Evaluation of a Novel Antimicrobial Solution (AMS) with Retail Marinades on Fresh Beef

by

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ABSTRACT

Pathogenic bacteria represent a public health concern when present on meat and result in recall of product from the market. Marination of meat and antimicrobial solutions are two technologies which reduce and prevent the growth of pathogenic bacteria. A novel antimicrobial solution has been developed by researchers at the USDA Agriculture Research Service, utilizing GRAS ingredients, and has shown favorable inhibition against pathogenic bacteria when evaluated on fruit rinds and vegetable stem scars. To date, this novel antimicrobial solution has not been evaluated on meat. The objective was to evaluate, in phases, the efficacy of this novel antimicrobial solution against pathogenic bacteria of concern on beef.

Phase one was conducted in two parts. First, the survivability of pathogenic bacteria grown on different mediums was evaluated. One strain each of pathogenic *Escherichia coli* and *Listeria monocytogenes* were grown in tryptic soy broth (TSB) tubes and on plate count agar (PCA) plates. The cells were harvested and used to inoculate the surface of meat samples. The survivability of cells from the two growth mediums were compared and were found to be similar. Cultures grown on plates were more costly, requiring additional time and resources for growth and harvest. This, in addition to the similar survivability, was the basis for using broth grown cultures in future phases.

In the second part of phase one the efficacy of the antimicrobial solution (AMS) against pathogenic bacteria was evaluated. The AMS was prepared and diluted to high,

medium, and low concentrations using distilled water as the diluent and the control. Meat samples were inoculated with a cocktail of either pathogenic *Escherichia coli* O157:H7, non-O157:H7 *E. coli* (STECs), *Salmonella* spp., or *Listeria monocytogenes*, treated with the assigned treatment (antimicrobial concentration), and stored. The high, medium, and low concentration of the AMS inhibited the growth of pathogenic bacteria inoculated on the surface of fresh beef top round steaks. The inhibitory capacity of the AMS increased with increasing concentration. The medium concentration was selected for further research because it was the lowest concentration which consistently inhibited the growth of pathogenic bacteria.

Phase two evaluated the efficacy of three retail marinades available against pathogenic bacteria. Marinades were chosen based on early 2014 market and food trends and included: 1) balsamic and roasted onion, 2) lemon pepper, and 3) classic steakhouse. Distilled water was used as the control. Meat samples were inoculated as previously described, treated with the assigned marinade, and stored. All three marinades inhibited the growth of pathogenic bacteria. The lemon pepper marinade was slightly more inhibitory than the balsamic and roasted onion and the classic steakhouse marinades which had similar inhibition of growth.

Phase three evaluated the efficacy of the lemon pepper and classic steakhouse marinades combined with the medium concentration of the AMS. The AMS was prepared and diluted to the medium concentration using the marinade as the diluent. Distilled water was used as the control. Meat samples were inoculated as previously described, treated with the assigned AMS + marinade solution, and stored. Both the lemon pepper marinade solution and classic steakhouse marinade solution were more

inhibitory of the growth of pathogenic bacteria than water. The lemon pepper marinade solution and the classic steakhouse marinade solution did not differ in the inhibition of growth of pathogenic bacteria. The combination of the marinade + AMS (marinade solution) was more inhibitory against pathogenic bacteria than water or marinade alone.

Phase four evaluated the sensory and objective color of beef top round steaks marinated in water, water+ antimicrobial solution, lemon pepper marinade solution, and classic steakhouse marinade solution for 0, 6, 24, and 48 hours. Steaks were marinated in the assigned treatment for the assigned time before measuring color. Steaks were then grilled and labeled for the sensory panel. Steaks marinated in water+ solution, lemon pepper marinade solution, and classic steakhouse marinade solution received higher ratings for initial juiciness, sustained juiciness, initial tenderness, sustained tenderness, and flavor intensity compared to steaks marinated in water alone. Steaks marinated in lemon pepper marinade solution received slightly higher ratings than the other marinades. Color was altered with marination. Steaks marinated in water were the lightest in color, followed by lemon pepper marinade solution, water+ solution, and classic steakhouse marinade solution. Steaks marinated in water+ solution were the most red in color followed by classic steakhouse marinade solution, lemon pepper marinade solution, and water. Steaks marinated in the classic steakhouse marinade solution were more yellow in color than the other marinades.

This research demonstrates the antimicrobial effects of this novel antimicrobial solution (AMS), determined an optimal concentration for application (medium), and demonstrates great potential for the meat industry in phase one. Phase two demonstrates that marination of meat has the potential to improve meat safety by inhibiting the growth

of pathogenic bacteria. Phase three demonstrates the inhibitory effect of the combination of retail marinades and the AMS against pathogenic bacteria on beef. It also demonstrates that the combined marinade and AMS is more inhibitory against pathogenic bacteria than water or the marinade alone. Finally, phase four demonstrates that marination of steaks in solution with the AMS improves juiciness, tenderness, and flavor compared to marination in water alone. It also demonstrates that the AMS should be used in combination with a flavorful marinade to minimize the development of off flavors. This research, as a whole, serves as a basis for additional research of antimicrobial solutions as an ingredient in marinades to enhance meat safety, tenderness, juiciness, and flavor.

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LIST OF ABBREVIATIONS

AMS = Term to describe the novel antimicrobial solution developed by USDA researchers and investigated in this research.

APC = Aerobic Plate Count

a_w = water activity

CDC = Centers for Disease Control and Prevention

CFU = Colony Forming Units

CO_2 = Carbon Dioxide

DAEC = Diffusely Adherent *E. coli*

DNA = Deoxyribonucleic Acid

EAEC = Enteroaggregative *E. coli*

EDTA = Ethylenediamine Tetraacetic Acid

Eh = Oxidation – Reduction Potential

EHEC = Enterohemorrhagic *E. coli*

EIEC = Enteroinvasive *E. coli*

ELISA – Enzyme Linked Immunosorbent Assay

EPEC = Enteropathogenic *E. coli*

ETEC = Enterotoxigenic *E. coli*

FDA = Food and Drug Administration

FSIS = Food and Safety Inspection Service

GMP = Good Manufacturing Practices

GRAS = Generally Recognized As Safe

HACCP = Hazard Analysis and Critical Control Point model or system

HUS = Hemolytic Uremic Syndrome

IMS = Immunomagnetic Separation

IU = International Units

LAE = Lauric Arginate Ester

MAP = Modified Atmospheric Packaging

MOX = Modified Oxford Medium

MSA = MacConkey Agar with Sorbitol

mV = millivolts

NASA = National Aeronautics and Space Administration

O_2 = Oxygen

O_3 = Ozone

PCA = Plate Count Agar

PCR = Polymerase Chain Reaction

PFGE = Pulse Field Gel Electrophoresis

rpm = Rotations per Minute

RTE = Ready-To-Eat

SPC = Standard Plate Count

STEC = Shiga Toxin producing *E. coli*

*stx*1 = Shiga Toxin 1 gene

*stx*2 = Shiga Toxin 2 gene

Taq = abbreviation for *Thermus aquaticus* bacterium from which it was isolated

TE = Tris EDTA

TSB = Tryptic Soy Broth

USDA = United States Department of Agriculture

VTEC = Verotoxigenic *E. coli*

XLT4 = Xylose-Lysine Tergitol 4 agar

CHAPTER I: Review of Literature

Introduction

The food industry, especially in regards to meat products, is under constant scrutiny in developed countries due to outbreaks of foodborne illnesses. While most foodborne outbreaks cause mild to moderate illness, sometimes serious illness results in death which is of particular concern in elderly and immunocompromised individuals. Meat, poultry, milk, and eggs are the primary foods of concern leading to human illness due to undercooking or cross contamination. While foodborne pathogens are subjected to physical, chemical, and nutritional stresses during processing (Yousef & Courtney, 2003), there are still instances when pathogens survive and a person may become ill following consumption of those pathogens. *Escherichia coli* O157:H7, non-O157:H7 shiga toxin producing *E. coli* (STEC) serotypes, and *Salmonella* spp. are the main pathogens of concern for meat companies and their allied industries, research scientists, and regulatory agencies in fresh meat products. The pathogen of main concern in ready-to-eat (RTE) meat products is *Listeria monocytogenes*.

Many control measures are effective in preventing or minimizing microbial contamination of foods and inhibiting the growth of, or destroying microbial contaminants (Marth, 1998). It is necessary for food processors to have dynamic programs in place to lessen the risk of microbial contaminants. One example of programs that can be effective in lessening those risks are good manufacturing practices (GMPs). One important concept in GMPs begins with the selection of high quality raw materials with low levels of microorganisms, particularly psychrotrophs. Hygienic conditions must be maintained during food processing. Food processing equipment design is also a consideration for GMPs. Equipment should be designed and constructed so that it: 1) is

inert to the product, 2) has smooth and nonporous product-contact surfaces, 3) is readily accessible for cleaning and inspection, 4) is self-emptying or self-draining, 5) has covers to prevent external contamination, and 6) has readily cleanable surfaces that do not contact the product and do not harbor contaminants (Marth, 1998). Equipment must be cleaned as necessary according to a sanitation schedule and sanitation standard operating procedures to prevent the development of a biofilm (Marth, 1998). Airborne contaminants are reduced by filtration of air entering food processing areas (Marth, 1998). Caution should be exerted with air conditioning systems to ensure the condensate drains properly and does not contaminate the product (Marth, 1998). Personnel must practice good hygiene and should not be permitted to move from raw product areas to finished product areas (Marth, 1998).

Processors are required to determine the potential microbiological hazards of ingredients, materials, and processes utilizing the Hazard Analysis and Critical Control Point (HACCP) model and microbiological specifications to minimize risk (Scallan, 2011). GMPs, sanitation, and hygiene are prerequisite programs for the implementation of an effective Hazard Analysis and Critical Control Point (HACCP) system which is the highest level of food safety assurance. HACCP was implemented in 1996 and was originally developed for National Aeronautics and Space Administration (NASA) to ensure a safe food supply for astronauts. HACCP is a systematic, preventative approach to food safety for the identification, evaluation, and control of physical, chemical, and biological hazards from raw material production to distribution and consumption of the finished product. The 7 principles of HACCP are: 1) conduct a hazard analysis, 2) determine critical control points, 3) establish critical limits, 4) establish monitoring

procedures, 5) establish corrective actions, 6) establish verification, and 7) establish record – keeping and documentation procedures. GMPs and HACCP have improved food safety but rely heavily upon individuals to maintain protocols. Development of new technologies to inhibit the growth of pathogenic bacteria is a dynamic field.

Hurdle technology is the concept of combining several factors at subinhibitory levels which effectively control microorganisms in refrigerated foods (Leistner & Gorris, 1995). Common hurdles include refrigeration, modified atmospheric packaging (MAP), heat treatment, water activity, and pH alterations. These and additional hurdles will be discussed in more detail in the following sections. When hurdles are used in combination, a synergism occurs, enabling use of lower quantities of each factor than is necessary when used singly.

Intrinsic Factors Affecting Bacteria

Water Activity

Microorganisms require water to grow in food products, a concept generally defined as the water activity (a_w) of foods. Water activity is defined as the ratio of the water vapor pressure of the food substrate to the vapor pressure of pure water at the same temperature (Christian, 1963, 1980; Christian & Scott, 1953; Christian & Waltho, 1966; Jay, 2006). In a more simplistic definition, a_w is the amount of "free" or "unbound" water which is available to microorganisms for chemical and biochemical reactions facilitating microbial growth (Jay, 2006; Leistner & Rodel, 1976). The a_w of pure water is 1.0 and most fresh foods have a a_w of >0.98 (Leistner & Rodel, 1976; Nester, 2001; Sperber, 1983). The a_w of foods can be altered by addition of salt or sugar, binding unbound water,

or removing unbound water through cooking, baking, or dehydrating the food. Most microorganisms require a a_w >0.90, although various classifications of bacteria may be more or less sensitive to changes in a_w (Nester, 2001). Generally, Gram negative bacteria are more sensitive than Gram positive bacteria to changes in a_w (Nester, 2001; Sperber, 1983). Slight changes in a_w can have profound or minimal affects on bacterial growth (Christian, 1963, 1980; Christian & Scott, 1953; Christian & Waltho, 1966; Gill & Newton, 1978; Sperber, 1983; Troller, 1986a, 1986b). Required a_w for select pathogens is presented in Table 1.

pH

The organisms which can survive on the surface of or within a food are often affected by the pH of the food (Nester, 2001). Although some exceptions exist, microorganisms generally do not grow below a pH of 4.6 (Chung & Goepfert, 1970; Jay, 2006). Lower pH foods, or foods of a more acidic nature, are less hospitable to bacterial growth. The pH of normal meat is between 5.5 and 5.7, yet pH differences exist between carcasses and different muscles within the carcass (Gill & Newton, 1978). Fermentation and addition of weak acids which decrease the pH, or increase the acidity, have been utilized as effective food preservation techniques for hundreds of years (Jay, 2006). The buffering capacity of meat, created by the protein content of the meat, allows it to resist pH changes better than other foods (Jay, 2006).

Nutrient Availability

Microorganisms require five nutrients to grow and maintain normal function; these nutrients, in order of importance, are: water, an energy source, a nitrogen source, vitamins and related growth factors, and minerals (Baron, Gautier, & Brule, 1997; Jay, 2006). If an organism requires a vitamin which it cannot synthesize, its growth may be impaired (Nester, 2001). Gram negative bacteria tend to have more stringent nutrient requirements than do Gram positive bacteria (Jay, 2006; Nester, 2001). Thus, Gram negative bacteria must be supplemented or receive nutrients from the food environment in order to stimulate growth (Jay, 2006). Nutrients vary greatly between different types of foods. The major energy sources include sugars, alcohols, and amino acids, although some microorganisms can utilize complex carbohydrates such as starch and cellulose as energy sources by degrading them to simple sugars (Jay, 2006; Nester, 2001). Very few microorganisms can utilize fat as an energy source (Jay, 2006). Amino acids are also one of the primary sources of nitrogen for microorganisms although a variety of other sources may be utilized (Jay, 2006; Nester, 2001).

