactate alone was effective to inhibit pathogen growth until 50 days of storage and when used in combination with the other antimicrobials, the inhibition was effective throughout 120 days of storage at 4^{o}C. In conclusion, it is better to use these antimicrobials in combination than to use them individually to inhibit *L. monocytogenes* on processed meat products.

Sodium levulinate and lactate (Turkey roll and Bologna)

Thompson et al. (2008) evaluated if the antimicrobial sodium levulinate (a GRAS flavoring agent) is effective in comparison to sodium lactate or to the combination of sodium lactate and sodium diacetate (industry standards) to control *L. monocytogenes* on turkey breast roll and bologna. The samples were prepared and added to the meat formulation at 1%, 2% or 3% sodium levulinate, or at 2% sodium lactate, and 2% combination of sodium lactate (1.875%) and sodium diacetate (0.125%). A five strain mixture of pathogen was prepared at 10^2 to 10^3 CFU/cm^2, and inoculated on previously sliced samples. Following this step, samples were vacuum-packaged and kept at 3.0 ±

1.1°C up to 12 weeks. Sodium levulinate added at 1% or 2% to turkey breast roll and bologna demonstrated effectiveness in completely preventing growth of *L. monocytogenes* during 12 weeks of storage. Sodium levulinate demonstrated to be as effective as the other antimicrobials that are currently used in the industry. There was no overall difference on taste after testing in a sensory panel on both samples.

Sodium lactate and diacetate (Beef Bologna)

Antimicrobial activities of sodium lactate and sodium diacetate were studied in combination against *L. monocytogenes* and *Salmonella enterica* inoculated onto the surface of commercial beef bologna with 57% moisture. Results from several studies have shown moderate effectiveness in delaying bacterial growth when the product is stored at 5 and 10°C. Based on this information, Mbandi and Shelef (2002) examined the use of single or multiple-strain mixture of 6 strains of pathogens (*Listeria* and *Salmonella*) inoculated on samples at 3 log CFU/gm. The antimicrobials where used at 2.5% for sodium lactate and 0.2% sodium diacetate, stored at 5 and 10°C for up to 60 days. The use of these organic acids were effective even when used alone against certain pathogens, however, their use in combination showed an enhanced inhibition against *L. monocytogenes* and *S. enterica*. Results of treatments using combinations of these antimicrobials with *L. monocytogenes* demonstrated that the inhibition was effective for 45 days when kept at 5°C, showing that this approach can prevent the proliferation of this pathogen. Results with *Salmonella* showed undetectable levels at 5 and 10°C up to 30 days of storage. These findings are conclusive that there is an enhancement in the inhibitory activity in beef bologna products when these antimicrobials are used in combination.

Nisin/ Organic acids (Pork Bologna)

In a recent study by Samelis et al. (2005), using cooked pork bologna dipped in solutions of nisin, with or without organic acids prior to vacuum packaging, demonstrated that the process can significantly offer protection against *L. monocytogenes*. The objectives were to evaluate nisin used alone or in combination with organic acids to be applied as dipping solutions before cooked pork bologna been packaged in order to control the pathogen. A mixture of 10 strains of *L. monocytogenes* was prepared and inoculated onto bologna slices after which samples were dipped into nisin (5000 IU/ml) or in 1%, 3%, or 5% acetic or lactic acid combined with nisin. Microbiological counts were analyzed at 0, 10, 20, 35, 50, 70, 90 and 120 days of storage. Results for this shelf life study shows that treatments with nisin in combination with acetic acid or lactic acid were the most effective against *L. monocytogenes* as growth was not observed for 90 days of storage.

Another study using the dip method of treating frankfurters with organic acids against *L. monocytogenes* prior packaging showed that 2.5% acetic acid combined with 2.5% citric acid was effective to combat growth of the pathogen for up to 90 days of storage at 5^{o}C (Palumbo and Williams, 1994).

Acidic calcium sulfate and lauric arginate ("Table Brown" Ham)

An intervention delivery method called "Sprayed Lethality In Container" (SLIC) was applied in an extensive study where lethality and shelf-life studies were performed (Luchansky et al., 2005). During this study two antimicrobials were used to evaluate the effectiveness against 5 strains of *L. monocytogenes* inoculated (7.0 $\log_{10}$) onto commercial "table brown" ham prior to packaging and stored at 4^{o}C. The antimicrobials

used were acidic calcium sulfate (ACS) in a 1:1 or 1:2 solution and lauric arginate (LAE) at 5% or 10% solution, spraying 0, 2, 4, 6, or 8 mL immediately before the ham was introduced into the bag. They obtained 1.2 $\log_{10}$ CFU/ham, 1.6 $\log_{10}$ CFU/ham, 2.4 $\log_{10}$ CFU/ham, and 3.1 $\log_{10}$ CFU/ham reduction when ASC was applied in 1:1 solution and 0.7 $\log_{10}$ CFU/ham, 1.6 $\log_{10}$ CFU/ham, 2.2 $\log_{10}$ CFU/ham, and 2.6 $\log_{10}$ CFU/ham reduction, respectively, when applied in a 1:2 solution. For 5% LAE, the results showed reductions of 3.3 $\log_{10}$ CFU/ham, 6.5 $\log_{10}$ CFU/ham, 5.6 $\log_{10}$ CFU/ham, and 6.5 $\log_{10}$ CFU/ham and when tested with 10% LAE they obtained 6.5 $\log_{10}$ CFU/ham reduction for all volumes sprayed. According to these results, it is possible to reduce levels of *L. monocytogenes* on ham product within 24 hours stored at 4^{o}C. For the shelf-life study, the initial inoculum was 3.0 or 7.0 $\log_{10}$ CFU/ham applied at the same conditions as in the lethality study, but only treated with 4, 6, and 8 mL of the 1:2 ACS or 5% LAE solution, and stored for 60 days at 4^{o}C. For samples inoculated with 7.0 $\log_{10}$ CFU and treated with 1:2 ACS solution had a reduction within 24 hours of 1.2 $\log_{10}$ CFU/ham, 1.5 $\log_{10}$ CFU/ham, and 2.0 $\log_{10}$ CFU/ham when treated with 4, 6, and 8 mL, respectively, of the antimicrobial. Samples treated with 5% LAE had reductions of 5.1 $\log_{10}$ CFU/ham, 5.4 $\log_{10}$ CFU/ham, and 5.5 $\log_{10}$ CFU/ham for 4, 6 and 8 mLs applied, respectively. Samples inoculated with 3.0 $\log_{10}$ CFU/ham of the pathogen observed a decrease of 1.3 $\log_{10}$ CFU/ham, 1.9 $\log_{10}$ CFU/ham, and 1.8 $\log_{10}$ CFU/ham for the 1:2 ACS treatments when compared to controls and for 5% LAE treatments the levels of pathogen were reduced below the detection level. After 60 days of storage at 4^{o}C, the samples treated with 1:2 ACS levels of the pathogen remained without change and there was an increase of 2.0 – 5.0 $\log_{10}$ CFU/ham on samples treated with 5% solution of LAE. The SLIC intervention

method to control *L. monocytogenes* on ham is a potentially effective method to apply antimicrobials directly to bags receiving RTE meats (Luchansky et al., 2005).

Sodium lactate and diacetate (Wieners and Bratwurst)

In a study involving wieners and cooked bratwurst, two antimicrobial agents (sodium lactate and diacetate) were analyzed in order to determine their efficacy against the pathogen *L. monocytogenes* (Glass et al., 2002). The product ingredients included pork, turkey and beef, and the cooked bratwurst contained beef and pork. Both product samples were inoculated on the surface with 0.2 ml of the pathogen at a target level of 10^5 CFU per package. After the treatments, samples were vacuum-packaged in gas-impermeable pouches and wieners were stored for 60 days at 4.5^{o}C and bratwurst were stored for 84 days at 3 and 7^{o}C. Treatments consisted of dipping the samples into the antimicrobial solutions at the point where possible contamination can occur: after chilling and before packaging. Although the results for these wieners samples were not satisfactory to delay pathogen growth, all surface treatments consisted of dipping wieners into different test solutions. In the first trial: 3% sodium diacetate solution, 6% sodium lactate solution, and 3% sodium diacetate plus 6% sodium lactate were used. For the second trial, wieners were dipped in solutions containing 6% sodium lactate plus 3% sodium diacetate and another treatment containing 3% sodium lactate plus 1.5% sodium diacetate. The treatments for the bratwurst samples, which had two types of samples: cured and naturally smoked bratwurst and uncured and unsmoked bratwurst, consisted of 3.4% sodium lactate plus 0.1% sodium diacetate (cured and naturally smoked), 2.0% sodium lactate, and 3.4% sodium lactate plus 0.1% sodium diacetate (uncured and unsmoked). Results for uncured and unsmoked bratwurst were effective in delaying *L.*

monocytogenes growth for 4 weeks at 7°C and 12 weeks at 3°C at 3.4% and 0.1% for sodium diacetate solution, respectively. At 2% sodium lactate, the bacterial growth was delayed by 1 week at 7°C and 2 weeks at 3°C. However, for the cured and smoked samples, the inhibition was delayed for as long as 12 weeks when 3.4% sodium lactate and 0.1% sodium diacetate were used (Glass et al., 2002).

Sodium chloride, sodium diacetate, and potassium lactate (Wieners, smoked-cooked ham, light bologna, cotto salami)

A response surface method was used to determine the effect of added sodium chloride, sodium diacetate, and potassium lactate on the growth of *L. monocytogenes* in cured RTE meat products. In the various treatments, the concentration of these antimicrobials differed: for sodium chloride, the concentration was in a range of 0.8 to 3.6%; for sodium diacetate, from 0 to 0.2%, and the concentration of potassium lactate was from 0.25 to 9.25%. The finished-product moisture was another parameter that was evaluated, varying from 45.5 to 83.5%. The sample products were wieners, smoked-cooked hams, light bologna, and cotto salami which were produced according to three different concentrations of potassium lactate and sodium diacetate. Three treatments were made to obtain 0% potassium lactate and sodium diacetate (treatment 1), 1.5% potassium lactate and 0.15% sodium diacetate (treatment 2), and 2.5% potassium lactate and 1.5% sodium diacetate (treatment 3). Five strains of *L. monocytogenes* where mixed at the desired inoculum level (1,000 CFU/g) and applied (100µl) directly to the surface of sliced samples weighting 100g, and after the inoculation, the pouches were vacuum sealed and kept for 18 weeks at 4°C. The samples were analyzed every week for the presence of pathogen, and results demonstrated that pathogen growth was reduced

significantly for all treatments during the 18 weeks. The study was useful to confirm proper use of sodium diacetate and potassium lactate in these specific products against *L. monocytogenes* (Seman et al., 2002).