Biological Structures

Some foods have inherent biological structures which protect them from entry and subsequent growth of pathogenic microorganisms. Such biological structures include the shell of eggs, rinds of fruit, hide or feathers of animals, and other outer coverings which form a protective layer (Baron et al., 1997; Jay, 2006). Once these structures have been damaged; however, pathogenic microorganisms may enter and grow as the protective layer is no longer functional (Jay, 2006). In meat animals and meat products, this protective structure exists in multiple forms. The hide of the animal serves as the external

protective layer which prevents entry of pathogenic bacteria into the muscle of the animal. Much as the skin protects humans from injury, so does the hide of the animal. Similarly, the outer portion of an intact muscle protects the inner portion from pathogenic contamination (Jay, 2006). In this instance, the surface of the muscle may become contaminated while the innermost portions remain sterile. The skin or hide barrier is broken when the animal is harvested and the hide is removed. The barriers within the muscle are destroyed when the meat is cut, chopped, or ground. These actions eliminate an inner and outer portion of the muscle, thus allowing the entry of bacteria into the interior portions of the meat (Jay, 2006).

Oxidation-Reduction Potential

The oxidation – reduction potential (Eh) is defined as the ease with which a substrate can gain (reduction) or lose (oxidation) electrons (Brown, 1980; Jay, 2006). Aerobic organisms require positive Eh values (oxidized), anaerobic organisms require more negative Eh values (reduced), while facultative microorganisms can survive and grow in both conditions (Jay, 2006; Walden & Hentges, 1975). Post rigor muscle has an Eh of -60 to -150 mV while cooked sausages and canned meat have an Eh of -20 to -150 mV (Jay, 2006). Specific Eh values vary depending upon the type of meat, cookery method, and ingredients. The Eh values for *E. coli, Salmonella,* and *Listeria* are broadly classified as being between -42 and -350 mV (Bagramyan, Galstyan, & Trchounian, 2000; Jay, 2006).

Antimicrobials

Antimicrobials may occur naturally in foods. One example is lysozyme which is naturally found in egg whites (Nester, 2001). Basil is a popular culinary herb which exhibits natural antimicrobial activity against bacteria (Arfat, Benjakul, Prodpran, Sumpavapol, & Songtipya, 2014; Koba, Poutouli, Raynaud, Chaumont, & Sanda, 2009; Suppakul, Miltz, Sonneveld, & Bigger, 2003; Synowiec et al., 2014). Many other culinary compounds have also been identified as having antimicrobial activity. Oregano and its essential oils, specifically carvacrol, are another example of an herb which exhibits antimicrobial activity against bacteria (Tajkarimi, Ibrahim, & Cliver, 2010). Soy sauce (Kataoka, 2005), red wine (Fernandes, Gomes, Couto, & Hogg, 2007; Vaquero, Alberto, & de Nadra, 2007), garlic and onion (Benkeblia, 2004), black pepper (Zarai, Boujelbene, Ben Salem, Gargouri, & Sayari, 2013), and olive extracts (Techathuvanan, Reyes, David, & Davidson, 2014) have all exhibited naturally occurring antimicrobial properties.

Wood smoke is considered a natural antimicrobial and is commonly used to add flavor and color to products (Gedela, Escoubas, & Muriana, 2007; Gedela, Gamble, Macwana, Escoubas, & Muriana, 2007; Holley & Patel, 2005; Sunen, Fernandez-Galian, & Aristimuno, 2001; Vitt, Himelbloom, & Crapo, 2001). The antimicrobial properties of wood smoke are due to naturally present phenols and carbonyl compounds (Holley & Patel, 2005; Sunen et al., 2001; Vitt et al., 2001). The phenols of wood smoke contribute to flavor and aroma of the product while the carbonyls primarily affect color. Organic acids present in wood smoke provide a preservation effect, help skin formation through coagulation of surface proteins, contribute to color, have antimicrobial properties, and accelerate the cure reaction.

Commercial liquid smoke products, such as Zesti Smoke offered by Kerry Ingredients and Flavors (Beloit, WI), are unique water-soluble combinations of natural smoke extracts with listeriostatic properties (Morey, Bratcher, Singh, & McKee, 2012). These are a result of a wood smoke being cooled and condensed to form a liquid. Liquid smoke products possess the same characteristics as wood smoke, only they are in a liquid form which is easily sprayed onto the surface of products to impart flavor, color, and aroma.

Liquid smoke products, in addition to wood smoke, can be included on labels as a natural extract, thus meeting current market trends for clean labeling. Additionally, liquid smoke can be included in the product formulation as an ingredient during batter mixing or can be added through a surface application during post-thermal processing. Both formulation and surface applications can reduce or eliminate *Listeria* while simultaneously imparting color and flavor to the final product (Gedela et al., 2007; Gedela et al., 2007; Morey et al., 2012; Vitt et al., 2001). Caution should be exerted when incorporating liquid smoke into the product formulation as the pH of the meat batter system will be lowered and meat emulsion, texture, and quality of the product are likely to be affected (Faith, Yousef, & Luchansky, 1992; Gedela et al., 2007; Gedela et al., 2007; Morey et al., 2012). Surface application to meat products requires additional equipment and drying time. A previous study found that liquid smoke suppressed growth of *L. monocytogenes* on frankfurters during storage, although no listeriocidal properties were observed (Morey et al., 2012). Researchers obtained frankfurters from Kelley Foods (Elba, AL) manufactured to contain 0, 2.5, 5, or 10% liquid smoke wt/wt. Two inoculation concentrations (high and low) were selected, sprayed onto the surface of

frankfurters, hand massaged for 15 seconds to evenly distribute inoculum, and vacuum packaged. Frankfurters were then stored at 4°C for up to 12 weeks. Listeriostatic activity increased with increasing concentrations of liquid smoke throughout the storage period (Morey et al., 2012).

Extrinsic Factors Affecting Bacteria

Storage Temperatures

Microorganisms have a specific range of temperatures for optimal growth, a selection of which can be found in Table 1. There are four classifications of microorganisms based on the optimal temperature range for growth: psychrotrophs, psychrophiles, mesophiles, and thermophiles (Jay, 2006). Psychrotrophs and mesophiles are of primary concern relating to foodborne illnesses. Psychrotrophs are bacteria, yeasts, and molds which grow, at a reduced rate, at refrigeration temperatures of less than 7°C (Marth, 1998; Ratkowsky, Olley, Mcmeekin, & Ball, 1982). Although psychrotrophs grow at refrigeration temperatures, the optimum temperature range for growth is above refrigeration temperatures, in the range of 25-30°C (Marth, 1998). Mesophilic pathogens may survive refrigeration temperatures and grow during temperature abuse of foods (Marth, 1998). Mesophiles may grow in a temperature range of 20-45°C, with optimum temperatures between 30-40°C (Jay, 2006; Ratkowsky et al., 1982). Nearly all human pathogens are included in the mesophilic bacteria classification. Storage temperatures should be determined with the quality of the food in mind (Jay, 2006). Mesophilic bacteria are inhibited by cold temperature storage; however, cold storage temperatures

facilitate growth of psychrotrophic organisms. Slight temperature changes can alter the microbial profile of meat (Sun & Holley, 2012).

The lag phase of microbial growth, that phase during which there is no increase or decrease in microbial numbers, and the generation time, the duration between formation of a daughter cell and its division into two new cells, increase with decreasing refrigeration temperature (Marth, 1998). Product shelf life at specific temperatures should be established and monitored to manage food safety and quality. Potential for temperature abuse of food products exists during handling, thus temperature indicators may be useful in determining when refrigeration temperatures or intended storage time have been exceeded (Marth, 1998). The recommended storage temperature for meat and poultry products is at or below 4°C because low temperatures affect membrane permeability, reduce nutrient uptake, protein synthesis, and enzyme functionality, all of which contribute to reduced pathogen growth (Graumann & Marahiel, 1999).

In addition to storage temperature, processing temperature is also important. Microbial population on foods is reduced by heating, one element of hurdle technology. The degree of reduction of microbial population is dependent upon the magnitude of the heat treatment, namely time and temperature (Marth, 1998). Pasteurization, which destroys vegetative pathogenic cells, is the commonly used magnitude of heating, as opposed to sterilizing (Marth, 1998). However, if food products are handled after a post-lethality treatment such as heating, the potential for microbial contamination of the food exists. For example, deli meats are fully cooked RTE meat products, yet slicing of these deli meats in grocery stores represents a potential for contamination of the food by microorganisms. Sterilized products, milk for example, must be aseptically handled

following the sterilization process to prevent recontamination of the product by microorganisms. Fresh meat products are most likely to be exposed to temperature abuse during shipping and handling and are more likely to be cooked to internal temperatures which are insufficient to kill pathogenic bacteria. Numerous factors influence the growth, or lack thereof, of microorganisms in food; some factors pertain to the environment in which the food is stored while others pertain to the food itself.

Atmospheric Composition of Packaging

Carbon dioxide (CO_2), ozone (O_3), and oxygen (O_2) are inhibitory to growth of certain pathogens and incorporating these gases into packaging of food provides an antimicrobial effect (Clark & Lentz, 1973; Gill & Newton, 1978; Nester, 2001; Stier et al., 1981). Modified atmospheric packaging (MAP) is one technology which controls the atmosphere within the packaging of meat and extends product shelf life by decreasing oxygen and/or increasing gases, such as carbon dioxide (Brody, 1996; Gill & Newton, 1978; Marth, 1998; Nester, 2001). MAP inhibits growth of aerobic spoilage microorganisms, such as *Pseudomonas* species, but permits growth of facultative anaerobes, such as lactic acid bacteria, in the food environment (Brody, 1996; Marth, 1998; Stier et al., 1981).

MAP, in combination with aseptic packaging, has experienced considerable growth in the minimally processed refrigerated foods sector (Brody, 1996; Clark & Lentz, 1973; Marth, 1998). Despite the benefits of MAP packaging, some risks still exist. Facultatively anaerobic organisms are capable of anaerobic respiration if oxygen is absent though they can utilize aerobic respiration in the presence of oxygen. Examples of

facultatively anaerobic organisms include *Listeria* spp. and *E. coli*. Anaerobic organisms are those organisms which do not require oxygen for growth. Examples of anaerobic organisms include *Clostridium* and *Propionibacterium.* Both facultatively anaerobic and anaerobic psychrotrophic pathogens may be able to grow until lactic acid bacteria sufficiently reduce the pH of the product to inhibitory levels (Brody, 1996; Marth, 1998; Nester, 2001). Further, growth of lactic acid bacteria may not coincide with overt evidence of spoilage (Clark & Lentz, 1973; Marth, 1998). Another technique to control atmospheric composition within packaging is vacuum packaging which restricts O_2 levels and allows for levels of approximately 20% CO_2 which inhibits growth of Gram negative aerobes (Gill & Newton, 1978).

Pathogens of Concern

Meat is one food source which has been linked with outbreaks of *E. coli*, *Salmonella*, and *Listeria*. Most illnesses associated with meat are due to consumption of under cooked meat or cross contamination in the home preparation of raw meats with ready to eat foods (Soon, Chadd, & Baines, 2011). It is reasonable to assume that, if foods were prepared in such a manner as to prevent cross contamination and meat were cooked to the appropriate internal temperature, many foodborne illness outbreaks would be eliminated. Strains of pathogens of concern are outlined in Table 2.

Pathogenic *Escherichia coli*

Escherichia coli (*E. coli*) is a Gram negative non-spore forming rod in the *Enterobacteriaceae* family. It is a facultative anaerobe which is part of the normal

microflora of the intestinal tract of most warm-blooded mammals, including humans (Marth, 1998). There are both pathogenic and non-pathogenic strains of *E. coli*, though most are harmless to the human and animal population. A primary function of *E. coli* in the gut microflora of warm-blooded mammals is to inhibit other pathogenic bacteria. Not considered to be true psychrotrophs, some strains of *E. coli* can grow at 6.9°C and below (Palumbo, Lee, & Boerman, 1994). Pathogenic strains are categorized into six groups: enteropathogenic (EPEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroaggregative (EAEC), and diffusely adherent (DAEC).

As few as 10 to 100 cells are needed as an infectious dose of EHEC with symptoms including nausea, abdominal cramps, vomiting, and watery or bloody diarrhea (Nester, 2001). The duration of the incubation period may last anywhere from 2 hours to 6 days (Nester, 2001). In extreme instances, hemorrhagic colitis can progress to hemolytic uremic syndrome (HUS). Six serovars of EHEC are pathogenic, making this classification the most commonly associated with foodborne outbreaks. Foods involved in pathogenic *E. coli* outbreaks include meat, poultry, fish, vegetables, apple cider, raw milk, Brie and Camembert cheese, water, and radish and alfalfa sprouts (Brooks et al., 2005). Some strains of *E. coli* are more tolerant of acidic environments, a growth phase dependent and inducible phenomenon, which may persist for extended periods of refrigeration (Marth, 1998).

In 2011 it was estimated that nearly 64,000 cases of O157:H7 EHEC and 113,000 cases of non-O157:H7 EHEC infections occurred on an annual basis (Scallan, 2011). Nearly 75% of all pathogenic *E. coli* related foodborne outbreaks worldwide are due to O157:H7; however, the United States is testing for the presence of non-O157:H7

pathogenic *E. coli* serotypes due to their association with human illness. Verotoxigenic *E. coli* (VTEC) and shiga toxin producing *E. coli* (STEC) are interchangeably used to describe the group of serotypes most commonly linked to human illness; the interchangeability of the terms is related to cellular cytotoxin production (Mathusa, Chen, Enache, & Hontz, 2010). So termed the "big six" non-O157:H7 serotypes are O26, O45, O103, O111, O121, and O145 (Brooks et al., 2005). These "big six" STECs account for approximately 70% of the non-O157:H7 infections, making them of greater importance than O157:H7 (Brooks et al., 2005). Because of the importance of the STEC strains in causing human illness, USDA has placed the big six on the zero tolerance adulterant list.

Growth of *E. coli* can be driven by aerobic or anaerobic respiration as it is a facultative anaerobic microorganism. It can survive in a wide variety of substrates. *E. coli* growth utilizes a variety of redox pairs, including oxidation of pyruvic acid, formic acid, hydrogen, and amino acids, and the reduction of substrates including oxygen, nitrate, fumarate, and dimethyl sulfoxide. Contaminated foods, especially undercooked ground beef and unpasteurized milk and juice, are common sources for infection. Most individuals will recover after 6-8 days of symptoms though the illness can last 5-10 days.

Control of pathogenic *E. coli* can be achieved by reducing water activity and lowering the pH of the substrate below the intracellular pH thereby disrupting the cell membrane (Chung, & Murdock, 1991; Morey et al., 2012). Membrane disruption may also lead to disturbance of vital cell functions and lead to cell lysis, also controlling growth of pathogenic *E. coli* (Rodriguez, Seguer, Rocabayera, & Manresa, 2004; Sharma & Beuchat, 2004). Cell membrane disruption may be achieved through association between positively charged amino groups and negatively charged anions on the surface of

the bacteria (Friedman & Juneja, 2010). Inhibition of pathogenic bacteria growth may be accomplished through interaction of anionic constituents of the negatively charged cell wall, resulting in rapid efflux of cytoplasmic constituents (Henning, 1986).

***Salmonella* spp.**

Salmonella are Gram negative, non-spore forming, motile rod-shaped, heterotrophic, mesophilic bacteria which belong to the *Enterobacteriaceae* family. These organisms grow well at 37°C and are able to utilize D-glucose as a carbohydrate source, generating organic acids and gas as byproducts (Li, 2013). Some strains are capable of reproduction at temperatures in excess of 54°C (Droffner & Yamamoto, 1992) while others can grow at refrigeration temperatures of 2-4°C (D'Aoust, 1991a, 1991b). *Salmonella* are commonly present in the environment and in the intestinal tract of warm-blooded animals. *Salmonella* are an important human pathogen associated with poultry and poultry products (Bryan, 1995). There are more than 2,400 serovars currently recognized as members of the *Salmonella* family (Popoff, Bockemuhl, & Brenner, 1998). While not all serovars are pathogenic, some can cause serious illness in humans, primarily the young, the elderly, and the immunocompromised. Enteritidis and Typhimurium serotypes are commonly identified subspecies within *S. enterica*. Nontyphoidal salmonellosis and typhoid fever, caused by *S.* Typhi or *S.* Paratyphi A., are the illnesses caused by *Salmonella* infection. These serotypes are only found in human hosts.

Human infection can lead to typhoid fever, entercolitis and systemic infections by nontyphoidal *Salmonellae* (D'Aoust, 1991a, 1991b). A 6 to 72 hour incubation period is

required in foodborne cases prior to expression of symptoms (Mccullough & Eisele, 1951). Salmonellosis symptoms include diarrhea, vomiting, prolonged fever, headache, abdominal pain, abscesses, shock, and an overall feeling of exhaustion (D'Aoust, 1991a, 1991b; Nester, 2001). Most salmonellosis cases are self-limiting with symptoms fading after several days. Diagnosis is carried out through isolation from a stool or blood sample.

Poultry, eggs, meat, meat products, peanut butter, cocoa, and produce have been identified as vehicles of salmonellosis (Mishu et al., 1994). The fat content of the food is important in the degree of clinical manifestation such that a lower infectious dose is associated with a higher fat content food (D'Aoust, 1989). Contamination of meat and poultry with *Salmonella* is of critical importance to these industries as contamination leads to recalls of product. Advances have been made in thermal intervention technologies which reduce *Salmonella* spp. in meat and poultry products, yet the ability of certain strains to survive and grow at temperatures of up to 54°C, survive in foods stored at 2-4°C, and grow over a wide pH range of 4.5 to 9.5 (D'Aoust, 1991a, 1991b) remains a concern during food storage, distribution, and preparation. Processing and storage temperatures are equally important in reducing instances of foodborne illness. The ability of pathogenic microorganisms to grow in the same temperature range as that in which processing and storage of foods occurs is a unique challenge for the meat industry and for consumers.

Risk of contracting *Salmonella* is increased with travel to countries with poor sanitation. The infectious dose of *Salmonella* spp. is higher than that of *E. coli* though it is dependent upon the age and health status of the individual. Most illnesses last 4-7 days

and often individuals do not require treatment. Control of pathogenic *Salmonella* can be achieved by reducing water activity and lowering the pH of the substrate below the intracellular pH thereby disrupting the cell membrane (Chung, & Murdock, 1991; Morey et al., 2012). Membrane disruption may also lead to disturbance of vital cell functions and lead to cell lysis, also controlling growth of pathogenic *Salmonella* (Rodriguez et al., 2004; Sharma & Beuchat, 2004). Cell membrane disruption may be achieved through association between positively charged amino groups and negatively charged anions on the surface of the bacteria (Friedman & Juneja, 2010). Inhibition of pathogenic bacteria growth may be accomplished through interaction of anionic constituents of the negatively charged cell wall, resulting in rapid efflux of cytoplasmic constituents (Henning, 1986).

Listeria monocytogenes

Listeria monocytogenes is a Gram positive, motile, rod-shaped, facultative anaerobe which is ubiquitous in the environment (Marth, 1998). Thirteen serotypes have been identified, though only three have been associated with the majority of foodborne illnesses. *L. monocytogenes* has been isolated from soil, silage, food processing environments, and healthy humans and animals and is known to be salt and cold tolerant (Scallan, 2011). *L. monocytogenes,* though not a leading cause of foodborne illness, is a leading cause of death from foodborne illness (Scallan, 2011). The CDC estimates 255 deaths resulting from 1,591 cases of foodborne illness due to *L. monocytogenes* (Scallan, 2011). The infectious dose is not known, although estimations indicate it to be fewer than 1,000 cells (Scallan, 2011).

Risk of contracting listeriosis is increased in young children, the elderly, and any individual with a compromised immune system (Marth, 1998; Morey et al., 2012). Symptoms generally include fever, muscle aches, diarrhea, and other gastrointestinal issues. Pregnant women are susceptible to spontaneous abortions and stillbirths in approximately one third of cases (Scallan, 2011). A variety of foods have been recalled from the market due to contamination by *L. monocytogenes,* though the most commonly associated foods are refrigerated ready-to-eat (RTE) foods (Mbandi & Shelef, 2002; Ryser & Marth, 1988, 1989).

A unique problem for the food industry is the ability of *L. monocytogenes* to grow and thrive at refrigeration temperatures. The unique ability of *L. monocytogenes* to thrive at refrigeration temperatures along with its high mortality rate have resulted in the USDA setting a "zero tolerance" policy for *L. monocytogenes* in RTE foods. Listeriosis incidences are caused by consumption of foods contaminated with *Listeria* and have been associated with coleslaw, soft Mexican-style cheese, milk, meat, poultry, meat sandwiches, meat salads, and many other refrigerated RTE foods (Mbandi & Shelef, 2001, 2002; Ryser & Marth, 1988, 1989). RTE lunch meats are an area of particular concern, as sandwiches featuring RTE lunch meats are often a quick and simple lunch option for children, the elderly, and working pregnant women.

RTE foods are contaminated with *L. monocytogenes* mainly during post-processing handling and further thermal applications are often not applied to these foods (Mbandi & Shelef, 2002). Despite containing sodium chloride, nitrites, and nitrates, growth of *L. monocytogenes* is not inhibited during storage in refrigerated temperatures (Mbandi & Shelef, 2002). Prevention of listeriosis requires application of intervention

strategies at all stages of the food chain, beginning with the processing facility and progressing to the consumer's home (Lianou et al., 2007). Evidence suggests the use of topical treatments such as lactic acid, acetic acid, organic acids, chitosan, nisin, and lauric arginate ester are effective against these pathogens (Avery, 1997; Gao, Zhu, & Zhang, 2013; Guo, Jin, Wang, Scullen, & Sommers, 2014; Huffman, 2002; Mani-Lopez, 2012; Mattick & Hirsch, 1947; Podolak, 1995a, 1995b; Ruckman, Rocabayera, Borzelleca, & Sandusky, 2004; Theron, 2007; Yoder et al., 2012).

Control of *Listeria monocytogenes* can be achieved by reducing water activity and lowering the pH of the substrate below the intracellular pH thereby disrupting the cell membrane (Chung, & Murdock, 1991; Morey et al., 2012). Membrane disruption may also lead to disturbance of vital cell functions and lead to cell lysis, also controlling growth of pathogenic *L. monocytogenes* (Rodriguez et al., 2004; Sharma & Beuchat, 2004). Cell membrane disruption may be achieved through association between positively charged amino groups and negatively charged anions on the surface of the bacteria (Friedman & Juneja, 2010). Inhibition of pathogenic bacteria growth may be accomplished through interaction of anionic constituents of the negatively charged cell wall, resulting in rapid efflux of cytoplasmic constituents (Henning, 1986).

Common Topical Treatments

Lactic Acid

Lactic acid is an organic acid that is "Generally Recognized As Safe" (GRAS) as a food additive and is commonly used in the meat industry. Lactic acid has been used as a hot carcass rinse on abattoirs (Huffman, 2002). At concentrations of 1-2% lactic acid has

been used to decontaminate red meat carcasses without negatively impacting meat quality (Theron, 2007). High temperature (>60°C) application of lactic acid has proven to effectively control pathogenic bacteria populations on carcasses (Theron, 2007). Lactic acid and other organic acids elicit bactericidal and bacteriostatic affects by reducing the pH of the substrate to a lower level than the internal cellular pH, thus disrupting the cell membrane (Chung, & Murdock, 1991).

Post-processing spray and dip applications of lactic acid have effectively reduced *L. monocytogenes* (Theron, 2007). Additionally, a 2% solution of lactic acid has been proven to reduce *E. coli* O157:H7 and *Salmonella* Typhimurium (Yoder et al., 2012). The acceptable limit for the use of lactic acid in products may exceed 4% without negative organoleptic consequences; however, the buffering capacity of the meat system will likely be reduced due to the acidic pH of lactic acid (Gill & Badoni, 2004). A spray application of a 4% lactic acid solution effectively reduced both non-O157:H7 and O157:H7 on inoculated beef flanks (Kalchayanand et al., 2012). Concentration and temperature of lactic acid influences the effectiveness at inhibiting growth of pathogens of concern. Research has been conducted to identify the optimal concentration and temperature of lactic acid for application to meats (DeGeer, 2014). This research found 1, 2, 3, and 4 % lactic acid inhibited growth of pathogenic bacteria on fresh beef. Additionally, the researchers reported 2, 3, 4, and 5% sodium metasilicate inhibited pathogenic bacteria growth.

Acetic Acid

Acetic acid, among other organic acids, is utilized in dairy and meat products to target yeast and bacteria growth (Mani-Lopez, 2012). It has also achieved GRAS status. The pungent odor and flavor of acetic acid, the primary component of vinegar, limits its application in many foods (Mani-Lopez, 2012). Applications for acetic acid in foods must grant consideration to the potential for off odors and flavors in the final product. Many pickled products include acetic acid (Mani-Lopez, 2012). Acetic acid has been shown to reduce growth of pathogenic bacteria on lean beef muscle over time (Podolak, 1995a, 1995b). *Escherichia coli* O157:H7 and *Listeria monocytogenes* were inoculated onto the surface of lean beef and then sanitized with fumaric, acetic, or lactic acid alone and in combined solutions of those acids at 55°C for 5 seconds. A 1% concentration of fumaric acid was the most effective acid in reducing the *L. monocytogenes* and *E. coli* O157:H7 populations. The researchers ranked the order of acids tested against the growth of *L. monocytogenes* and *E. coli* O157:H7 and reported fumaric acid as the most effective followed by lactic and acetic acids. Fumaric acid at concentrations of 1% and 1.5% was reported to be more effective than any of the combined solutions of acids. Acetic acid and other organic acids, are known to be more effective inhibitors of pathogen growth than hot water, though the discoloration and off odor properties associated with acetic acid in particular will determine the concentration(s) which should be utilized (Sun & Holley, 2012). Researchers reported a reduction of surface shininess resulting from including sodium acetate injected at 0.1%, which made beef steaks treated with 1.5% potassium lactate more attractive to consumers because steaks had a better "fresh beef" appearance (Knock et al., 2006a, 2006b).

Emerging Topical Treatments

Lauric Arginate Ester

Lauric arginate ester (LAE), classified as GRAS by USDA, is verified nontoxic and is metabolized to naturally occurring amino acids, primarily arginine and ornithine, following consumption (Ruckman et al., 2004). It is a cationic preservative which is derived from lauric acid, L-arginine, and ethanol (Kang et al., 2014; Ruckman et al., 2004). LAE is a surfactant typically used in food manufacturing as a processing aid; it also has antimicrobial properties in foods (Martin et al., 2009). It is believed that the antimicrobial action of LAE originates from its ability to cause membrane disruption and disturbance of vital cell functions (Rodriguez et al., 2004). Known as a potent antimicrobial agent in foods, LAE tends to concentrate in the aqueous phase of products because of its low oil-water interaction equilibrium (Bakal, 2005).

One study conducted *L. monocytogenes* challenge trials in brain heart infusion and on salmon disks that were supplemented with bactericidal compounds nisin, lauric arginate, epsilon-polylysine, and chitosan (Kang et al., 2014). Researchers reported varying degrees of effects; however, nisin decreased initial *L. monocytogenes* populations on salmon compared to control. Other researchers sought to validate combinations of antimicrobials that would produce an immediate lethality of at least 1 log of *L. monocytogenes* on artificially contaminated frankfurters, and suppress growth to less than 2 logs throughout the extended shelf life at refrigerated temperatures (Martin et al., 2009). These researchers reported 22 ppm LAE gave more than a 1 log reduction of *L. monocytogenes* inoculated frankfurters within 12 hours. The combination of potassium

lactate or sodium diacetates with 22 ppm LAE caused more than a 2 log reduction at 12 hours.