CHAPTER III

METHODOLOGY

Bacterial cultures

All strains were obtained from the bacterial culture collection in the food microbiology laboratory of Dr. Peter M. Muriana in the Food and Agricultural Products Center (FAPC) at Oklahoma State University. For storage purposes, each strain was grown in sterile tryptic soy broth (TSB; Difco™ Becton-Dickenson Laboratories, Sparks, MD), centrifuged, and the pellet was resuspended in TSB plus 10% glycerol. Stock cultures were maintained in cryovials and stored at -80^{o}C. Prior to use in experiments, each strain was grown overnight for two consecutive days at 30^{o}C in TSB (re-transferred into fresh media each day).

Mixed strain "cocktails" were prepared comprising either 6 strains of *Salmonella* (*Salmonella* Thompson 120, *Salmonella* Heidelberg F5038BG1, *Salmonella* Montevideo FS1S051, *Salmonella* Hadar MF60404, *Salmonella* Enteritidis H3527 and *Salmonella* Typhimurium H3380), 4 strains of *E. coli* O157:H7 (ATCC 43894, ATCC 43895, ATCC 35150, and ATCC 43890), and 5 strains of *L. monocytogenes* (Scott A-2, 39-2, CW-62, CW-77 and CW-50).

Bacterial antibiotic resistance

Antibiotic resistant variants were recovered for each strain by surface plating 100 µl of overnight culture onto tryptic soy agar (TSB, Difco) containing the various antibiotics. Plates were then incubated at 30ºC for 3-5 days to allow for the appearance of spontaneous resistant variants which were re-streaked and isolated again from the same medium to insure the isolation of a resistance phenotype. Resistance to a second antibiotic was obtained in the same manner in order to obtain a double resistance phenotype that would provide a good selective advantage for cultures that may be used for inoculated studies, and subsequent recovery, from non-sterile foods. In this manner, variants of the 6 *Salmonella* strains were obtained that were resistant to 10 µg/ml spectinomycin (Sigma-Aldrich Inc., St. Louis, MO) and 100 µg/ml novobiocin (Sigma-Aldrich Inc.). Those for the 4 *E. coli* O157:H7 strains were resistant to 10 µg/ml rifamycin and 20 µg/ml gentamycin (Sigma-Aldrich Inc.) and the 5 strains of *L. monocytogenes* were resistant to 10 µg/ml rifamycin and 100 µg/ml streptomycin (Mediatech, Inc., Herndon, VA). Stock solutions of spectinomycin, novobiocin, rifamycin, gentamycin, and streptomycin were prepared (separately) in sterile water as recommended by the manufacture. The antibiotic solutions were filter sterilized using 0.45 µm sterile syringe filters (Pall Corporation, Newquay, Cornwall, UK) and a sterile 30 ml syringe (Becton Dickinson, Franklin Lakes, NJ). All antibiotic working stocks were maintained refrigerated and protected from light to maintain stability.

Before use in antimicrobial kinetic growth assays, these strains were evaluated for confirmation of their specific antibiotic resistances to facilitate a selective plating regimen and to insure that the selection for antibiotic resistance did not affect growth rate

in comparison to growth on antibiotic–free medium. The 6 strains of *Salmonella*, 4 strains of *E. coli* O157:H7, and 4 strains of *L. monocytogenes* (Scott A2, 39-2, V7-2, 383-2) were inoculated into TSB from frozen stock cultures and were transferred for two consecutive days at 30°C before use. The strains were then serially diluted 10-fold with sterile buffered peptone water (BPW; Becton, Dickinson and Company, Sparks, MD) and 0.1 ml of sample was surface plated in duplicate onto TSA and onto TSA containing the two antibiotics for which each strain was resistant. Plates were incubated at 30º C for 24 h and counted manually.

Antimicrobial solutions

Ten antimicrobials were used in this study. They include acetic acid, citric acid, lactic acid (88%), peroxyacetic acid, PuraQ XTend FX25 (Table 3), AFTEC 3000, AvGard® XP, Cytoguard, Protect M, and Syntrx 3300 (Table 4). Calculations for dilution of antimicrobials were performed using the following equation: $C_1 \times V_1 = C_2 \times V_2$, where C is concentration and V is volume. This equation was used to calculate how much volume of the concentrated stock was necessary to add and obtain a desired final antimicrobial concentration (or dilution of a commercial blend) of the antimicrobial for further inclusion with the bacterial inoculum. The antimicrobials were diluted and mixed appropriately in sterile TSB solution at a desired concentration as follows. Acetic acid was prepared from 17.4 M solution to an 8% solution, Aftec 3000 was prepared to a 5% final solution, AvGard® XP (powdered form) was prepared to a 6% solution, citric acid (powder) was prepared to a final concentration of 2% solution, Cytoguard LA was prepared to a final concentration of 5%, lactic acid (88%) was prepared to a 2% solution, Protect M was prepared to 1.19% solution, peroxyacetic acid (15%) was prepared to a

final concentration of 4%, PuraQ XTend FX25 (25%) to a 1% final solution, and Syntrx 3300 was prepared to a 4% solution (Table 3). The antimicrobials concentration of acetic acid, citric acid, lactic acid (88%), peroxyacetic acid, and PuraQ XTend FX25 were based on the main active ingredients (Table 3), while AFTEC 3000, AvGard® XP, Cytoguard, Protect M, and Syntrx 3300 antimicrobials concentration were prepared based on the commercial solutions (Table 4). The sterilization of the solutions was performed by filtration using 0.45 μm sterilized syringe filter (Pall Corp.) and a 30 ml Syringe (Becton Dickinson).

Determination of minimum inhibitory concentration (MIC) of antimicrobials

Mixed strains "cocktails" were prepared comprising either 6 strains of *Salmonella,* 4 strains of *E. coli* O157:H7, or 5 strains of *L. monocytogenes* which were separately cultured overnight at 30ºC in TSB (all were resistant to two antibiotics as per our previous selective regimen). Immediately prior to use in kinetic growth assays, one ml from each individually grown culture was added together for each specific pathogen cocktail and mixed by vortexing. The mixture (i.e., the 'cocktail') was then diluted with sterile TSB to obtain a concentration of approximately 10^6 CFU ml $^{-1}$.

The kinetic growth assays were performed in 96-well flat bottom microplates (Becton Dickinson, Franklin Lakes, NJ) in which three separate solutions were added in stages in order to obtain the desired effect serial dilution of antimicrobial while maintaining a constant inoculation level for the microbial cocktail. Both antimicrobial and bacterial cocktail were inoculated into sterile broth media. Initially, 100 μl of sterile TSB was aliquoted into a series of wells in microplates. Then, 100 μl of antimicrobial solution (in TSB) was added sequentially to make 1:2 serial dilutions of the antimicrobial

solution along the series of wells by carrying 100 µl from one well to the next (with mixing in between) to give the desired 2-fold serial dilution series of the antimicrobial. Finally, each well plate was inoculated with 100 µl of the cocktail prepared from the bacterial strains (also in fresh TSB). The 96-well microplates were then incubated using the Tecan GENios^{TM} (Phenix, Austria, Europe) microplate reader using Magellan v.5.0 data analysis software (Magellan^{TM}, Switzerland). The settings for kinetic growth curve analyses were as follows: (1) measurement mode: absorbance, (2) measurement wavelength: 595_{nm}, (3) number of flashes: 1, (4) valid temperature range: 33 - 35° C, (5) shake duration (orbital normal): 10 sec, (6) kinetic interval: 1800 sec, (7) unit: OD. The 96-well plates were sealed with UltraClear film (Axygen Inc., Union City, CA) to prevent evaporation of the liquids as well as well to well contamination. Measurements were taken for up to 48 hours. Controls included in this series of experiments were culture without added antimicrobial. Each antimicrobial series was performed with all three pathogens. Three replicates were performed for each treatment. The addition of all substances during the entire experiment was done by using an 8-channel pipetter, which was capable of inoculating multiple wells simultaneously. The experimental disposition of a 96-well microplate format is shown in Figure 1.

Statistical analysis

Growth level comparisons on media with, and without, antibiotics were used to calculate the mean (log CFU ml^{-1}) from three replicates performed for each analysis. Kinetic growth inhibition assays were also performed in triplicate and mean values of O.D. were plotted over time for each concentration of antimicrobial. Data were analyzed using one way repeated measures analysis of variance (ANOVA) to compare the

significance level between growth curves of different antimicrobial concentrations. Pairwise multiple comparisons was applied using the Holm-Sidak method at a level of significance (*p*-value) set at 0.05. The data were analyzed using SigmaPlot v.11 software (Systat Software, Chicago, IL).

CHAPTER IV

RESULTS

We evaluated some common and proposed antimicrobials in head-to-head comparisons against 3 banks of pathogens (*Salmonella, E. coli* O157:H7, and *L. monocytogenes*) to identify MIC levels. It is hoped that this would provide initial data that could be used for making recommendations of concentrations that would be useful for applications in the beef industry to enhance safety and increase shelf-life of food products. Since subsequent application of this data is intended to be performed with actual food products, and surviving pathogenic bacteria would need to be recovered from non-sterile foods, we needed to insure that the antibiotic resistant strains we isolated were not physiologically impaired relative to growth on nonselective media. The MIC kinetic growth assay determinations were then tested at different concentrations of antimicrobials against mixtures of the antibiotic-resistant strains of *Salmonella*, *E.coli* O157:H7, and *L. monocytogenes* we isolated.

Evaluation of antibiotic resistance of pathogenic strains

Prior to the testing of food antimicrobials against *Salmonella*, *E.coli* O157:H7, and *L. monocytogenes*, the growth levels of antibiotic resistant variants was evaluated to insure that the same counts could be obtained with media containing antibiotics as without. This was done in preparation for future food applications that would be performed on non-sterile food products and the use of antibiotics would allow selective enumeration for inoculated strains. It is not unusual in some instances that an 'antibiotic resistant' isolate is obtained that is not completely resistant and may show differences in plate counts between selective and nonselective media that could be as large as 1-3 logs. In this experiment, analyses were performed to compare treatments with and without antibiotic. For this purpose, TSA enumeration plates were prepared with (selective), and without (non-selective) antibiotics. For *Salmonella* strains, selective TSA plates were prepared containing spectinomycin (10 μg ml^{-1}) plus novobiocin (100 μg ml^{-1}). For *E.coli* O157:H7 strains, selective TSA plates were prepared containing rifamycin (10 μg ml^{-1}) plus gentamycin (20 μg ml^{-1}). Lastly, for *L. monocytogenes* strains, selective TSA plates were prepared containing rifamycin (10 μg ml^{-1}) plus streptomycin (100 μg ml^{-1}) (Table 5).

The bacterial antibiotic resistance study for *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* strains did not vary with serotype and all strains obtained similar log (CFU ml^{-1}) counts when comparing plate counts on TSA plates without antibiotics and TSA plates containing antibiotics. The results confirmed that all strains tested were unaffected by the specific antibiotics to which they were resistant and therefore did not affect the microbial counts obtained. The use of selective plating on media containing

antibiotics would be useful when performing antimicrobial trials on actual inoculated food samples whereby the double antibiotic resistance would exclude any indigenous bacteria from being counted.

Kinetic growth curve and antimicrobial inhibition assays

The pathogenic groups of strains described earlier were used in kinetic growth assays in the presence of as many as 7 different concentrations of each antimicrobial (2-fold dilutions). Select groupings of *Salmonella, E. coli* O157:H7, and *L. monocytogenes* (i.e., the 'cocktail' inoculum showed differences in inhibitory activity with various antimicrobials). The objective was to evaluate growth of mixtures of pathogens at different concentrations of antimicrobials which would help in establishing Minimum Inhibitory Concentration (MIC) that could possibly be helpful in antimicrobial applications with actual food products. The effect of the antimicrobials tested (acetic acid, Aftec, AvGard, citric acid, Cytoguard, lactic acid, Protect M, peroxyacetic acid, PuraQ, and Syntrx 3300) were found to be different for each microorganism, hence the MIC was dependent on the interaction between pathogen and antimicrobial (Table 3 and 4). We determined that the MIC would be the concentration that resulted in near complete suppression of growth as observed in the kinetic growth curve assays.

For the *Salmonella* strains tested, the results show that the MIC's were 4% acetic acid (Fig. 2), 1.25% AFTEC solution (Fig. 3), and 0.75% AvGard required a concentration of 0.75% (Fig. 4). Citric acid, lactic acid, and PuraQ XTend (Figures 5, 6, 7, respectively) were found to control *Salmonella* growth at a concentration (or dilution of commercial product) of 0.5%. Moreover, Cytoguard effectively inhibit microbial growth with a 2.5% concentration (Fig. 8) whereas peroxyacetic acid solution

concentration as low as 0.063% controlled growth of *Salmonella* (Fig. 9). Protect M inhibited microbial growth at 0.07% concentration (Fig. 10). Lastly, Syntrx (Fig. 11) showed MIC level at 0.125% concentration.

Similar to the results for *Salmonella*, when acetic acid, AFTEC, AvGard, citric acid, Cytoguard, lactic acid, Protect M, and Syntrx (Figs. 2, 3, 4, 5, 8, 6, 10, and 11) were evaluated against *E. coli* strains the MICs were 4%, 1.25%, 0.75%, 0.5%, 2.5%, 0.5%, 0.07%, and 2.00% respectively. Even though the response of *E. coli* and *Salmonella* to many of the antimicrobials tested was similar, when peroxyacetic acid and PuraQ XTend were tested against *E. coli,* lower levels of antimicrobial concentration were able to inhibit *E. coli* as compared to *Salmonella*. The concentration of peroxyacetic acid and PuraQ XTend (Figs. 9 and 7) that controlled *E. coli* was 0.063% and 0.25%, respectively.

Results for *L. monocytogenes* showed that acetic acid, AFTEC, AvGard, citric acid, and Syntrx (Figs. 2, 3, 4, 5, and 11) controlled *L. monocytogenes* at the same MIC concentrations that were observed for *E. coli* and *Salmonella*. However, Cytoguard, lactic acid, Protect M, and PuraQ XTend were effective at lower concentrations for *L. monocytogenes* than were needed to inhibit *E. coli* or *Salmonella*. The MIC for Cytoguard, lactic acid, Protect M, and PuraQ XTend for complete inhibition of *L. monocytogenes* were: 1.25%, 0.25%, 0.04%, and 0.016%, respectively (Figs. 8, 6, 10, and 7). Peroxyacetic acid MIC for *L. monocytogenes* was 0.125% (Fig. 9).

We observed that peroxyacetic acid (Fig. 9) required the lowest concentration (0.063%) to control the mixed *Salmonella* serovars, followed by Protect M 0.07% (Fig. 10), and 0.5% for citric acid, lactic acid, and PuraQ XTend (Figs. 5, 6, and 7). For the mixed *E. coli* O157:H7 strains tested, the lowest concentration of antimicrobial necessary

to inhibit microbial growth was provided by peroxyacetic acid 0.063% (Fig. 9), followed by 0.07% Protect M (Fig. 10), and 0.25% PuraQ XTend (Fig. 7). For the *L. monocytogenes* strains tested, PuraQ XTend provided the lowest concentration necessary to inhibit microbial growth (0.016%) (Fig. 7), followed by 0.04% Protect M (Fig. 10) and 0.125% peroxyacetic acid (Fig. 9). On the contrary, the highest concentration of antimicrobial necessary for complete growth inhibition of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* was 4% acetic acid as an antimicrobial (Fig. 2).

CHAPTER V

DISCUSSION

Using the kinetic growth assay for antimicrobial inhibition studies, we were able to identify the MIC of each antimicrobial that inhibited *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* growth (Table 6 and 7). During these experiments it was observed that 4% acetic acid was required to inhibit the growth of the three groups of pathogens (Fig. 3), due to its ability to lower pH and cause instability of bacterial cell membranes (Stivarius et al., 2002). Delmore et al. (1998) reported that acetic acid at 2% concentration, applied on adipose tissue from beef carcasses samples through pre-evisceration washing, was effective in reducing aerobic microbial and *E. coli* counts. The method used consisted of rinsing with 2% acetic acid followed by final washing, and hot water rinse treatments. In another study, Bell et al. (1997) applied 1% acetic acid, 3% hydrogen peroxide, and 1% sodium bicarbonate using a spray application, in combination or individually, with the objective to reduce *E. coli*, *L. innocua*, and *Salmonella* Wentworth counts on beef carcasses. They proved that acetic acid was most effective when used in combination with hydrogen peroxide. However, in a study conducted by Anderson and Marshall (1989), a factorial treatment of three acetic acid concentrations

(0, 1, 2, and 3%) and four temperatures (25, 40, 55 and 70°C) were applied as dip method of beef semitendinosus muscle contaminated with *E. coli* and *Salmonella* Typhimurium. Their research demonstrated that 3% acetic acid at 70° C was the most effective treatment in reducing microbial counts.

When citric acid was tested, we observed that 0.5% concentration was effective in inhibiting growth of the three groups of pathogens (Fig. 5). Algino et al. (2007) used a 2.5% acetic acid and Fresh Bloom (mixture of 5% citric acid, ascorbic acid, and erythorbic acid) applied onto beef carcasses before chilling, and showed that all interventions used were effective and decreased levels of *E. coli* O157:H7, coliforms, and *Enterobacteriaceae*. A 2.5% citric acid solution combined with 2.5% acetic acid, applied as dip treatments to control possible contamination of *L. monocytogenes* on the surface of frankfurters was demonstrated to be an effective method to control *L. monocytogenes* growth during 90 day storage at 5°C (Palumbo and Williams, 1993).

When lactic acid was tested against *Salmonella* and *E. coli*, a 0.5% concentration was sufficient to inhibit growth, however for *L. monocytogenes*, even 0.25% lactic acid was an effective growth inhibitor (Fig. 6). Calicioglu et al. (2002) conducted a study using lactic acid at 2% concentration for beef carcass decontamination using a spray method against strains of *E. coli* O157:H7. In their experiment, a series of treatments were conducted including pre-spraying a solution of 5% concentration of Tween 20 (TW20) that resulted in increased effectiveness of lactic acid. Gill and Landers (2003) applied spray treatment using 2% lactic acid, vacuum hot water, trimming, washing, and pasteurizing (with steam or hot water) for decontamination of beef carcasses, providing evidence that 2% lactic acid, vacuum hot water, and trimming were not effective in

reducing *E. coli* counts, but washing and pasteurization were satisfactory as far as reducing bacterial counts on carcasses. These findings are in agreement with previous studies reported using lactic acid at 2% solution. Lactic acid at 4% and 2% concentrations were used in a study conducted by Castillo et al. (2001b) using spray systems on beef carcass surfaces inoculated with *E. coli* O157:H7 and *Salmonella* Typhimurium and obtained significant reductions counts of these pathogens. Further, treatments of 2.5% and 5% lactic acid at 50 and 55^{o}C, respectively, as well as 2% activated lactoferrin followed by 5% lactic acid at 55^{o}C were evaluated on subprimal cuts applied before blade tenderization and moisture enhancement indicated that all treatment interventions were effective in decreasing *E. coli* population on the interior of beef subprimal cuts when applied prior to mechanical tenderization (Heller et al., 2007). Byelashov et al. (2010) demonstrated that it was possible to achieve a 2-log reduction on frankfurters inoculated with *L. monocytogenes* using 3% lactic acid at 25º C applied for 120 seconds, and/or 1% lactic acid at 55 º C for 60 seconds.

Protect M (lauric arginate) antimicrobial showed significant inhibition of *Salmonella*, *E. coli*, and *L. monocytogenes* at low concentration (Fig. 10). Although, the inhibition of *Salmonella* and *E. coli* was achieved with 0.07% concentration, 0.04% Protect M was required to suppress growth of *L. monocytogenes*. Taormina and Dorsa (2009) conducted a study using Sprayed Lethality in Container (SLIC) method, and showed that at 5% solution of lauric arginate resulted in a 5-log reduction of *L. monocytogenes* counts, concluding that lauric arginate solution is an effective intervention at this concentration. Even though a 5% lauric arginate solution using the SLIC method reduced microbial growth, the concentration used was higher than the

concentration used to inhibit microbial growth during our MIC determination (this study). The contrast between these results is likely to happen due to an interaction effect between concentration, method used (intervention and MIC determination), specific antimicrobial, and the effect of the food product upon which is applied. Our approach was only able to determine levels that prevent growth, even at optimal growth temperatures. A future application would be to utilize the data obtained herein as a starting point to examine log reduction as well as interactions when several antimicrobials are combined.

Peroxyacetic acid (PAA) antimicrobial tested against *Salmonella* and *L. monocytogenes* inhibited microbial growth at a concentration of 0.125% whereas only 0.063% was necessary to kill *E. coli* (Fig. 9). Penney et al. (2007) demonstrated that PAA reduced the microbial load of *E. coli* O157:H7 by 97.4 to 99.9%, and supported the potential of this antimicrobial for beef carcass decontamination.

With additional antimicrobials, our results showed that 2.5% concentration of Cytoguard antimicrobial was needed to inhibit *Salmonella* and *E. coli* growth, whereas for *L. monocytogenes* growth inhibition was achieved at 1.25% concentration (Fig. 8). Sommers et al (2009) demonstrated that Ultraviolet Light (UV-C) irradiation applied with 5% lauric arginate (Cytoguard) to the surface of frankfurters immediately prior sealing inactivated *L. monocytogenes*, *Staphylococcus aureus*, and *Salmonella* spp. by 1.39–1.65 log when the initial log was 10^6 cfu/g. Lauric arginate used in combination with potassium lactate and sodium diacetate, and UV-C light inactivated 2.32–2.80 log of the pathogens, showed more effectiveness than individual treatments.

Aftec at 1.25% was able to inhibit growth of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* (Fig. 3) while only a 0.75% solution of AvGard was able to inhibit the

three groups of pathogens (Fig. 4). Salvat et al (1997), demonstrated that AvGard, applied at 10% for poultry carcass decontamination through manual dip method, had significant effects against aerobic mesophilic counts, thermo tolerant coliforms, *Pseudomonas*, *Campylobacter*, *L. monocytogenes*, and *Salmonella*. A reduction of 2 log was observed for *Salmonella* counts while for *L. monocytogenes* it was also reduced bacterial counts in poultry carcasses.