Chitosan

Chitin is the major constituent of the exoskeletons of crustaceans. Chitosan is a natural polymer which is obtained by deacetylation of chitin. Chitosan has been verified nontoxic, biodegradable, and biocompatible (Guo et al., 2014). Although the antimicrobial mechanism of chitosan remains unclear, it is thought to involve disruption of the cell membrane as a result of the association between positively charged amino groups of chitosan and negatively charged anions on the surface of bacteria (Friedman & Juneja, 2010). Broad application potential exists for chitosan in the meat industry, particularly as an edible coating and as an ingredient for antimicrobial solutions (Gao et al., 2013). Chitosan coatings create a semi-permeable barrier which may reduce loss of moisture and alter gas exchange, reducing respiration and inhibiting microbial decay (Gao et al., 2013).

In one study, freshly harvested grapes were treated with chitosan, glucose, chitosan-glucose complex, or water (control) for up to 60 days at 0°C followed by 3 days in the air at 20°C (Gao et al., 2013). The researchers reported coated samples were effective in terms of inhibition and postharvest disease prevention with chitosan-glucose complex showing better effects compared to pure chitosan or glucose. In addition, the complex coating treatment ensured better berry texture and higher sensory scores, compared with those treated with chitosan or glucose alone. Another study evaluated edible antimicrobial coating solutions incorporating chitosan, lauric arginate ester (LAE)

and nisin (Guo et al., 2014). Deli meat samples were directly coated with the solutions, or treated with solution-coated polylactic acid films. Antimicrobial coatings containing 1.94 mg/cm^2 of chitosan and 0.388 mg/cm^2 of LAE reduced *L. innocua* by 4.5 log CFU/cm^2.

Nisin

Bacteriocins are proteins produced by certain bacteria and are known to have antimicrobial properties against other bacteria (Marth, 1998). Nisin is a particularly well known bacteriocin which is produced by certain strains of *Lactobacillus lactis* subspecies *lactis*. Nisin and salts of organic acids inhibit pathogens and extend the shelf-life of shrimp when used in dip treatments (Al-Dagal & Bazaraa, 1999). It is a reasonable assumption that the inhibitory effects of nisin and salts of organic acids may also be observed in red meat and poultry. Nisin is generally recognized as safe (GRAS) by the United States Food and Drug Administration (FDA) and has been used as a food preservative since the 1940s (Mattick & Hirsch, 1947). The effects of nisin have been well studied and it has been found to be effective against a wide range of Gram positive bacteria, including *L. monocytogenes* and spore formers, as well as Gram negative bacteria in combination with food grade chelators such as ethylenediamine tetraacetic acid (EDTA) (Delves-Broughton, Blackburn, Evans, & Hugenholtz, 1996). Nisin has been approved as a food preservative in more than 50 countries (Surekha, 2000). Potassium sorbates, sodium benzoate, and sodium diacetates are examples of salts of organic acids which act as antimicrobials in food (FDA, 2014; Thomas, 2000). Such salts of organic acids are available commercially, are inexpensive, and are widely approved food additives (Surekha, 2000). Studies on the application of nisin and/or salts of organic

acids have been conducted for decades on dairy and processed foods (Delves-Broughton et al., 1996) with recent focus on the application to fresh meat and poultry (Avery, 1997; Gonzalez-Fandos & Dominguez, 2006).

One study used three hemolytic pathogenic strains of *Listeria monocytogenes* stored at 4°C in phosphate-buffered saline, pH 5.5, containing a combination of sorbate (0.2% wt/vol) and nisin (40 IU/mL) to assess antilisterial effects (Avery, 1997). Sterile beef steaks were inoculated with a cocktail of the three strains at approximately 5 log CFU/cm^2 and the surface of half the steaks was treated with the antimicrobial solution 1.0% sorbate plus 1,000 IU of nisin per mL. The meat was packaged under vacuum or 100% carbon dioxide and stored at 4°C for 4 weeks. Populations decreased by 0.54 log in CO_2 packages while vacuum packaged meat populations decreased during storage to the extent that 96.5% of the initial pathogen load was eliminated. Treatment with the sorbate-nisin combination did not significantly affect pathogenicity of the *L. monocytogenes* cocktail recovered from vacuum or carbon dioxide packages after storage, in contrast to the in vitro study, where pathogenicity was clearly attenuated.

Nisin inhibits growth of Gram positive bacteria through interaction with anionic constituents of the negatively charged cell wall, resulting in a rapid and specific efflux of cytoplasmic constituents (Henning, 1986). In at least one study, the application of nisin alone reduced numbers of *L. monocytogenes* by 0.95 log CFU g^{-1} on vacuum packaged shrimp (Wan Norhana, Poole, Deeth, & Dykes, 2012). The authors postulate the relative inefficiency of nisin may have been due to the very low concentration used in the dipping solution due to the expense of nisin for industrial application (Wan Norhana et al., 2012). The presence of protease in meats may partially explain the reduced inhibition of nisin

applications in comparison to lower lipid content foods such as cabbage, broccoli, and bean sprouts which exhibited higher reductions of *L. monocytogenes* (Bari, 2005). Nisin is known to be more efficient at lower pH whereas the relatively high pH of meat (5.5-6.0) may reduce the activity (Delves-Broughton et al., 1996). Nisin may not be stable in meat and the activity can be rapidly lost from nisin binding to meat particles (Henning, 1986; Wan Norhana et al., 2012).

Nisin (50 µg/mL) and pediocin (100 IU/mL) individually or in combination with 2% sodium lactate, 0.02% potassium sorbate, 0.02% phytic acid, and 10 mM citric acid were tested as sanitizer treatments for reducing *Listeria monocytogenes* on cabbage, broccoli, and bean sprouts (Bari, 2005). Cabbage, broccoli, and bean sprouts were inoculated with a five-strain cocktail of *L. monocytogenes* and left at room temperature (25°C) for up to 4 hours prior to antimicrobial treatment. Washing treatments were applied to inoculated produce for 1 minute, and surviving bacterial populations were determined. All compounds resulted in 2.20 to 4.35 log reductions of *L. monocytogenes* on bean, cabbage, and broccoli when tested alone. Combination treatments of nisin-phytic acid and nisin-pediocin-phytic acid caused significant reductions of *L. monocytogenes* on cabbage and broccoli but not on bean sprouts.

Antimicrobial Solution (AMS)

The antimicrobial effects of these ingredients used singly suggests that an additive effect may be observed if the ingredients are combined. Researchers at the USDA Agriculture Research Service have developed a novel antimicrobial solution incorporating lactic acid, acetic acid, levulinic acid, lauric arginate ester (LAE), and

chitosan into MilliQ water (Guo et al., 2013, 2014). Previous research has evaluated the efficacy of the AMS on samples directly coated with the solution or treated with solution-coated polylactic films (Guo et al., 2013, 2014). The application of AMS has shown favorable inhibition against bacteria when applied to RTE pre-sliced turkey deli meat and frozen RTE shrimp. Similar AMS applications have also been evaluated in tomato stem scars and cantaloupe rinds and have yielded favorable results as well (Chen, Jin, Gurtler, Geveke, & Fan, 2012; Jin & Gurtler, 2012). Additional research is needed to evaluate the efficacy of AMS in application to fresh meats.

Other Topical Treatments

Many organic acids are currently utilized in the food industry to impart characteristic flavors and to inhibit growth of microorganisms. Lactates and diacetates have effectively reduced *Listeria monocytogenes* in RTE meat and poultry products (Bedie et al., 2001; Choi & Chin, 2003). One study indicated that 2 – 3% potassium lactate as an ingredient showed a listeriostatic effect on inoculated frankfurters over a 90 day vacuum storage period at 4°C (Porto et al., 2002). Lactates and diacetates reduce water activity and lower intracellular pH, thereby impairing cell function and growth (Morey et al., 2012). Another study found the addition of lactate did not affect meat pH, addition of diacetate reduced meat pH, yet addition of both lactate and diacetates increased the meat pH (Mbandi & Shelef, 2001, 2002; Stekelenburg & Kant-Muermans, 2001). Both lactate and diacetates were found to have listeriostatic properties rather than listeriocidal properties, the combination of the two was found to be more inhibitory than

either singly, and similar inhibition was noted in samples inoculated with *Salmonella* (Mbandi & Shelef, 2002).

Acetates and diacetates are used in dairy and meat products to target growth of yeast and bacteria while sodium propionate is used to target mold growth (Mani-Lopez, 2012). Other food preservatives include propionic, citric, and benzoic acids (Theron, 2007). A combination of lactic acid or acetic acid with fumaric acid, or fumaric acid alone, is also effective against *L. monocytogenes* (Podolak, 1995a, 1995b). The poultry industry commonly adds citric acid and citrates to chill tanks to control pH and thus control *Salmonella* spp. (Mani-Lopez, 2012). Malic, propionic, and tartaric acids are organic acids which are not as commonly utilized by the food industry but which may offer antimicrobial effects (Mani-Lopez, 2012). *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* Typhimurium counts on lean beef have been reduced over time by the application of fumaric, lactic, and acetic acids (Podolak, 1995a, 1995b). *Escherichia coli* O157:H7 and *Listeria monocytogenes* were inoculated onto the surface of lean beef and then sanitized with fumaric, acetic, or lactic acid alone and in combined solutions of those acids at 55°C for 5 seconds (Podolak, 1995a, 1995b). A 1% concentration of fumaric acid was the most effective acid in reducing the *L. monocytogenes* and *E. coli* O157:H7 populations. The researchers ranked the order of acids tested against the growth of *L. monocytogenes* and *E. coli* O157:H7 and reported fumaric acid as the most effective followed by lactic and acetic acids. Fumaric acid at concentrations of 1.0% and 1.5% was reported to be more effective than any of the combined solutions of acids. Hot water is less effective at reducing bacteria than organic acids; however, discoloration and off odors may develop with the addition of organic acids (Sun & Holley, 2012). Thus, this

should be the main concern when determining the concentration(s) of organic acid(s) to be utilized (Sun & Holley, 2012).

Sorbate, propionate, and benzoate have antibacterial and antifungal properties (Elshenawy & Marth, 1988a, 1988b; Park & Marth, 1972a, 1972b, 1972c, 1972d). Salt is a preservative, a flavor enhancer, and has antimicrobial properties, although it is not used in high enough concentrations to be an effective antimicrobial, particularly in "low-sodium" foods (Marth, 1998). Use of salt and other ingredients will reduce the water activity to 0.98 or below, lengthening the lag phase of most bacteria and further reducing the rate of any subsequent growth (Elshenawy & Marth, 1988a, 1988b; Park & Marth, 1972a, 1972b, 1972c, 1972d). Like organic acids, salt is self limiting due to organoleptic properties.

Sodium metasilicate is approved as an antimicrobial in RTE meat and poultry products, up to 6% as stated by the USDA FSIS directive 7120.1 Rev. 20 which became effective September 9, 2014 (Carlson et al., 2008). Although little research has been conducted with sodium metasilicate in meat applications, it has proven to effectively reduce Gram negative bacteria on the surface of meat and meat products (Carlson et al., 2008). The effectiveness of sodium metasilicate is derived from is ability to act on the cytoplasmic membrane and cause cell lysis (Sharma & Beuchat, 2004). Although research on the effect of sodium metasilicate on Gram positive bacteria remains limited, one *in vitro* study found it reduced *L. monocytogenes* (Sharma & Beuchat, 2004). Another study used sodium metasilicate at 2, 3, 4, and 5% and found it reduced *E. coli* O157:H7, STECs, *Salmonella* spp., and *L. monocytogenes* when applied to inoculated bottom beef rounds and deli meats (DeGeer, 2014). Results indicate the effectiveness of

sodium metasilicate against pathogens of concern without negative meat quality concerns (DeGeer, 2014; Quilo et al., 2010). Any antimicrobial additives must be declared on the product label under the purview of the FDA; such a declaration is against current "clean label" and "green label" market trends (Morey et al., 2012).

Meat Marination

Marinades are typically a water-oil emersion containing a combination of sugar, salt, acids (acetic and citric acids), additives (Xanthan and guar gum), spices, sorbates, benzoates, and aroma enhancers (Bjorkroth, 2005). Commercial marination practices rely primarily on salt-water and phosphate formulations which increase tenderness, juiciness, and yield with current applications including injection technology, immersion, and vacuum tumbling (Alvarado & Mckee, 2007). A variety of spices and spice extracts are utilized in the industry to cater to specific flavor profiles and preferences. Marinated meat products, particularly poultry products, represent a growing segment of the food industry on a global scale. Yogurt is gaining attention as a possible marinade ingredient as it has shown 2 log reductions of *Campylobacter jejuni* on pork medallions (Birk & Knochel, 2009). Studies indicate that marinade sauces prevent the growth of spoilage organisms based on a low pH, high salt concentration, sorbates and benzoates, and various spices (Bjorkroth, 2005). Studies have shown marinades with paprika, garlic, coriander, salt (NaCl), and sodium phosphates have been effective in reducing survival of *Campylobacter* cells (Perko-Makela, Koljonen, Miettinen, & Hanninen, 2000). Teriyaki marinades are typically thick and highly acidic sauces with powdered onion, garlic,

spices, and powdered soy sauce as the dominant ingredients which may contribute to its bactericidal activity (Pathania et al., 2010).

One study used three strains (Heidelberg, Typhimurium, and Senftenberg) to determine the effect of commercially available teriyaki and lemon pepper marinades on the survival of *Salmonella* (Pathania et al., 2010). Teriyaki and lemon pepper marinades were inoculated with nalidixic acid resistant *Salmonella*, homogenized, and divided into aliquots. Aliquots were then stored at 4 or 25°C for up to 32 hours. Serial dilutions were performed and plated onto XLT4 agar. Non-inoculated aliquots of each marinade served as the negative controls. Both teriyaki and lemon pepper marinades showed reduction of *Salmonella* spp. during the 32 hour storage period with greater reductions observed in marinades maintained at 4°C (Pathania et al., 2010). Survival populations were lower in the teriyaki marinade compared to the lemon pepper, though no differences in growth patterns of the three strains were observed (Pathania et al., 2010). The pH of lemon pepper marinade was less acidic compared to the teriyaki with oils of lemon, ground black pepper, and lemon peel granules as its primary ingredients (Pathania, McKee, Bilgili, & Singh, 2010). Other researchers have indicated that Gram positive bacteria are more sensitive to citrus essential oils *in vitro* than Gram negative bacteria (Fisher & Phillips, 2006). Additional reports indicate a higher activity of orange, lemon, grapefruit, and mandarin citrus oils and their derivatives *in vitro* (Dabbah, Edwards, & Moats, 1970).