The PuraQ XTend antimicrobial inhibited *Salmonella* at 0.5% whereas *E. coli* was inhibited at 0.25% and *L. monocytogenes* by 0.016% (Fig. 7). Finally, for the Syntrx antimicrobial that we tested, the results showed that as high as 2% was necessary to inhibit the three groups of pathogens (Fig. 11).

Our data defines the relative level of each antimicrobial solution/mixture and their inhibitory effect on the 3 mixtures of different pathogens, *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes*. Although complete inhibition or microbial growth under permissive conditions was demonstrated by the MIC, it does not identify microbial reduction yet it provides a basis from which future studies can draw upon to examine lethality and define potential synergistic activities. The data provided herein will be useful to future studies by applying the antimicrobials in challenge trials on raw meat products as surface interventions as well as shelf life inhibitors. However, if used as shelf life inhibitors, the presence of extended activity during shelf life may require the antimicrobials to be listed as an ingredient as opposed to being considered as a 'processing aid' and remain unlisted as an ingredient.

CHAPTER V

CONCLUSION

Currently, *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* have been problematic to the raw and processed meat industries, causing foodborne illness, product recalls, and even deaths. *Salmonella* and *E. coli* O157:H7 are major problems for raw meat and poultry products, while *L. monocytogenes* has been recognized as the main pathogen of concern for RTE meat products. The problems associated contamination of meats during slaughter, with the persistence of these pathogens in meat processing plants that sometimes ending up on the products themselves, has led to numerous regulatory directives and control measures including antimicrobial treatments to reduce pathogens and/or prevent their growth during the shelf life of a product. Data obtained from the literature may not be as clear as to the effect of antimicrobials on target pathogens. Most studies examine only a few antimicrobials. Different studies may use different strains of pathogens, so it is not clear if the sensitivity of the different strains of the same pathogens can be compared, let alone the conditions for treatment. This study was meant to compare several antimicrobials against mixed-strain 'cocktails' of 3 different pathogens using the same conditions and antimicrobial solutions for each of the 3 pathogens. This identifies the MIC in head-to-head comparisons. In conclusion, this research suggests

what levels the various antimicrobials may be used to inhibit pathogen growth and be beneficial for consumers. These levels may be used in further studies to determine lethality effects on the same types of pathogens to establish microbial reduction conditions for post-process lethality regimens.

TABLES

Table 1. The seven principles of HACCP program

Principle 1. Conduct a hazard analysis.
Principle 2. Identify the critical control points (CCPs) in the program.
Principle 3. Establish critical limits for preventive measures associated with each identifiable CCP.
Principle 4. Establish monitoring requirements and procedures for using monitoring results to adjust processes and maintain control.
Principle 5. Establish corrective actions to be taken when monitoring indicates that there is a deviation from an established critical limit.
Principle 6. Establish effective record-keeping procedures that document the HACCP plan.
Principle 7. Establish procedures to verify that the HACCP system is working correctly.

(Hulebak and Schlosser, 2002)

Table 2. Safe and suitable antimicrobials used in the production of meat and poultry products.

Substance	Product	Amount	Reference	Labeling Requirements
Acetic acid (0.5%)	Cooked meat products	Not to exceed 0.5% of finished product formulation	Acceptability determination	Listed by common or usual name in the ingredients statement
Citric acid	Bologna in an edible casing	Up to a 10 percent solution applied prior to slicing	Acceptability determination	Listed by common or usual name in the ingredients statement
Citric acid	Bologna in an inedible casing	Up to a 10 percent solution applied prior to slicing	Acceptability determination	None under the accepted conditions of use
Citric acid	Fully cooked meat and poultry products in fibrous casings.	Up to a 3 percent solution is applied to the casing just prior to removal	Acceptability determination	None under the accepted conditions of use
Lactic acid	Livestock carcasses prior to fabrication (i.e., pre- and post-chill), variety meats	Up to 5 percent lactic acid solution	Acceptability determination	None under the accepted conditions of use
Lactic acid	Beef and pork sub-primals and trimmings	2 - 5 percent lactic acid solution	Acceptability determination	None under the accepted conditions of use
Lauramide arginine ethyl ester (LAE)	RTE meat and poultry products	Applied to the inside of the package via "Sprayed Lethality in Container" (SLIC) up to 44 ppm	Acceptability determination	None under the accepted conditions of use
Lauramide arginine ethyl ester (LAE) dissolved in either water, propylene glycol, or glycerin	Non-standardized and standardized RTE comminuted meat products that permit the use of any safe and suitable antimicrobial agent	Not to exceed 200 ppm LAE by weight of the finished product	Acceptability determination	When applied to the surface of RTE products listed by usual name and when applied to the surface of fresh cuts of meat and poultry none under the accepted conditions of use

Substance	Product	Amount	Reference	Labeling Requirements
Lauramide arginine ethyl ester (LAE) dissolved in either water, propylene glycol, or glycerin	Fresh cuts of meat and poultry and various non-standardized and standardized RTE meat and poultry products that permit use of any safe and suitable antimicrobial agent	Applied to the surface of the product at a rate not to exceed 200 ppm LAE by weight of the finished food product	GRAS Notice No. 000164	None under the accepted conditions of use
Organic acids (i.e., lactic, acetic and citric acid)	As part of carcass wash applied pre-chill	At up to 2.5 percent of a solution	FSIS Notice 49-94	None under the accepted conditions of use
Peroxyacetic acid, octanoic acid, acetic acid, hydrogen peroxide, peroxyoctanoic acid, and 1-hydroxyethylidene-1, 1-diphosphonic acid	Meat and poultry carcasses	Maximum concentration for meat carcasses: Peroxyacetic acids 220 ppm, hydrogen peroxide 75 ppm; Maximum concentration for poultry carcasses: Peroxyacetic acids 220 ppm, hydrogen peroxide 110 ppm	21 CRF 173.370	None under the accepted conditions of use

(FSIS-USDA, 2009)

Table 3. Description of antimicrobials evaluated in this study, trade name, supplier, main active ingredient(s), and maximum product application strength. Product application strength calculated based on concentration of the main active ingredient.

Trade Name	Supplier	Main Active Ingredient(s)	Maximum Product Application Strength
Acetic acid	Pharmco Products Inc., Brookfield, CT	Acetic acid	8% Acetic acid
Citric acid	Spectrum Chemicals & Laboratory Products, Gardena, CA	Citric acid	2% Citric acid
Lactic acid FCC	Birko Corporation, Henderson, CO	Hydroxypropanoic acid (88%)	2% Lactic acid
Peroxyacetic acid	Enviro Tech Chemical Services, Inc., Modesto, CA	Peroxyacetic acid (15% PAA)	4% Peroxyacetic acid
PuraQ XTend FX25	Purac America Inc., Lincolnshire, IL	25% Polylysine	1% PuraQ XTend

Table 4. Description of antimicrobials evaluated in this study, trade name, supplier, main active ingredient(s), and maximum product application strength. Product application strength calculated based on the dilution factor of the supplier commercial solution.

Trade Name	Supplier	Main Active Ingredient(s)	Maximum Product Application Strength
Aftec 3000	Advanced Food Technologies, LLC, Shreveport, LA	Buffered sulfuric acid	5% Aftec 3000
AvGard XP	Danisco A/S, Copenhagen, Denmark	Sodium Metasilicate, anhydrous	6% AvGard XP
Cytoguard	A&B Ingredients, Fairfield, NJ	Lauric arginate & peroxyacetic acid	5% Cytoguard
Protect M	Purac America Inc., Lincolnshire, IL	Lauric arginate (10.5%)	1.19% Protect M
Syntrx 3300	Synergy Technologies Inc., Shreveport, LA	Hydrochloric & citric acids	4% Syntrx 3300

Table 5. *Salmonella, E. coli* O157:H7, and *L. monocytogenes* strains tested for plate count enumeration on media with, and without, antibiotics. Control plates were prepared without (non-selective) antibiotics. Plates were incubated at 30ºC for 24 h.

Bacterial strain	TSA		TSA + Antibiotics	
	Log CFU/ml	S.D.	Log CFU/ml	S.D.
Salmonella Hadar MF60404	9.09[a]	0.05	9.03[a]	0.01
Salmonella Typhimurium H3380	9.09[a]	0.03	9.01[a]	0.10
Salmonella Thompson 120	9.08[a]	0.05	9.04[a]	0.01
Salmonella Montevideo FSIS 051	8.66[a]	0.02	8.77[a]	0.04
Salmonella Heidelberg F5038BG1	9.06[a]	0.05	9.07[a]	0.01
Salmonella Enteritidis H3527	9.02[a]	0.07	9.09[a]	0.01
E. coli O157:H7 ATCC 43894	7.79[a]	0.08	7.88[a]	0.01
E.coli O157:H7 ATCC 43895	7.73[a]	0.06	7.85[a]	0.03
E. coli O157:H7 ATCC 35150	7.60[a]	0.00	7.48[a]	0.05
E. coli O157:H7 ATCC 43890	7.76[a]	0.02	7.83[a]	0.04
L. monocytogenes V7-2	7.59[a]	0.02	7.62[a]	0.08
L. monocytogenes 383-2	8.20[a]	0.05	8.19[a]	0.01
L. monocytogenes Scott A-2	8.22[a]	0.05	8.30[a]	0.01
L. monocytogenes 39-2	8.16[a]	0.00	8.15[a]	0.06

[a] Data means in the same row with the same lowercase superscript letter designation are not significantly different ($P > 0.05$). S.D., standard deviation.

Table 6. Minimum Inhibitory Concentrations (%) of various mixed-strain cocktails of foodborne pathogens.

	Salmonella	***E.coli* O157:H7**	***L.monocytogenes***
Antimicrobials	**MIC**	**MIC**	**MIC**
Aftec	1.25	1.25	1.25
AvGard XP	0.75	0.75	0.75
Cytoguard	2.50	2.50	1.25
Protect M	0.07	0.07	0.04
Syntrx 3300	2.00	2.00	2.00

Where: MIC = Minimum Inhibitory Concentration (minimum concentration at which no growth was observed for 48 h at 30^{o}C over the course of the assay).

Table 7. Minimum Inhibitory Concentrations (%) of various mixed-strain cocktails of foodborne pathogens.

	Salmonella	***E.coli* O157:H7**	***L.monocytogenes***
Antimicrobials	**MIC**	**MIC**	**MIC**
Acetic acid	4.00	4.00	4.00
Citric acid	0.50	0.50	0.50
Lactic acid	0.50	0.50	0.25
Peroxyacetic acid	0.063	0.063	0.125
PuraQ XTend FX25	0.50	0.25	0.016

Where: MIC = Minimum Inhibitory Concentration (minimum concentration at which no growth was observed for 48 h at 30^{o}C over the course of the assay).

FIGURES

A	1	2	3	4	5	6	7	8	9	10	11	12
A	controls →			*Salmonella* (control; AM 0x)			*E. coli* O157:H7 (control; AM 0x)			*L. monocytogenes* (control; AM 0x)		
B	Blanks (sterile media)			AM (1x)			AM (1x)			AM (1x)		
C				AM (0.5x)			AM (0.5x)			AM (0.5x)		
D				AM (0.25x)			AM (0.25x)			AM (0.25x)		
E				AM (0.125x)			AM (0.125x)			AM (0.125x)		
F				AM (0.063x)			AM (0.063x)			AM (0.063x)		
G				AM (0.033x)			AM (0.033x)			AM (0.033x)		
H				AM (0.016x)			AM (0.016x)			AM (0.016x)		

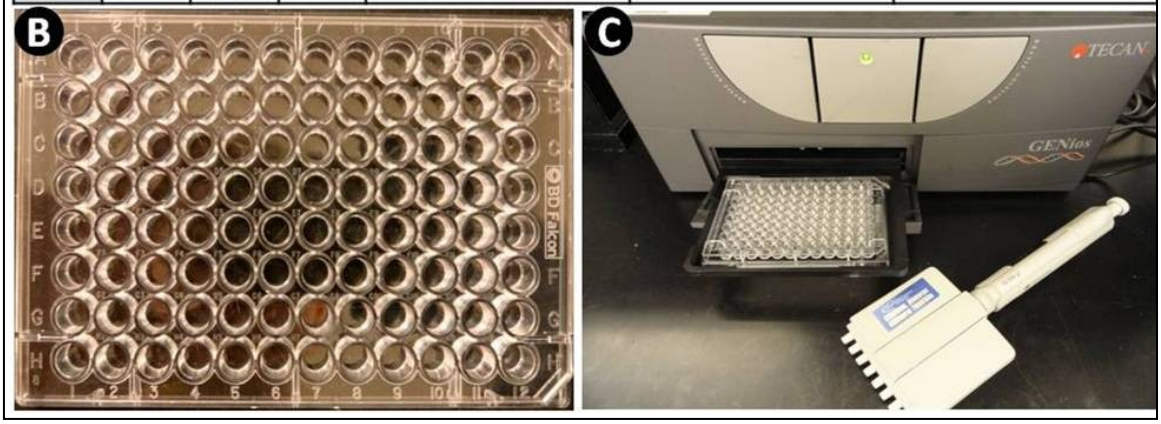

Figure 1. Panel A, experimental design of 96-well plates. Each antimicrobial series was performed with all three groups of bacteria (*Salmonella*, *E. coli* and *L. monocytogenes*). All microbiological experiments were performed in triplicate replication. Antimicrobial, AM. Panel B, flat bottom microplates that were sealed with optical film; plates were read from the bottom side. Panel C, multichannel pipetter and temperature-controlled plate reader; the plate reader was programmed to shake plates before reading.

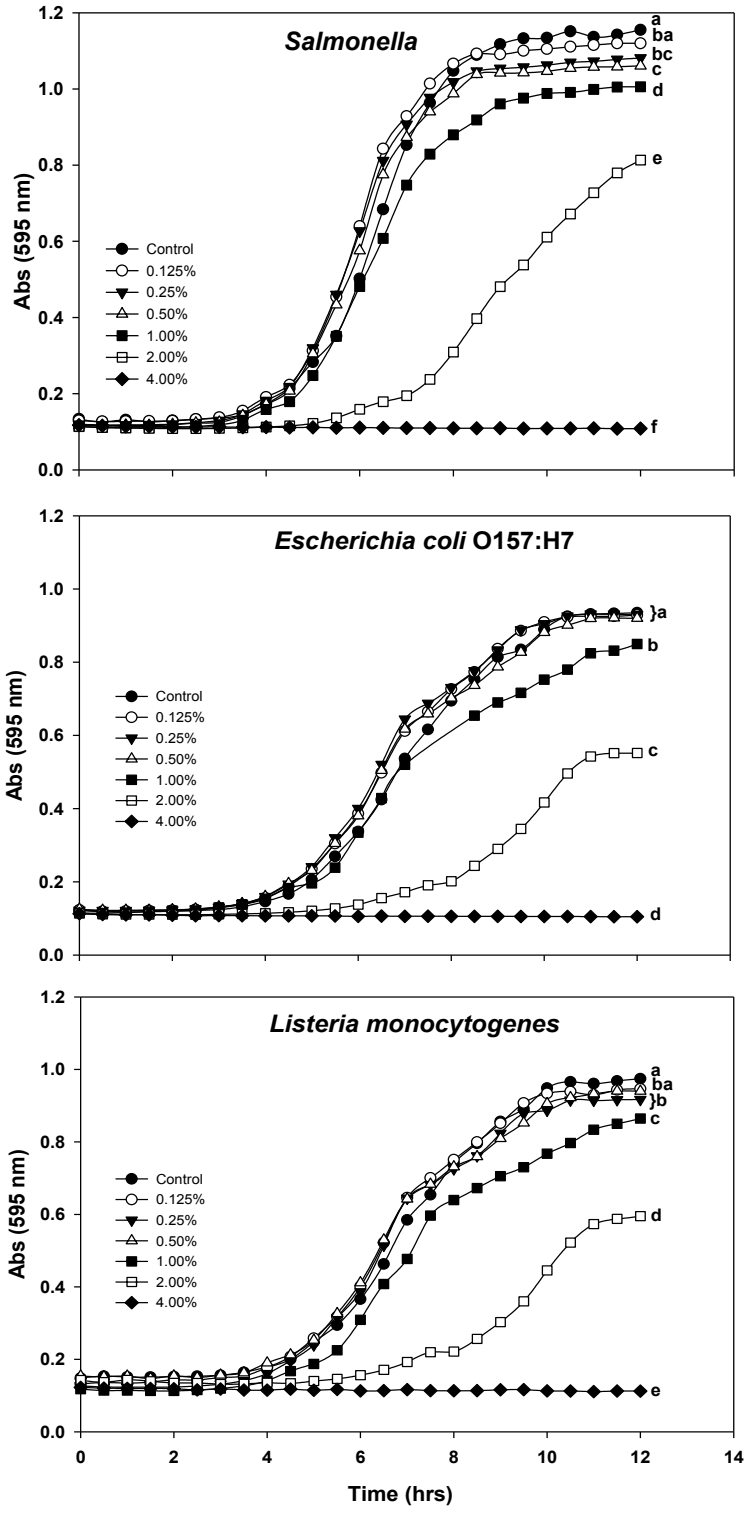

Figure 2. The effect of acetic acid on *Salmonella*, *E. coli*, and *L.* monocytogenes. Statistical significance was determined from end-point values. End point values with the same lowercase letter designation are not significantly different ($P > 0.05$).

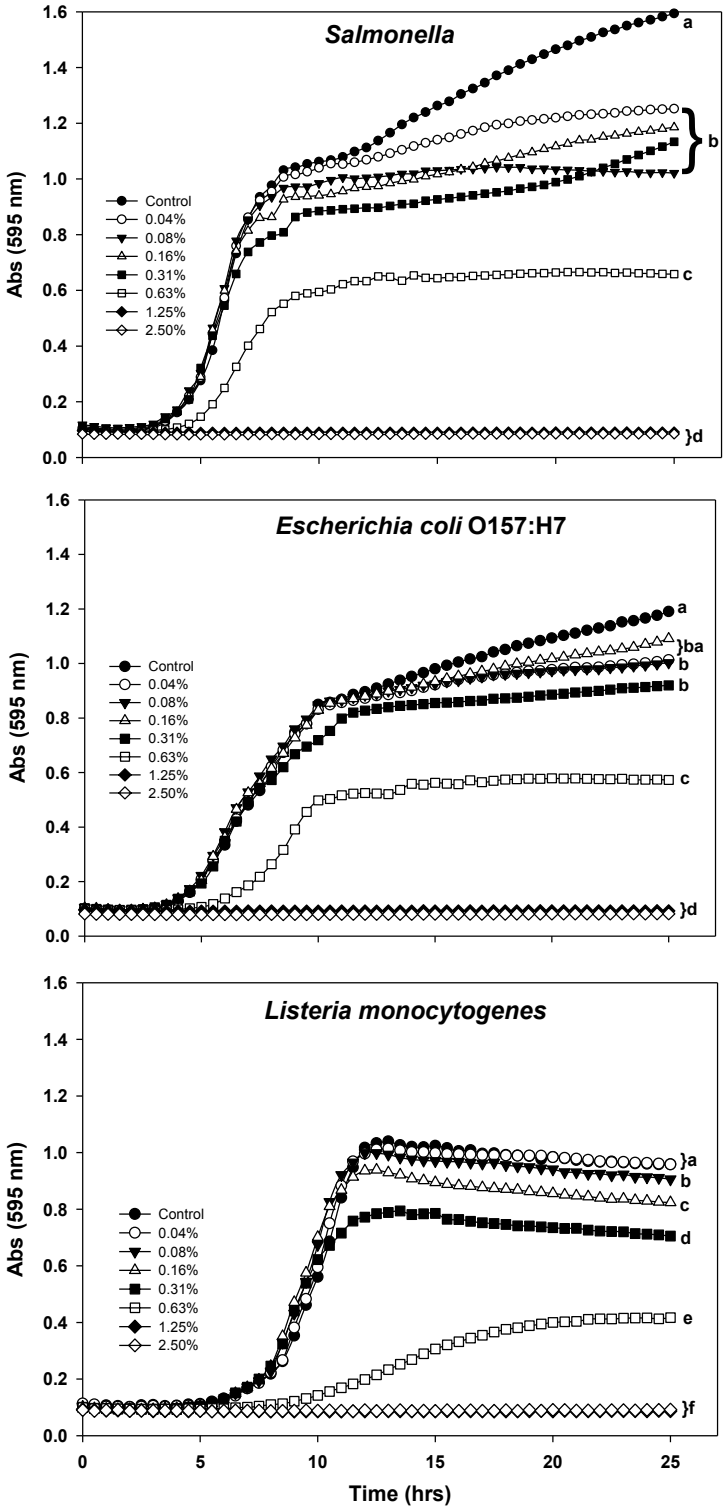

Figure 3. The effect of AFTEC on *Salmonella*, *E. coli*, and *L. monocytogenes.* Statistical significance was determined from end-point values. End point values with the same lowercase letter designation are not significantly different ($P > 0.05$).