As shown by these previous studies, marination has the potential to increase the shelf life of meat and poultry products as one non-thermal intervention technology. It can enhance the safety and quality of meat and poultry products by acting as an additional

hurdle for the bacterial growth while simultaneously improving flavor, juiciness, and convenience (Pathania et al., 2010).

Meat Quality Evaluation

Sensory Evaluation

The meat industry relies on a combination of techniques to determine product shelf life. These techniques include aerobic plate counts, anaerobic plate counts, color evaluation, and sensory evaluation. Sensory evaluation is a set of techniques for accurate measurement of human responses to foods which minimizes potential bias effects (Lawless, 2010). Sensory evaluation is important to the meat industry because it helps relate consumer perceptions to the quality of the meat product. Sensory can be defined as a scientific method to evoke, measure, analyze, and interpret responses to products as perceived by the senses: sight, touch, smell, taste, and sound (Lawless, 2010). Panel preparation includes decisions about environment, number of sessions, and physical condition of the samples (AMSA, 1995). The validity of the sensory panel is dependent upon the control of various factors within the testing environment (AMSA, 1995). The specific parameters measured during a sensory evaluation panel are selected by the individual organizing the panel and are designed to answer a specific research question about the product. Trained sensory panels are used to identity and quantify specific parameters.

In situations where cookery method or treatment may create variation in color, red filtered lights are necessary to provide uniform and adequate lighting (AMSA, 1995). Panelists are provided a standardized amount of each sample and an evaluation form with

a numerical scale for responses. Samples are held in a warmer to maintain the appropriate temperature for tasting and are presented to panelists in a randomized design (AMSA, 1995). The number of samples presented during each panel should be managed to prevent panelist fatigue but also should be a function of product characteristics, experience of the panelists, and number of attributes to be measured per sample (AMSA, 1995).

Panelists are recruited and trained prior to the sensory panel. Objectives of training are to: 1) familiarize the individual with test procedures, 2) improve an individual's ability to recognize and identify sensory attributes, and 3) improve an individual's sensitivity and memory, permitting precise and consistent sensory judgments (AMSA, 1995). Numerous decisions must be made prior to the panel. Some decisions include the amount of sample panelists will receive, if samples are to be swallowed, rinsing should be standardized, and the temperature of the water provided should be standardized (AMSA, 1995). Unsalted crackers may be provided for panelists when considerable aftertaste is present in the samples; however, caution should be exerted as the mouth feel may be impacted (AMSA, 1995). During training, panelists are provided reference samples along with a corresponding score for each parameter such as tenderness, juiciness, flavor intensity, and aroma intensity. Additional parameters which may be evaluated in a sensory panel include initial tenderness, initial juiciness, sustained tenderness, sustained juiciness, flavor intensity, aroma intensity, off flavor intensity, and off flavor descriptors. Panelists provide a numerical response corresponding with the provided scale; these responses are then analyzed statistically.

Color

One of the most important factors consumers evaluate when selecting meat is color because the consumer associates color with quality (Carpenter, Cornforth, & Whittier, 2001). The color of meat is determined by myoglobin which is the red pigment in meat. A prescribed color is expected from various meat types: a bright, cherry – red from beef, a gray – pink from pork, and a white – pink from poultry. Consumers rely on the eye to evaluate color while researchers use instrumental colorimeters to objectively evaluate color. The AMSA Meat Color Measurement Guidelines are a comprehensive review of meat color measurements (Hunt, 2012). Briefly, instrumental color is expressed in three dimensional terms using the CIE L* a* b* scale. a* values cover the X axis, b* values cover the Y axis, and L* values cover the Z axis (Hunt, 2012). A visual depiction of the color scale has been provided in Figure 1. The center of the color scale is neutral gray. Positive a* values represent red and negative a* values represent green. Similarly, positive b* values represent yellow and negative b* values represent blue. The scale for L* is somewhat different in that a value of 100 represents white while a value of 0 represents black. The L* scale is used to determine the darkness or lightness of the sample. Meat color, as well as the expectation of the color, is adjusted when the meat is marinated. It is expected that the meat color will be changed to reflect the color properties of the marinade. Thus, dark marinades, such as soy sauce based marinades, will alter the color of the meat such that it is darker, more like the marinade. If a lighter colored marinade is chosen, Italian dressing for example, the meat will appear lighter on the surface. Consumers evaluate the visual appeal of these color changes while a colorimeter objectively detects differences.

Detection and Enumeration of Bacteria

Detection and enumeration of food associated pathogenic bacteria is complicated by emerging strains which are not routinely encountered and may have an unclear transmission route (Mead et al., 1999). Additionally, high throughput screening of a diverse array of fresh and processed foods requires that food safety practices be dynamic, sensitive, specific, versatile, and cost effective for large numbers of samples (Gracias & McKillip, 2004). No single method or assay for culture based techniques optimally addresses these criteria. Molecular approaches offer the capacity for near-time or real-time detection of bacteria and are rapid, sensitive, and specific for target pathogens or the virulence determinants of that pathogen (Feng, 2001; Rijpens & Herman, 2002; Smith, O'Connor, Glennon, & Maher, 2000). Despite these advantages of molecular techniques, their adoption into food microbiology laboratories and scale up in food processing facilities may be limited due to concerns of reliability, cost, and novelty; thus many laboratories will be obligated to rely on traditional techniques (Jaykus, 2003). The premise of these methods is the recovery and enumeration of viable bacteria in the food matrix. Food microbiology laboratories which lack necessary resources to utilize emerging molecular based technologies rely on methods such as the standard plate count (SPC) and selective/differential media for bacterial isolation and enumeration in addition to commercially available biochemical profiling systems for identification of specific food isolates (Gracias & McKillip, 2004). Novel detection and enumeration techniques are continually reported and, while the majority involve molecular biological approaches, many remain classified as conventional (Gracias & McKillip, 2004).

Standard Plate Counts and Relevant Variations

Traditionally, detection of viable bacteria is performed by culturing or measuring growth of individual microorganisms. Hundreds of bacteriological media are commonly utilized in the food industry and are uniquely applied to best monitor for spoilage and pathogenic bacteria in food (Harrigan, 1998). Standard plate counts (SPC) or aerobic plate counts (APC) are accomplished through use of routine nonselective media such as trypticase soy agar or standard methods agar.

The sensitivity of SPCs can be increased with the application of a selective agar overlay which is designed to recover a larger proportion of bacteria from food matrices, compared to straight plating onto selective media, following sublethal stressors during processing such as heat, cold, acid, or osmotic shock (Harrigan, 1998; Speck, Ray, & Read, 1975). Detection of sublethally damaged yet viable pathogenic bacteria is of dire importance to the food industry as these cells may continue to pose a threat to human health. The selective agar overlay aides in resuscitation of damaged but viable cells. The technique is to pour-plate the inoculum with a base layer of trypticase soy agar, or comparable nonselective media, and incubate for 1-4 hours. The incubation allows sublethally damaged bacteria to recover and begin growing prior to the application of an appropriate selective media overlay (Hurst, 1977; Ray, 1986). This technique has been proven effective with a variety of bacteria including *E. coli, Salmonella, Listeria monocytogenes, Bacillus cereus,* and *Staphylococcus areus* (Golden, Beuchat, & Brackett, 1988; Hajmeer, Fung, Marsden, & Milliken, 2001; Hara-Kudo et al., 2000; Kang & Fung, 1999, 2000; Kang & Siragusa, 1999; Kang et al., 2014; McKillip, 2001; Sandel, Wu, & Mckillip, 2003; Wu, Fung, & Kang, 2001).

Another strategy to increase the numbers of damaged but viable target bacteria to detectable levels is to perform a pre-enrichment of the food sample (Zhao & Doyle, 2001). The primary disadvantage of pre-enrichment is that, depending upon the food being analyzed, it may require an additional 8-24 hours prior to enumeration or detection. Enrichment in the appropriate selective media can yield densities of at least 4 log of heat-damaged pathogens, confirming the presence of the pathogen (Zhao & Doyle, 2001). Despite the familiarity, ease of use, and low cost, assay sensitivity is lacking in comparison to molecular-based applications such as polymerase chain reaction, and the time required to obtain data omits inclusion as a "rapid method" (Gracias & McKillip, 2004).

Immunomagnetic separation (IMS) employs the use of antibodies linked to magnetic beads placed in food slurry and allowed to interact with specific epitopes on the cell surface of the bacteria. The material is exposed to a magnetic field, essentially pulling the bacteria out of suspension for plating or molecular-based detection and enumeration (Jinneman et al., 1995; Tomoyasu, 1998). Dynabeads™ (Dynal Botech, Oslo, Norway) has been effective for isolation of pathogenic *L. monocytogenes, E. coli* O157:H7, and *Salmonella* spp. (Chandler et al., 2001; Hsih & Tsen, 2001; Hudson, Lake, Savill, Scholes, & McCormick, 2001; Ogden, Hepburn, & MacRae, 2001).

An array of chromogenic and fluorogenic culture media have been developed for selective isolation and differentiation of food associated pathogens. Enzyme or substrate inclusion into selective media may eliminate or expedite follow-up biochemical confirmation of bacterial identity. Fluorogenic enzyme substrates consist of a fluorogen conjugated to a sugar or amino acid (Manafi, 1996, 2000). One commonly utilized for

coliforms, including *E. coli* O157:H7, is methylumbelliferyl. A blue fluorescence is observed when suspect colonies are exposed to long-wave ultraviolet light following cleaving by enzymes produced from specific target species (Alonso, Soriano, Carbajo, Amoros, & Garelick, 1999; Alonso, Soriano, Amoros, & Ferrus, 1998; Berg & Fiksdal, 1988). Virtually all coliforms are positive for the methylumbelliferyl-β-D-glucoronase enzyme, with the exception of enterohemorrhagic *E. coli* O157:H7 (Hartman, 1992). Although additional steps are required, this distinction is important for determining the presence of enterohemorrhagic *E. coli* O157:H7 in water and food samples (Bettelheim, 1998; Manafi, 2000).

Dry plate culturing, such as the 3M Petrifilm product, is another widely utilized means to assess microbiological quality of a diverse range of foods for coliforms, aerobic mesophilic bacteria, psychrotrophs, and staphylococci (Blackburn, Baylis, & Petitt, 1996; Ellis & Meldrum, 2002; Linton, Eisel, & Muriana, 1997; Silbernagel & Lindberg, 2001). Multiple layers of a plastic film encase a dehydrated disc of the appropriate medium. A single sheet of plastic is pulled back, aseptically, and 1 mL of inoculum is applied to rehydrate the medium while the film is replaced and pressed flat. Dry media culture plating techniques have been applied to predicting shelf life and monitoring the microbiological quality of milk and to assessing surface contamination of meat and poultry (Erdmann, Dickson, & Grant, 2002; Guthrie, Dunlop, & Saunders, 1994; Hughes & Sutherland, 1987; Park, Seo, Ahn, Yoo, & Kim, 2001; Phillips & Griffiths, 1990). Additionally, dry plate culturing is an approved method of quality control in food microbiology. Petrifilm plates are small, convenient for large sample sizes, and are common in quality control laboratories. Though they require less incubator space than

other methods, Petrifilm plates have the same limitations of SPCs in terms of poor sensitivity and likelihood of false negative results from sublethally injured yet viable bacteria (Gracias & McKillip, 2004).

Immunoassays

The enzyme linked immunosorbent assay (ELISA) potentially offers greater specificity compared to SPC due to the interaction between the antibody and the target molecule (Gracias & McKillip, 2004). A suspect sample is added to wells in a microtiter plate containing a primary antibody with specificity for the target molecule. The target molecule may be a component of the pathogen, such as a cell or flagellar antigen, or a product of the bacteria, such as an enterotoxin (Notermans & Wernars, 1991). An incubation step is performed, after which unbound material is washed away and a secondary antibody is added to "sandwich" the antigen between two antibodies. A second rinse is performed and the assay is then developed per the conjugate or tag bound to the secondary antibody. ELISA has been used to detect whole-cell antigen targets or products for *Salmonella* spp., *E. coli* O157:H7, *Campylobacter* spp., *B. cereus,* and *L. monocytogenes*, among other pathogens (Bolton, Sails, Fox, Wareing, & Greenway, 2002; Chen, Ding, & Chang, 2001; Daly, Collier, & Doyle, 2002; De Paula, Gelli, Landgraf, Destro, & Franco, 2002; Peplow, Correa-Prisant, Stebbins, Jones, & Davies, 1999; Valdivieso-Garcia, Riche, Abubakar, Waddell, & Brooks, 2001; Yeh, Tsai, Chen, & Liao, 2002). ELISA is automatable and is convenient for large sample sizes; however, they may lack the desired sensitivity with a typical detection limit of 10^4 CFU/mL, depending on the food being analyzed (Cox, 1987; Hartman, 1992).

Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a technique which is useful to determine the origin of foodborne illness outbreaks by analyzing DNA of pathogenic strains of bacteria. Presence of the shiga toxin 1 (*stx*1) and shiga toxin 2 (*stx*2) genes indicate the presence of potentially pathogenic STEC. Polymerase chain reaction (PCR) is used to test for the presence of these genes. PCR amplifies a length of DNA millions of times during a relatively short time period. A thermo-cycler progresses through various temperatures for predetermined times to create the environment necessary for amplification. The machine will first increase the temperature to approximately 95°C to denature DNA strands into single-stranded DNA chains. The machine will then decrease the temperature to approximately 60°C to allow the primers, one forward and one reverse, to anneal to the complimentary length of base pairs on the strands of DNA. Annealing temperature is critical to the process of PCR. If the temperature during the annealing step is too high or too low, primers will lose specificity or not bind at all (Bartlett, 2003). Specificity of primers varies as does the melting temperature. Next, the temperature will be increased to 72°C for at least one minute. This step allows the *Taq* polymerase to bind each priming site and extend or synthesize a new strand of DNA. The thermo-cycler then increases the temperature, beginning the process again. Generally, 30 cycles are required to generate sufficient copies of the DNA, although additional cycles may be included.

DNA for PCR must first be extracted from cells. Multiple methods exist for DNA extraction with varying expense and success of extraction. A simple and cost effective method is to boil the cell suspension in water for ten minutes (Wasilenko et al., 2012).

Using a TE buffer method produces a lower cycle threshold value, indicating strong positive reactions. Both boiling and TE buffer are acceptable techniques for extracting DNA for PCR when considering cost and quality of extracted DNA (Wasilenko et al., 2012). Reliable rapid detection methods for identifying STEC strains are currently being researched (Fratamico, DebRoy, Miyamoto, & Liu, 2009). PCR has become a standard technique in microbiology and food safety laboratories, using amplification of known virulent genes to test for presence of certain pathogens. *stx*-positive and *stx*-negative versions of bacteria may exist within a single serotype (Fratamico et al., 2009). *stx* genes are of primary interest to researchers due to their capacity to cause human illness. However, some researchers are interested in *wxz* and *wxy* gene primers. These primers are O-antigen gene clusters specific to the antigen and are useful for identifying individual O serogroups (Wang et al., 2010). The *wxz* and *wxy* gene primers are more useful when identifying a specific, known serogroup within a material (Wang et al., 2010).

Pulse Field Gel Electrophoresis

Pulse field gel electrophoresis (PFGE), in addition to PCR, is a widely utilized technique to identify contamination sources. PFGE is similar to gel electrophoresis and is used to separate large DNA pieces in an agarose gel matrix. The unique aspect of PFGE is that the voltage path is altered and conducted in three different directions while the DNA continues along the central axis of the gel matrix, pulling apart larger DNA fragments. Restriction enzymes used in preparation of the DNA cut it at restriction sites resulting in fragments. The fragments are loaded onto the agarose gel and are pulled apart

by electrophoresis over a period of hours. After the electrophoresis is complete, larger DNA fragments will be in the top portion of the gel while the smaller fragments will be located in the bottom portion. PFGE is frequently used to compare strains of the same serogroup, such that if the strains are identical the result would be two identical gels. PFGE can also be used to compare the genetic similarity of pathogenic outbreak strains to farm strains (Miko et al., 2013). This allows pathogenic strains to be traced to the point of origination. PFGE can serve as the evidence to initiate a recall of meat products from the market due to contamination.

Summary and Conclusions

The United States has the safest food supply in the world; however, consumers continually demand safer products. The meat industry continues to meet these demands through technological advancements and development of processing aides. Thermal processing, refrigeration, freezing, vacuum packaging, and modified atmospheric packaging are current and viable strategies to maintain and enhance the safety of meat and meat products. Topical applications of organic acids, specifically lactic and acetic acids, effectively inhibits growth of pathogenic bacteria on the surface of the carcass. The decrease in pH at the surface of the carcass due to the application of organic acids is the primary effect of the inhibition of pathogen growth. Despite the inhibitory effect of organic acids, new technologies are being developed which are more inhibitory and more cost effective.

Emerging scientific research suggests the application of antimicrobial solutions may be a viable, cost effective, and value addition strategy. This is an area of food safety

research which is gaining much attention. Antimicrobial solutions address food safety concerns while simultaneously creating potential to add value to the product through increased juiciness and tenderness. This is also a hurdle technology as antimicrobial solutions may be incorporated as an ingredient in marinades, thus adding flavor to the product as well. Based on currently available literature, multiple antimicrobial solutions are being developed, each with unique application potential. With consumers and companies moving toward clean and green labels, particular focus will be on those antimicrobial solutions which meet natural labeling requirements. Compounds with natural antimicrobial properties are currently gaining much attention in research and industry settings. Nisin, chitosan, acetic acid, lactic acid, and lauric arginate ester will likely continue to gain attention as applications for meat products are developed.

Marination of meat and meat products is one way in which the consumer can obtain a more convenient and value-added product. Marination can extend the shelf life of the product, inhibit growth of pathogenic bacteria, and improve tenderness, juiciness, and flavor. Marinades can be selected based on the taste preferences of the individual or family for whom the product is intended. Marinades, much like recipes, can also be created or altered such that new ingredients may be included. This is one unique application of antimicrobial solutions for meat products. In addition to inhibition of growth of pathogenic bacteria, antimicrobial solutions incorporated into marinades may enhance flavor development and improve tenderness and juiciness of the product. When applied to beef and poultry, an antimicrobial marinade could severely inhibit growth of pathogenic bacteria such as *E. coli, Salmonella,* and *Listeria monocytogenes* while simultaneously creating a product which is more convenient and flavorful for consumers.

As with all new technology, the full inhibitory effects of the antimicrobial solution against pathogens of concern in meat must be evaluated. Antimicrobial solution efficacy against pathogens of concern must first be determined. The optimal concentration of the antimicrobial for pathogen inhibition must then be identified. Ideally, the optimal concentration would be low, such that the solution could be incorporated into a product batter or into a marinade without any perceived off flavors or odors. Application techniques must also be evaluated. Topical spray application and incorporation as a batter or marinade ingredient are two potential application strategies. With a spray application, additional equipment may be required and should be tested to ensure compatibility with current industry practices. Once the inhibitory effects of the solution have been confirmed and the optimal concentrations and application strategies identified, the incorporation of an antimicrobial solution can be implemented.

It is reasonable to assume that some pathogenic bacteria cells would survive after the application of antimicrobial solutions. In addition to traditional detection techniques, the new molecular techniques, such as PCR and PFGE, will not only help to confirm the survival of those inoculated pathogens, but also trace the source of the contamination. However, one challenge exists with the application of these molecular techniques in studies such as antimicrobial treatment evaluation. The question of "how to better extract the DNA from the inoculated and antimicrobial treated samples" must be answered.

Novel antimicrobial solutions are currently being developed and are gaining much attention (Guo, Jin, Scullen, & Sommers, 2013; Guo, Jin, Geveke, et al., 2014; M. Guo, Jin, Wang, et al., 2014). One novel solution containing acetic acid, lactic acid, levulinic acid, lauric arginate ester, nisin, and chitosan is of particular interest as all components

have achieved GRAS status and potentially classify as natural. In combination, these ingredients would create low pH solution which would significantly inhibit growth of pathogenic bacteria.

Research Objectives

The meat industry utilizes a variety of antimicrobial solutions for reducing pathogenic contamination of meat and meat products. Optimizing the use of existing antimicrobial solutions and identifying emerging antimicrobial solutions may decrease foodborne pathogenic bacteria. Identifying cross-functional applications and solutions which can be implemented in the meat industry may improve the safety of the meat supply. Additionally, these applications and solutions may be extended to other food types which will reduce foodborne pathogenic bacteria in those foods as well as further improve the safety of the food supply. Researchers at the USDA Agriculture Research Service have developed a novel antimicrobial solution (AMS) which has yielded promising results against pathogenic bacteria (Guo et al., 2013; Guo, Jin, Wang, et al., 2014). Thus, the objectives of this research address the efficacy of the AMS and potential applications in the meat industry.

The objective of the first study was multifaceted. The first objective was to determine differences in survivability of pathogens of concern grown in broth or on plates. *E. coli* (Gram negative) and *Listeria monocytogenes* (Gram positive) were selected for this portion of the study. Cultures were grown in tryptic soy broth (TSB) or on plate count agar (PCA) plates; the survivability of the two growth mediums was then compared. The broth grown cultures were selected for the remaining portion of this study

as well as future studies as this method showed similar survivability compared to PCA plates and offered a more time and resource efficient technique.

Once the survivability was determined, our objectives were: 1) to determine the effectiveness of the AMS at inhibiting pathogen growth on inoculated meat samples, and 2) to determine the optimal concentration of the antimicrobial solution. The AMS was applied to the surface of the meat samples at a high (stock), medium (1:5 dilution), and low (1:10) dilution. Distilled water was used as the diluent for the medium and low concentrations of the AMS as well as the control. The medium concentration of the AMS was chosen for further studies because it was the lowest concentration with the most consistent inhibition of pathogen growth. Top round beef samples were inoculated with one of four pathogen cocktails and allowed 30 minutes of contact time prior to application of the assigned antimicrobial concentration treatment. Samples were then stored at 4°C for 0, 6, 24, or 48 hours.

The objective of the second study was to determine the effectiveness of three retail marinades at inhibiting pathogen growth on inoculated meat samples. Marinades chosen were: 1) Ken's Steakhouse Marinade & Sauce, Balsamic & Roasted Onion, 2) KC Masterpiece 30 Minute Marinade, California Style Lemon & Cracked Pepper, and 3) KC Masterpiece 30 Minute Marinade, Classic Steakhouse. All bottles of marinade were obtained from the local Publix Super Market location (Auburn, AL) and were selected based on market and food trends in early 2014. During the course of this research, the balsamic and roasted onion marinade was discontinued in the area. As a result, this marinade was removed from later studies due to the lack of availability in the area and the similar pH and performance of the classic steakhouse marinade. Top round beef

samples were inoculated with one of four pathogen cocktails and allowed 30 minutes of contact time prior to application of the assigned marinade. Samples were then stored at 4°C for 0, 6, 24, or 48 hours.

The objective of the third study was multifaceted. The first objective was to determine the effectiveness of the marinade combined with the AMS at inhibiting pathogen growth on inoculated meat samples. The AMS was prepared and mixed with the marinade prior to application to the meat. The medium concentration (1:5 dilution) of the AMS was chosen due to the consistent inhibition of pathogen growth. The marinade served as the diluent for the AMS. Top round beef samples were inoculated with one of four pathogen cocktails and allowed 30 minutes of contact time prior to application of the assigned marinade + AMS treatment. Samples were then stored at 4°C for 0, 6, 24, or 48 hours.

The second objective was: 1) to determine if pathogens survive following application of the marinade + AMS, and 2) to determine what genetic markers are present which may allow pathogens to survive. Samples were prepared for polymerase chain reaction to amplify DNA extracted from the pathogenic cells. PFGE was then used to separate the genetic material, in the form of DNA fragments, based on size. Gels were then compared to determine similarity in genetic material of surviving pathogens.

The objective of the fourth study was also multifaceted. The first objective was to identify and quantify any organoleptic (sensory) attributes which may be perceived by consumers when this AMS is incorporated into a retail marinade. Un-inoculated one inch thick top round beef steaks were prepared and marinated in Ziploc bags with water, water + AMS, or one of the two marinade + AMS combinations for 0, 6, 24, or 48 hours. The

AMS was prepared and diluted with water or the marinades prior to application to the meat. The medium concentration (1:5 dilution) of the AMS was chosen due to the consistent inhibition of pathogen growth as well as concerns about the high concentration overwhelming the marinade flavor.

The second objective was to objectively evaluate color differences in steaks marinated 0, 6, 24, or 48 hours in water only, water + AMS, lemon-pepper marinade + AMS, or classic steakhouse marinade + AMS. One inch thick top round beef steaks were prepared and marinated in Ziploc bags for the assigned treatment and time combination. Steaks were not inoculated, as they were consumed by sensory panelists. Three steaks were prepared for each time and treatment combination. A colorimeter was used to measure color on each of the three steaks.

Table 1: Selected intrinsic factors affecting growth of pathogens of concern in beef.

Microorganism	a_w values for growth		pH values for growth			Temperatures for growth (°C)		
	Min.	**Opt.**	**Min.**	**Opt.**	**Max.**	**Min.**	**Opt.**	**Max.**
Escherichia coli	0.96	0.99	3.7	6.0-7.0	9.2	7	35-40	46
Salmonella spp.	0.94	0.99	4.2	7.0-7.5	9.5	5	35-37	45-47
Listeria monocytogenes	0.92		4.2	7.0	9.8	0	30-37	45

Adapted from: Jay, J.M., Loessner, M.J., & Golden, D.A. (2006). Chapter 3 *Modern Food Microbiology.* New York: Springer.

Minimum (Min.), Optimal (Opt.), and Maximum (Max.) known values are presented.

Table 2: Strains of microorganisms used in cocktails.

Microorganism	ATCC number or ID Code	Source
Escherichia coli O157:H7	ATCC 35150	Human – HC
Escherichia coli O157:H7	ATCC 43894*	Human – HC
Escherichia coli O157:H7	AU – 1	Laboratory Strain (301)
Escherichia coli O157:H7	AU – 2	Laboratory Strain (505B)
Escherichia coli O157:H7	AU – 3	Laboratory Strain
Non-O157:H7 STEC (O145)	TWO9356	Human - HUS
Non-O157:H7 STEC (O26)	TWO7814	Human – HUS
Non-O157:H7 STEC (O121)	TWO8039	Human
Non-O157:H7 STEC (O45)	TWO14003	Human
Non-O157:H7 STEC (O111)	TWO7926	Human – HC
Non-O157:H7 STEC (O103)	TWO8101	Human
Salmonella	AU – Enteritidis	Laboratory Strain
Salmonella	AU – Kentucky	Laboratory Strain
Salmonella	AU – Montevideo	Laboratory Strain
Salmonella	AU – Thompson	Laboratory Strain
Salmonella	AU – Stanley	Laboratory Strain
Listeria monocytogenes	ATCC 49594	Petite Scott A
Listeria monocytogenes	ATCC 19115	Human – Serotype 4b
Listeria monocytogenes	ATCC 7644*	Human
Listeria monocytogenes	AU – 4	Laboratory Strain (101M serotype 4b)
Listeria monocytogenes	AU – 5	Laboratory Strain (108M serotype 1/2b)

*Indicates strains used for Experiment 1: Optimum Growth Medium which is detailed in Chapter 2.