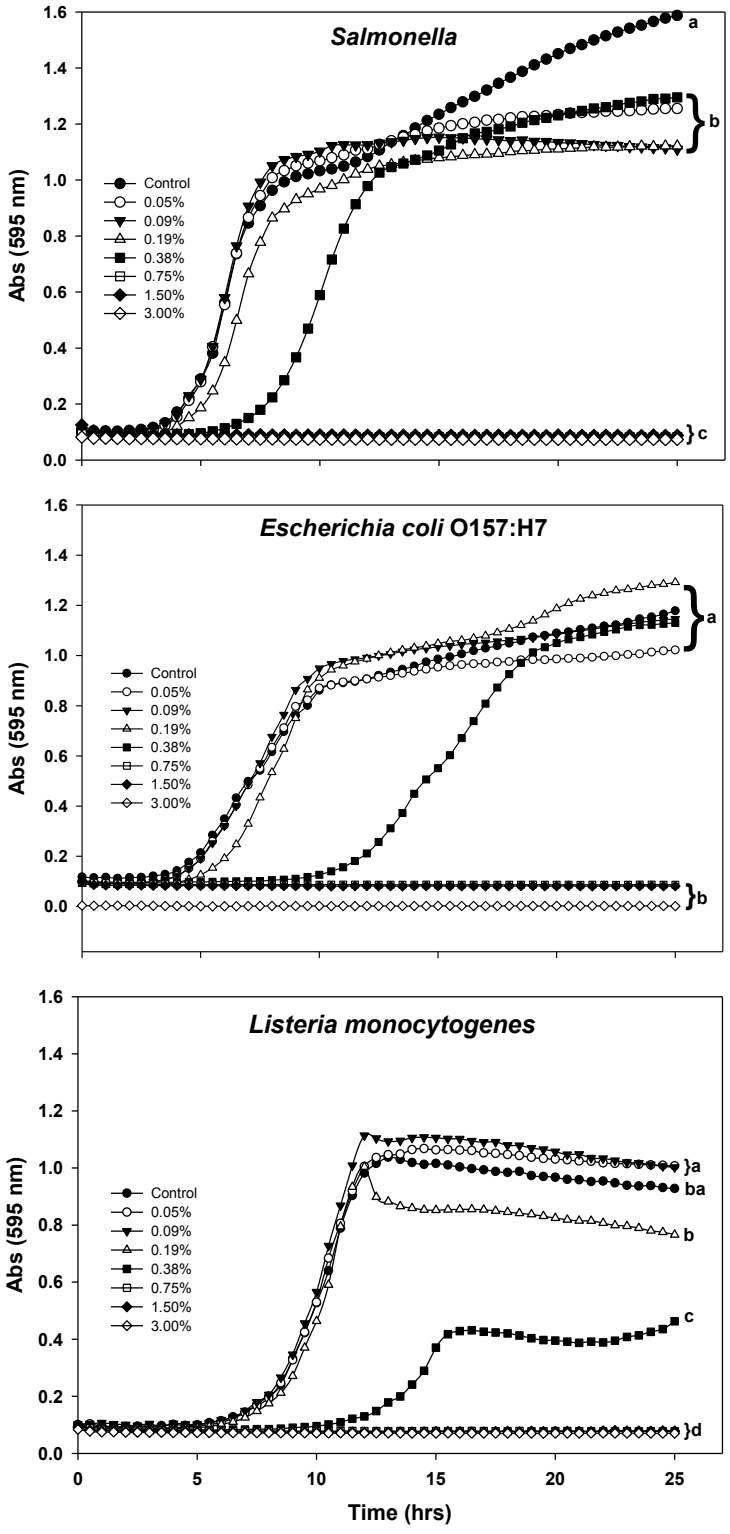

Figure 4. The effect of AvGard on *Salmonella*, *E. coli*, and *L. monocytogenes.* Statistical significance was determined from end-point values. End point values with the same lowercase letter designation are not significantly different ($P > 0.05$).

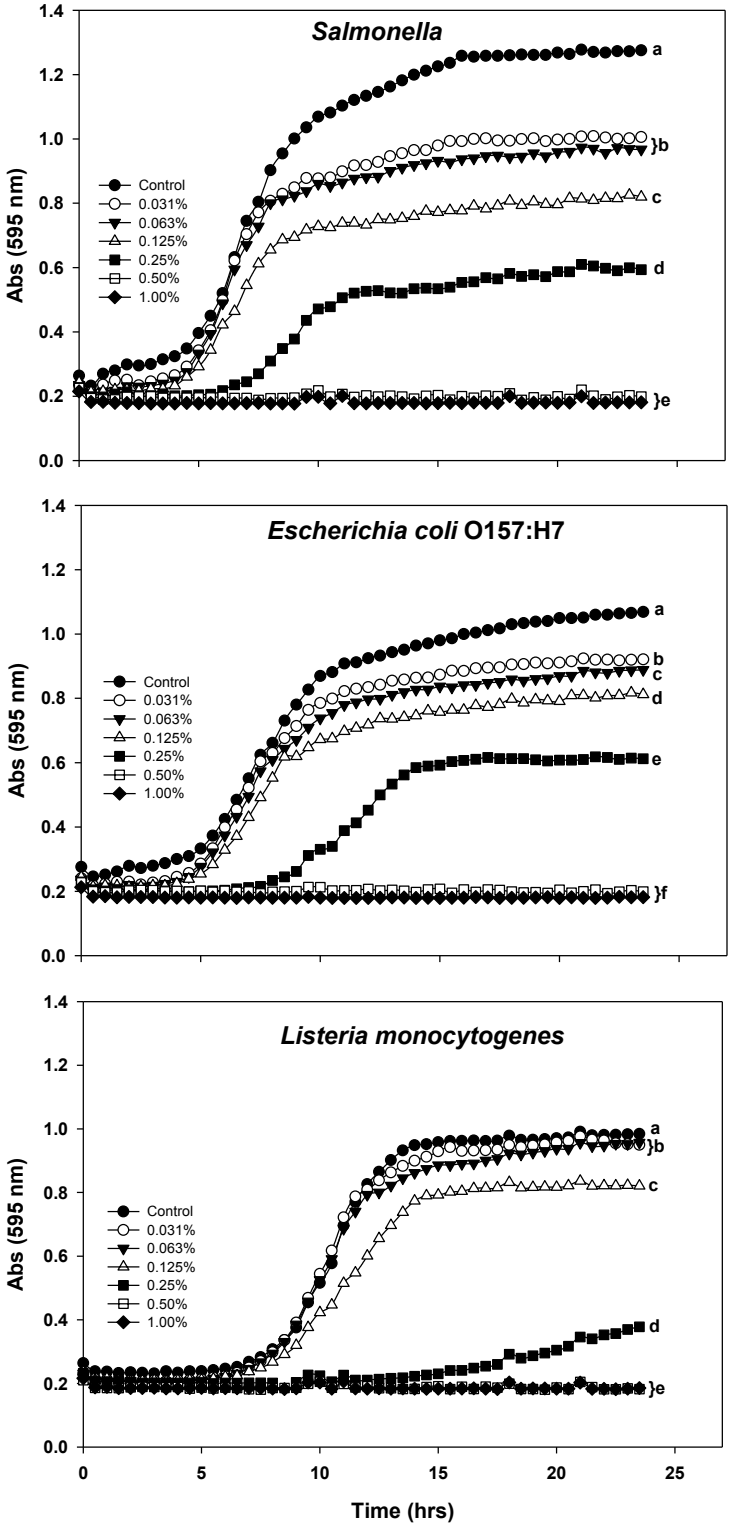

Figure 5. The effect of citric acid on *Salmonella*, *E. coli*, and *L. monocytogenes.* Statistical significance was determined from end-point values. End point values with the same lowercase letter designation are not significantly different ($P > 0.05$).

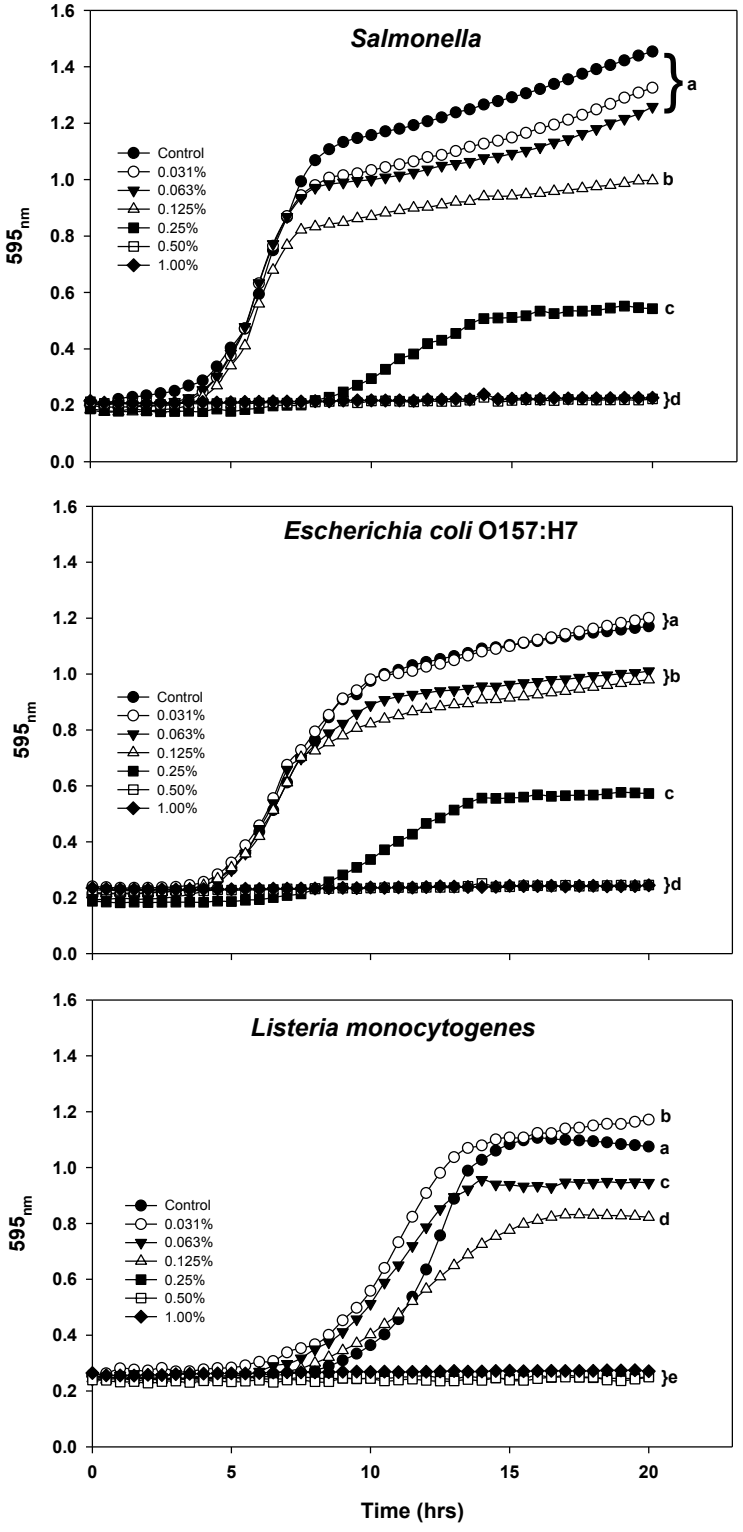

Figure 6. The effect of lactic acid on *Salmonella*, *E. coli*, and *L. monocytogenes.* Statistical significance was determined from end-point values. End point values with the same lowercase letter designation are not significantly different ($P > 0.05$).