Figure 1: Color scale for CIE L*a*b* color space.

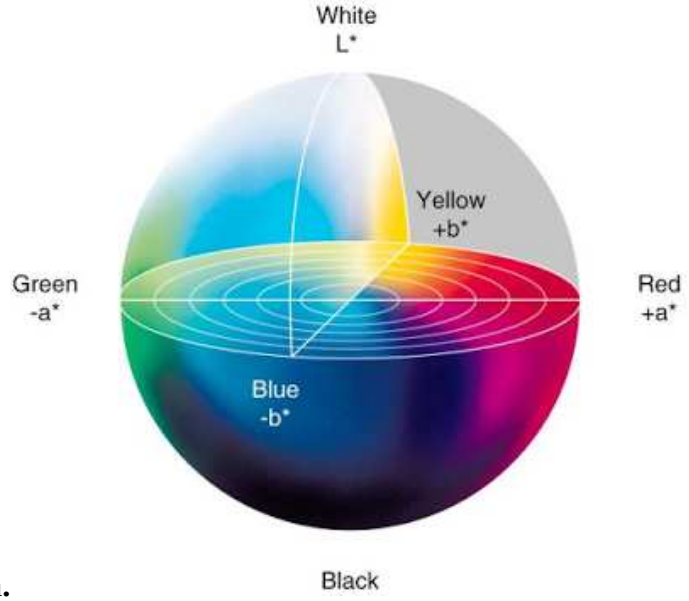

Figure 1a.

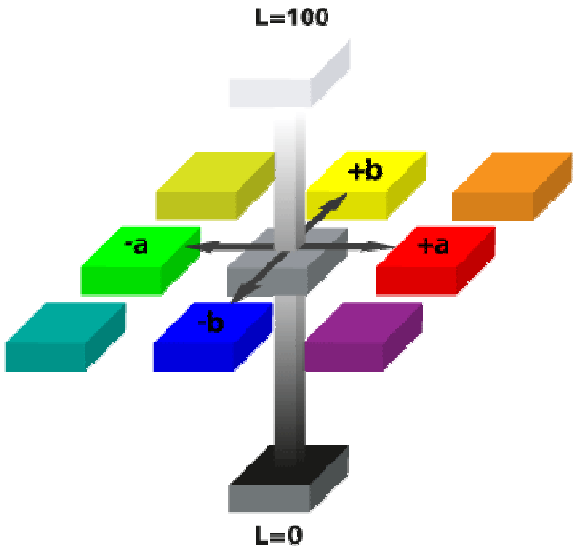

Figure 1b.

Figures adapted from AMSA Meat Color Measurement Guidelines (Hunt, 2012) and www.lump.co/lab-color-space.

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CHAPTER II: Evaluation a Novel Antimicrobial Solution (AMS) against Pathogens of Concern on Fresh Beef

Abstract

Pathogenic bacteria represent a public health concern when present on meat and meat products. Numerous strategies and technologies for reducing and preventing contamination by pathogenic bacteria have been evaluated and applied. A novel antimicrobial solution (AMS) has been developed using GRAS ingredients with potential antimicrobial properties. The objective of this study was to 1) determine survivability of pathogens of concern grown in broth or on plates, 2) to determine the effect of the novel AMS on growth of pathogens inoculated on meat samples, and 3) to determine the optimal concentration of the antimicrobial solution. Pathogens were individually cultured in sterile tryptic soy broth (TSB) tubes or on plate count agar (PCA) plates. Broth grown cells were then harvested by centrifugation while plate grown cells were harvested by first pipetting 1 mL 0.1% peptone onto the plate surface, followed by gentle scraping motions to remove the cells from the plate, and then centrifugation. Pathogens grown in broth and on plates had similar survivability when inoculated onto the surface of beef top round steaks (P=0.31). Based on the similar survivability, broth grown cultures were selected for the remainder of the study. The AMS was prepared at Auburn University and was then diluted to high (stock), medium (1:5 dilution), and low (1:10 dilution) concentrations using distilled water as the solvent. Distilled water also served as the control treatment. Thirty milliliters of the assigned concentration was applied to inoculated meat samples. Samples were placed in sterile stomacher bags and stored at 4°C until 0, 6, 24, or 48 hour sampling. The AMS inhibited the growth of pathogenic bacteria on fresh beef top round steaks (P<0.0001) at all concentrations evaluated. As the concentration of the AMS increased, so did the inhibitory capacity (P<0.0001). The low

concentration was less inhibitory than the high and medium concentrations, but was more inhibitory than the water control. This novel antimicrobial solution (AMS) showed antimicrobial effect and has great application potential for the meat industry.

Introduction

Foodborne illnesses create a concern for public safety. Although the United States has the safest food supply in the world, consumers continue to demand safer products free from pathogenic contamination. The meat industry, most notably the beef and poultry industries, is particularly involved in research and development of strategies to produce safer products. Pathogens of concern on meat and meat products commonly include *Escherichia coli, Salmonella* spp., and *Listeria monocytogenes.*

Escherichia coli (*E. coli*) is a part of the normal microflora of the intestinal tract of most warm-blooded mammals, including humans (Marth, 1998). As few as 10 to 100 cells are required as an infectious dose of enterohemorrhagic *E. coli* (EHEC) (Nester, 2001). Non- O157:H7 serotypes, the "big six" shiga toxin producing *E. coli* (STECs), associated with human illness are O26, O45, O103, O111, O121, and O145 (Brooks et al., 2005). *Salmonella* are commonly present in the environment and in the intestinal tract of warm-blooded animals. *Salmonella* are an important human pathogen associated with meat, particularly poultry and poultry products (Bryan, 1995). *Listeria monocytogenes* is ubiquitous in the environment (Marth, 1998) and is known to be salt and cold tolerant (Scallan, 2011). The infectious dose is not known, although estimations indicate it to be fewer than 1,000 cells. Individuals with compromised immune systems, including newborns, the elderly, pregnant women, and immunocompromised individuals are most susceptible to listeriosis (Marth, 1998; Morey, Bratcher, Singh, & McKee, 2012). Symptoms may be similar for *E. coli, Salmonella,* and *Listeria monocytogenes* illnesses and include abdominal cramps, vomiting, diarrhea, fever, headache, and an overall feeling of exhaustion (D'Aoust, 1991a, 1991b; Nester, 2001).

Evidence suggests the use of topical treatments are effective against *E. coli*, *Salmonella* spp., and *L. monocytogenes* (Avery, 1997; Gao, Zhu, & Zhang, 2013; Guo, Jin, Wang, Scullen, & Sommers, 2014; Huffman, 2002; Mani-Lopez, 2012; Mattick & Hirsch, 1947; Podolak, 1995a, 1995b; Ruckman, Rocabayera, Borzelleca, & Sandusky, 2004; Theron, 2007; Yoder et al., 2012). Some of the antimicrobial compounds which are being developed for food applications include chitosan, nisin, and lauric arginate ester in addition to compounds such as lactic and acetic acid which have been widely utilized in the meat industry for decades.

Lactic acid is "Generally Recognized As Safe" (GRAS) as a food additive and is commonly used in the meat industry as a hot carcass rinse (Huffman, 2002). It has been used to decontaminate red meat carcasses at concentrations of 1-2% without negatively impacting meat quality (Theron, 2007). Lactic acid and other organic acids elicit bactericidal and bacteriostatic affects by reducing the pH of the substrate to a lower level than the intracellular pH, thus disrupting the cell membrane (Chung, 1991). Post-processing spray and dip applications of lactic acid have effectively reduced *L. monocytogenes* (Theron, 2007), *E. coli* O157:H7 and *Salmonella* Typhimurium (Yoder et al., 2012). Similarly, acetic acid, another GRAS organic acid, is utilized in dairy and meat products to target yeast and bacteria growth (Mani-Lopez, 2012). Acetic acid has been shown to reduce growth of pathogenic bacteria on lean beef muscle over time (Podolak, 1995a, 1995b). Acetic acid and other organic acids, are known to be more effective inhibitors of pathogen growth than hot water, though the discoloration and off odor properties associated with acetic acid in particular will determine the concentration(s) which should be utilized (Sun & Holley, 2012). Though lactic and acetic acids have

inhibitory effects on pathogen growth, emerging antimicrobial compounds may offer additional inhibitory capabilities when used in combination with organic acids.

Lauric arginate ester (LAE) is GRAS, is verified nontoxic, and is metabolized to naturally occurring amino acids following consumption (Ruckman et al., 2004). It is a derivative of lauric acid, L-arginine, and ethanol (Kang et al., 2014; Ruckman et al., 2004). LAE causes membrane disruption and disturbance of vital cell functions (Rodriguez, Seguer, Rocabayera, & Manresa, 2004). LAE application in food packaging films has been performed in combination with chitosan (Guo, Jin, Scullen, & Sommers, 2013; Guo et al., 2014). Chitosan is a natural polymer obtained by deacetylation of chitin, the primary component of crustacean shells. Chitosan is verified nontoxic, biodegradable, and biocompatible (Guo et al., 2014). Although the antimicrobial mechanism of chitosan remains unclear, it is thought to involve disruption of the cell membrane as a result of the association between positively charged amino groups of chitosan and negatively charged anions on the surface of bacteria (Friedman & Juneja, 2010). At least one study has evaluated the combined effects of LAE, chitosan, and nisin in a food packaging application (Guo et al., 2014).

Nisin is a well known bacteriocin produced by certain strains of *Lactobacillus lactis* subspecies *lactis*. Nisin has also achieved GRAS status and studies have proven the effectiveness of nisin against a wide range of Gram positive bacteria, including *L. monocytogenes* and spore formers, as well as Gram negative bacteria (Delves-Broughton, Blackburn, Evans, & Hugenholtz, 1996; Henning, 1986). Studies on the application of nisin have been conducted for decades on dairy and processed foods (Delves-Broughton

et al., 1996) with recent focus on the application to fresh meat and poultry (Avery, 1997; Gonzalez-Fandos & Dominguez, 2006).

The known inhibitory effect of these compounds used singularly suggests that a combination would produce improved inhibitory effects. A novel antimicrobial solution (AMS) containing acetic acid, lactic acid, levulinic acid, LAE, and chitosan has been developed with potential application to meats. The current study sought to determine the effects of this novel AMS against pathogens of concern on fresh beef top round steaks. Thus, our objectives were multifaceted. The first objective was to determine differences in survivability of pathogens of concern grown in broth or on plates. *E. coli* (Gram negative) and *Listeria monocytogenes* (Gram positive) were selected for this portion of the study. The broth grown cultures were selected for the remaining portion of this study as well as future studies as this method showed similar survivability compared to PCA plates and offered a more time and resource efficient technique. Once the survivability was determined, our objectives were: 1) to determine the effectiveness of the AMS at inhibiting pathogen growth on inoculated meat samples, and 2) to determine the optimal concentration of the AMS.

Materials and Methods

Experiment 1: Optimum Growth Medium

Culture Strains

Escherichia coli O157:H7 and *Listeria monocytogenes* were selected to represent Gram negative and Gram positive bacteria, respectively. The strains used for this portion of the study are indicated by an asterisk in Table 2. All media was obtained from Neogen

Corporation, (Lansing, Michigan) unless otherwise stated. Cultured microorganisms were transferred individually into 9 mL sterile tryptic soy broth, vortexed (Labnet International, Inc., Edison, New Jersey), and incubated (Jeio Tech, Inc., Des Plaines, Illinois) at 37°C for 24 hours. Approximately 9 log CFU/mL culture suspensions were produced following the overnight incubation and were used for inoculation. Cultures were centrifuged at 37°C with 3650 rpm for 20 minutes (5810R Eppendorf, Hauppauge, New York). The supernatant was discarded and cells were gently washed then resuspended in 0.1% peptone water (Becton Dickinson and Company, Sparks, Maryland).

Growth Medium Preparation

Sterile 9 mL tubes of tryptic soy broth and plate count agar (Becton Dickinson and Company, Sparks, Maryland) petri dishes were prepared according to manufacturer directions. Tubes and plates were labeled for *E. coli* or *Listeria monocytogenes*. Following the 24 hour incubation of the culture strains and preparation of the culture cocktail, broth and plates were inoculated by pipetting 100 µL of the culture cocktail into the broth or onto the surface of the plate. Tubes were gently swirled and plate surfaces were spread using an L-shaped disposable cell spreader (VWR International, LLC, Radnor, Pennsylvania). Two PCA plates and one TSB tube were inoculated for each cocktail; inoculations were performed in triplicate. Tubes and plates were then incubated at 37°C for 24 hours.

Cells were harvested from TSB tubes by centrifugation at 37°C with 3650 rpm for 20 minutes. The supernatant was discarded and cells were gently washed with 0.1% peptone then resuspended in 0.1% peptone water. The resulting cell suspension served as

the “tube grown” inoculum. Cells were harvested from PCA plates by pipetting 1 mL of 0.1% peptone onto the surface of the plate; a cell spreader and gentle circular, scraping motions were used to harvest cultures from the agar surface. The cell suspension created from this process was then pipetted into a tube and brought to a 9 mL volume with 0.1% peptone water. The cell suspension was then centrifuged at 37°C with 3650 rpm for 20 minutes. The supernatant was discarded and cells were gently washed with 0.1% peptone then resuspended in 0.1% peptone water. The resulting cell suspension served as the “plate grown” inoculum. Serial dilutions of each inoculum, broth and plate grown, were performed in 9 mL tubes of 0.1% peptone and surface plated onto PCA plates; inoculum tubes were held for use the following day. Culture plates were enumerated following 24 hours incubation at 37°C to determine number of cells harvested and inoculums were diluted to 8 log CFU/mL as determined by a spectrometer (Amersham Biosciences Corporation, Piscataway, New Jersey) absorbance reading of 0.60. Results are presented in CFU/cm^2.