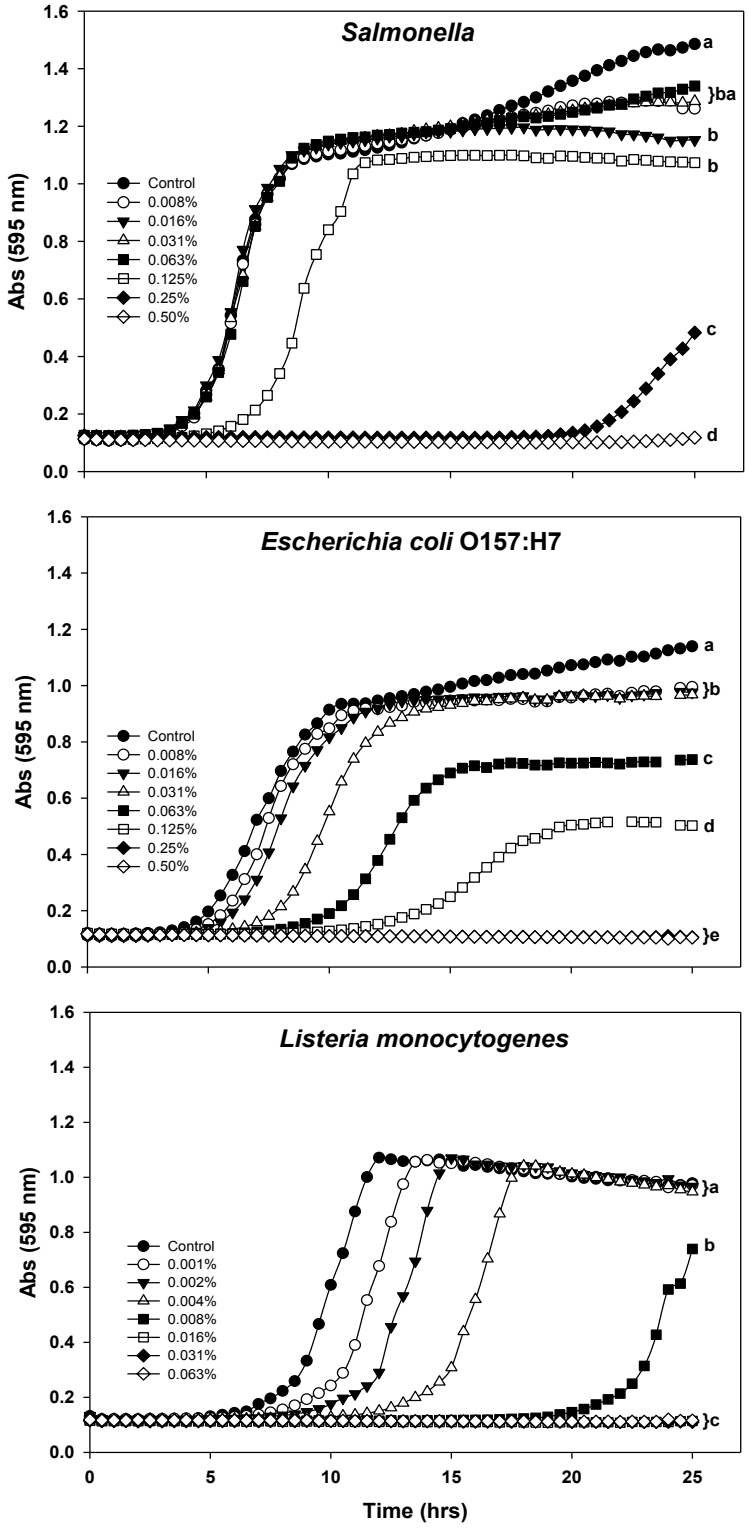

Figure 7. The effect of PuraQ on *Salmonella*, *E. coli*, and *L. monocytogenes.* Statistical significance was determined from end-point values. End point values with the same lowercase letter designation are not significantly different ($P > 0.05$).

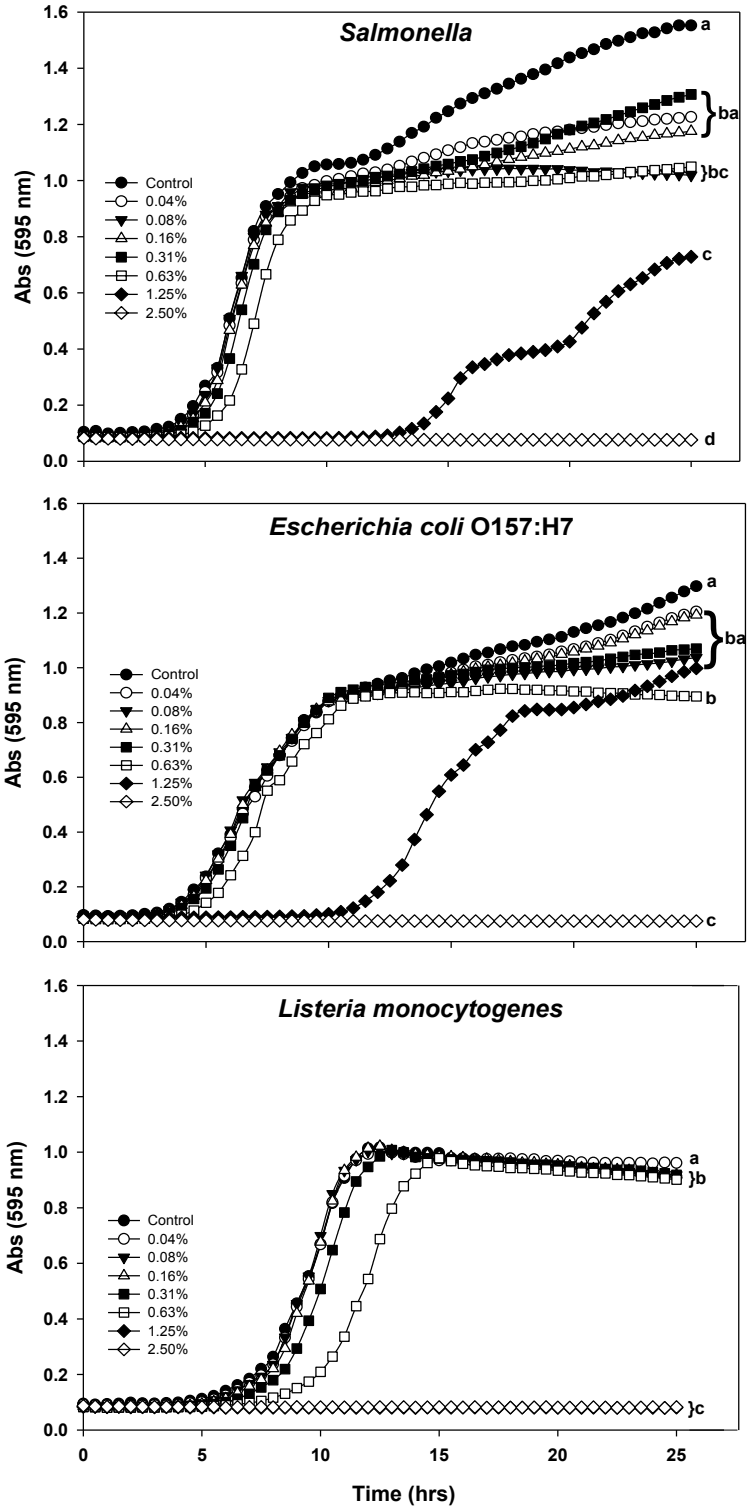

Figure 8. The effect of Cytoguard on *Salmonella*, *E. coli*, and *L. monocytogenes.* Statistical significance was determined from end-point values. End point values with the same lowercase letter designation are not significantly different ($P > 0.05$).

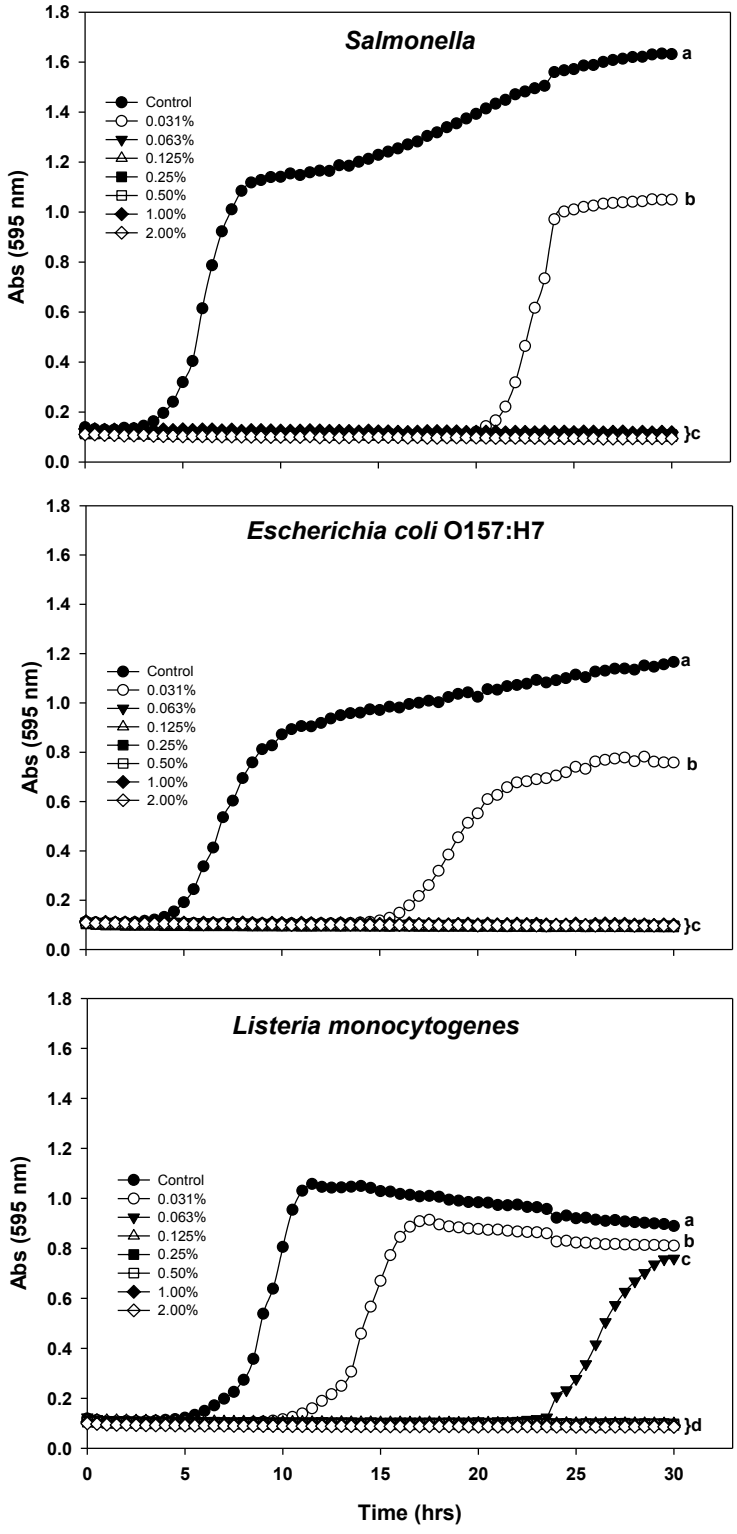

Figure 9. The effect of peroxyacetic acid on *Salmonella*, *E. coli*, and *L. monocytogenes.* Statistical significance was determined from end-point values. End point values with the same lowercase letter designation are not significantly different ($P > 0.05$).

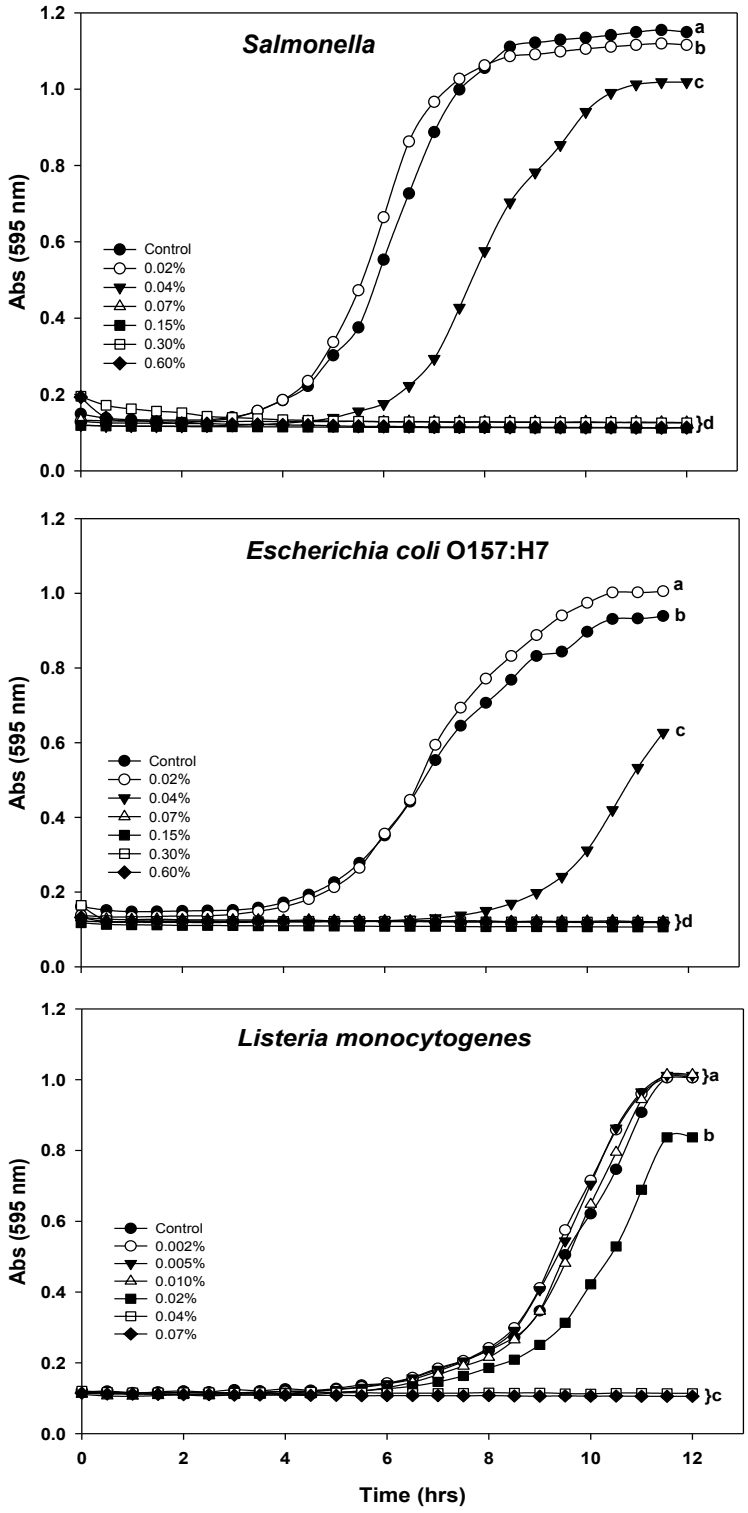

Figure 10. The effect of Protect M on *Salmonella*, *E. coli*, and *L. monocytogenes.* Statistical significance was determined from end-point values. End point values with the same lowercase letter designation are not significantly different ($P > 0.05$).

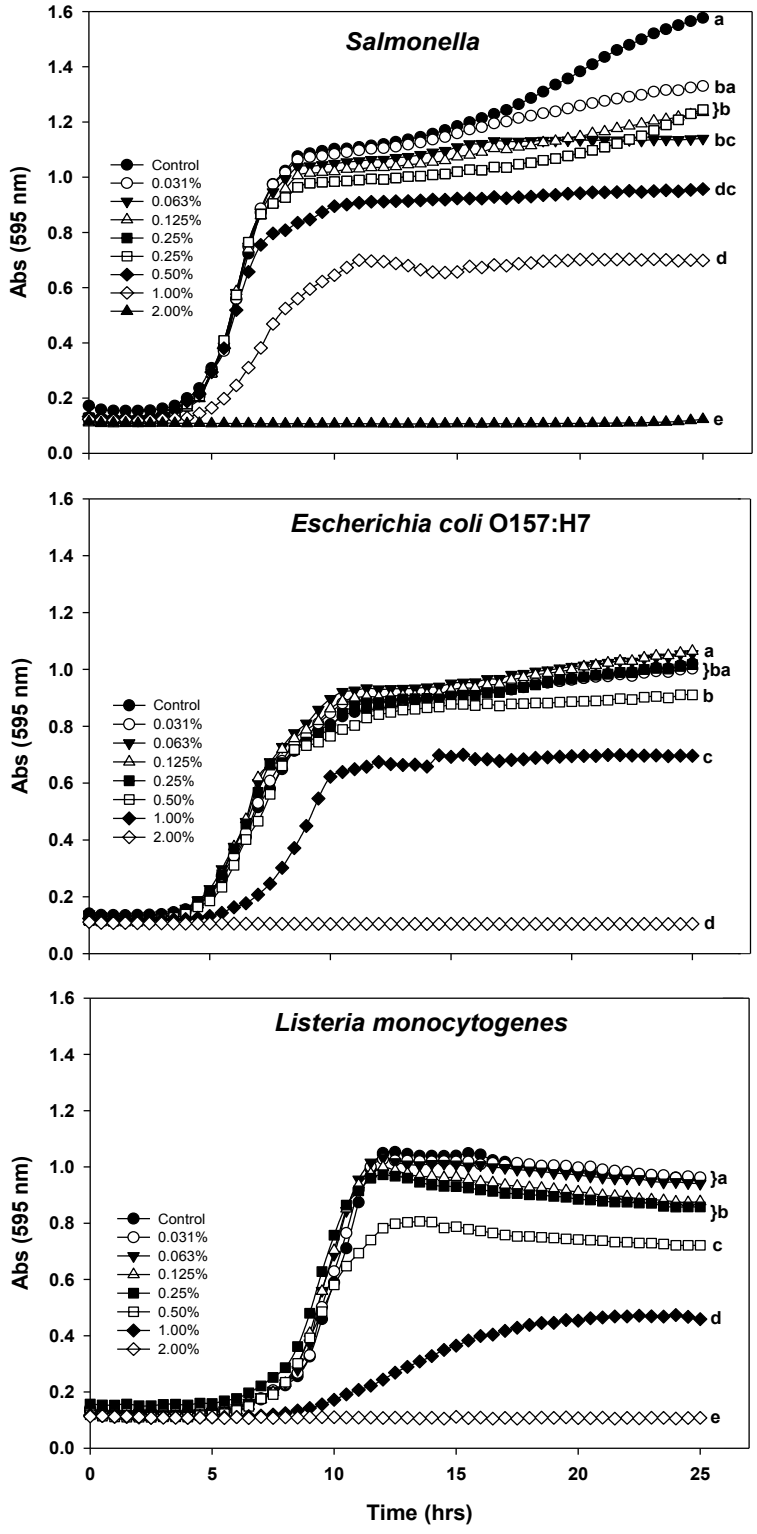

Figure 11. The effect of Syntrx on *Salmonella*, *E. coli*, and *L. monocytogenes.* Statistical significance was determined from end-point values. End point values with the same lowercase letter designation are not significantly different ($P > 0.05$).

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VITA

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Master of Science

Thesis: EVALUATION OF ANTIMICROBIALS AGAINST MULTI-STRAIN COCKTAILS OF *SALMONELLA*, *E. COLI* O157:H7 AND *LISTERIA MONOCYTOGENES* USING A KINETIC GROWTH INHIBITION ASSAY

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Title of Study: EVALUATION OF ANTIMICROBIALS AGAINST MULTI-STRAIN COCKTAILS OF *SALMONELLA*, *E. COLI* O157:H7 AND *LISTERIA MONOCYTOGENES* USING A KINETIC GROWTH INHIBITION ASSAY

Pages in Study: 78 Candidate for the Degree of Master of Science

Major Field: Food Science

Scope and Method of Study: The objective of this work was to evaluate the effect of antimicrobials that are commercially available for the food industry using a kinetic growth assay. The antimicrobials used in our study were tested to evaluate various concentrations in head-to-head trials against multi-strain cocktails of *Salmonella, E. coli* O157:H7, and *L. monocytogenes*. We also determined the Minimum Inhibitory Concentration (MIC). Mixed strains "cocktails" were prepared comprising either 6 strains of *Salmonella,* 4 strains of *E. coli* O157:H7*,* and 5 strains of *L. monocytogenes* which were separately cultured using TSB broth and used to inoculate antimicrobials performed in 96-well flat bottom microplates. The microplates were incubated using the Tecan GENious™ microplate reader using Magellan data analysis software to obtain growth curves at different concentrations of the antimicrobials. Measurements were taken as optical density (OD_{595nm}) readings every 30 minutes up to 48 hours.

Findings and Conclusions: The following antimicrobials were tested against *Salmonella, E. coli* 0157:H7, and *L. monocytogenes* with their respective results as Minimum Inhibitory Concentration (MIC): acetic acid (4%, 4% and 4%), Aftec (1.25%, 1.25% and 1.25%), AvGard XP (0.75%, 0.75% and 0.75%), citric acid (0.5%, 0.5% and 0.5%), Cytoguard (2.5%, 2.5% and 1.25%), lactic acid (0.5%, 0.5% and 0.25%), Protect M (0.07%, 0.07% and 0.04%), peroxyacetic acid (0.063%, 0.063% and 0.125%), PuraQ XTend (0.5%, 0.25% and 0.016%), and Syntrx 3300 (2%, 2% and 2%). The data identifies the MIC levels of antimicrobial that gives complete inhibition of the various pathogen cocktail mixtures. Our research suggests that, the levels of the various antimicrobials may be used to inhibit pathogen growth and be beneficial for consumers.

ADVISER'S APPROVAL: Peter M. Muriana