Sample Preparation

Fresh beef top round steaks were fabricated at the Lambert Powell Meats Laboratory at Auburn University without the use of antimicrobial solutions. Lean meat samples were cut to 100 cm^2 pieces. Each piece was individually inoculated with 100 µL of inoculum (either broth grown or plate grown) which was then spread using a cell spreader. Thirty minutes of contact time was allowed for cell adhesion to the meat surface. After the allowed contact time, samples were stored in sterile stomacher bags (Nasco Whirl-Pak, Fort Atkinson, Wisconsin) for 0, 24, 48, or 72 hours.

A modified plating technique using 0.1% peptone was utilized (Podolak, 1995a). One hundred mL of 0.1% peptone was added to each meat sample in stomacher bags prior to stomaching for 2 minutes at 300 rpm (400 Circular Seward Medical, London, England). Serial dilutions with 9 mL 0.1% peptone were created and dilutions were plated onto PCA plates. Plates were enumerated following 24 hours of incubation at 37°C. Results are presented in CFU/cm^2.

Statistical Analysis

A completely randomized design was used to conduct these experiments. Each experiment was conducted in triplicate with three replications performed in separate weeks. All data were converted to $log_{10}CFU$ prior to performing statistical analysis. The independent variables were treatment (growth medium), time, and pathogen/inoculum level and $log_{10}CFU$ was the dependent variable evaluated. Statistics were completed using the Proc Mixed procedure in SAS version 9.2 (SAS Institute, Inc., Cary, NC). All appropriate two and three way interactions were evaluated. In the event that no interactions were observed, main effects were evaluated. Least squares means were used to separate mean differences. There were no differences in replications and no treatment by replication interactions were included as no practical differences observed. Tukey pairwise comparisons were utilized due to potential unequal sample size resulting from the removal of data points due to contamination. Data are presented with pooled standard error.

Experiment 2: Effects of Antimicrobial Solution (AMS)

Culture Preparation

Five strains of *Escherichia coli* O157:H7, one strain of each of the big six STECs, five strains of *Salmonella* spp., and five strains of *Listeria monocytogenes* were used for this study as detailed in Table 2. All media was purchased from Neogen Corporation (Lansing, Michigan) unless otherwise stated. Cultured microorganisms were transferred individually to 9 mL sterile tryptic soy broth, vortexed (Labnet International, Inc., Edison, New Jersey), and incubated at 37°C for 24 hours (Jeio Tech, Inc., Des Plaines, Illinois). The overnight culture produced approximately 9 log CFU/mL culture suspensions. Equal parts of each strain were combined and vortexed to create the culture cocktail. Cells were harvested by centrifugation at 3650 rpm for 20 minutes at 37°C. The supernatant was discarded and the resulting pellet was gently washed before being resuspended in 0.1% peptone. The cell suspension was then diluted to a concentration of 4 or 6 log which was used to inoculate meat samples.

AMS Preparation

Food grade LAE (CytoGuard LA 2X; A&B Ingredients, 24 Spielman Road, Fairfield, NJ), levulinic acid (natural, 99%, FG; Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO), chitosan (low molecular weight; Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO), acetic acid (natural, ≥99.5%, FG; Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO), and lactic acid (natural, ≥85% FG; Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO) were obtained. Compounds were weighed and mixed into MilliQ water (Milli-Q Integral Water Purification System, Darmstadt, Germany) at Auburn University.

The AMS was allowed to mix overnight on a stir plate (VWR International, LLC, Radnor, Pennsylvania) and refrigerated at 4°C prior to use. On the day of use, the AMS was diluted using deionized distilled water (Barnstead Mega-Pure System Automatic Water Distillation Apparatus, Thermo Scientific, Waltham, MA) as the solvent to a high (stock), medium (1:5 dilution), or low (1:10 dilution) concentration. The pH values of the AMS were: high concentration pH=3.04, medium concentration pH=4.15, low concentration pH=5.63, and distilled water pH=6.72.

Sample Preparation

Fresh beef top round steaks were fabricated at the Lambert Powell Meats Laboratory at Auburn University without the use of antimicrobial solutions. Lean meat samples were cut to 100 cm^2 pieces. Each piece was individually inoculated with 1 mL of the assigned inoculum (either 4 or 6 log of *E. coli*, STECs, *Salmonella* spp., or *L. monocytogenes*) which was then spread using a disposable L-shaped cell spreader (VWR International, LLC, Radnor, Pennsylvania). Thirty minutes of contact time was allowed for cell adhesion to the meat surface. After the allowed contact time, samples were treated with 30 mL of the assigned concentration of the AMS. A control treatment of distilled water was also tested. Samples were then stored at 4°C in sterile stomacher bags (Nasco Whirl-Pak, Fort Atkinson, Wisconsin) for 0, 6, 24, or 48 hours.

A modified plating technique using 0.1% peptone was utilized (Podolak, 1995a). One hundred mL of 0.1% peptone was added to each meat sample in stomacher bags prior to stomaching for 2 minutes at 300 rpm (400 Circular Seward Medical, London, England). Serial dilutions with 9 mL 0.1% peptone were created and dilutions were

plated onto MacConkey Agar with Sorbitol (MSA; *E. coli*), XLT4 (*Salmonella* spp.), or Modified Oxford Medium (MOX; *L. monocytogenes*) plates. Plates were enumerated following 24 hours incubation at 37°C. Results are presented in $\log_{10}$CFU.

Statistical Analysis

A completely randomized design was used to conduct these experiments. Each experiment was conducted in triplicate with two replications performed in separate weeks. All data were converted to $\log_{10}$CFU prior to performing statistical analysis. The independent variables were treatment (concentration of AMS), time, and pathogen/inoculum level and $\log_{10}$CFU was the dependent variable evaluated. Statistics were completed using the Proc Mixed procedure in SAS version 9.2 (SAS Institute, Inc., Cary, NC). All appropriate two and three way interactions were evaluated. In the event that no interactions were observed, main effects were evaluated. Least squares means were used to separate mean differences. There were no differences in replications and no treatment by replication interactions were included as no practical differences observed. Tukey pairwise comparisons were utilized due to potential unequal sample size resulting from the removal of data points due to contamination. Data are presented with pooled standard error.

Results and Discussion

Experiment 1: Optimum Growth Medium

No differences were observed in time (P=0.92), indicating that pathogens survived the same on the meat samples regardless of storage time following inoculation.

Interestingly, there were no differences observed in survival of pathogens from the two different growth mediums (P=0.31). Although the plate grown cultures had slightly higher counts at the 24 hour sampling, this was only a numerical increase and the counts were not different (P=0.31). The initial hypothesis was that the plate grown cultures would be slightly more hardy. This was hypothesized due to the necessity to adhere to the plate surface, growth on an agar medium requires structural adaptations to attach whereas a liquid medium allows for free growth, and would thus better survive when inoculated onto the surface of the meat samples.

Mean CFU counts and standard errors are presented in Figure 2. The broth growth medium was chosen for future studies for several reasons. Primarily, the expense and required resources to grow pathogenic cultures in glass tubes with TSB broth are less than the requirements to grow cultures on plates. Additionally, the time required to prepare the plates, plate the cultures, and harvest cells from each individual plate is more than twice the amount of time required for broth grown cultures. Finally, given that the survivability of broth grown cultures is similar to plate grown cultures, the broth medium was selected as a more economic and efficient growth medium.

Experiment 2: Effects of Antimicrobial Solution (AMS)

The pH values as well as the inhibitory capacity of the AMS at high, medium, and low concentrations are outlined in Table 3. As expected, the pH of the AMS increased as the concentration decreased such that the pH of the low concentration of AMS was 5.63, the pH of the medium concentration of AMS was 4.15, the pH of the high concentration of AMS was 3.04, and the pH of the distilled water was 6.72. The pH of all three

concentrations of AMS evaluated were different from the pH of the distilled water (P<0.0001). The pH of each of the three concentrations of AMS also differed from one another (P=0.0017). This is in agreement with previous research which demonstrates that bacteria generally do not grow below a pH of 4.6 (Chung & Goepfert, 1970). As the concentration of the AMS increased, so did its ability to inhibit the growth of pathogenic bacteria of concern (P<0.0001; Table 3). The low concentration differed from the high and medium concentrations (P<0.0001) and the water control treatment (P<0.0001; Table 3).

An interaction of pathogen by AMS (P<0.0001) was observed. The different pathogens utilized in this research behaved differently when exposed to varying concentrations of the AMS, as was expected. The effects of the AMS against *E. coli* O157:H7 (Figure 3), the non-O157:H7 STECs (Figure 4), *Salmonella* spp. (Figure 5), and *L. monocytogenes* (Figure 6) are presented. The high and medium concentrations of AMS were more inhibitory (P<0.0001) against *E. coli* O157:H7 and the non-O157:H7 STECs as determined by the least squares means. All three concentrations evaluated were inhibitory against *Salmonella* spp (P<0.0001). The AMS inhibited *L. monocytogenes* growth; however, the inhibition was less clearly defined compared to the other pathogenic bacteria evaluated. It is believed that the AMS exhibited bactericidal activity towards Gram negative pathogenic bacteria but may exhibit inhibitory activity toward Gram positive pathogenic bacteria. As previously stated, the medium and high concentration of AMS demonstrated consistent inhibitory capacity against pathogenic bacteria of concern on fresh beef. Based on these findings, the medium concentration of

AMS was chosen for future research as it is the lowest concentration which exhibited consistent inhibitory capacity.

Meat samples in this study were stored at 4°C which may have affected membrane permeability and reduced nutrient uptake, protein synthesis, and enzyme functionality, contributing to reduced pathogen growth (Graumann & Marahiel, 1999). Meat subjected to temperature abuse may respond to treatment with AMS slightly differently. However, previous research conducted on ready-to-eat frozen shrimp and ready-to-eat presliced turkey deli meat suggest that temperature of the food product when AMS is applied does not negatively impact the inhibition of bacteria (Chen, Jin, Gurtler, Geveke, & Fan, 2012; Jin & Gurtler, 2012). This study confirms previous findings of the inhibitory affect of the AMS against pathogenic bacteria (Chen et al., 2012l Jin & Gurtler, 2012; Guo, Jin, Scullen, & Sommers, 2013; Guo et al., 2014; Guo, Jin, Wang, Scullen, & Sommers 2014).

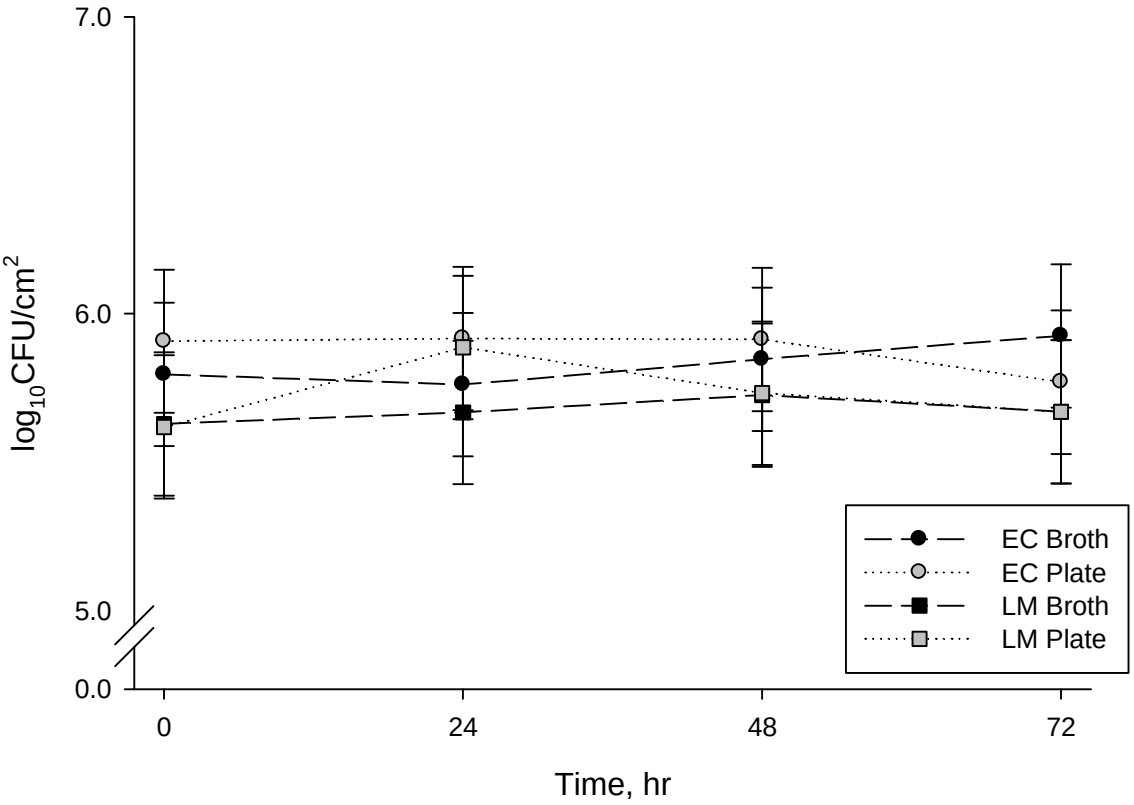
Conclusions

Cultures grown in TSB tubes and on PCA plates have comparable survivability when inoculated onto the surface of meat samples. The 30 minute adhesion time produced similar results in the 0 hour samples which were serially diluted and plated immediately following the adhesion time. Samples stored for 24, 48, and 72 hours prior to serial dilutions and plating also produced comparable results. Though the initial hypothesis was that the plate grown cultures would be more hardy, it is possible that our technique of harvesting and plating negated some potential differences in cell hardiness and adhesion.

This study indicates the survivability of broth and plate grown cultures to be similar, given the technique employed herein. The broth medium is more time efficient, requires fewer resources, and produces a consistent culture growth. Though the survivability of plate grown cultures is comparable to broth grown cultures, plate growth requires additional time and resources. At least ten plates are required to sufficiently recover 9 mL of cell suspension, a feat accomplished with one TSB tube. This formed the basis of the decision to utilize cultures grown in broth medium for future studies.

The AMS effectively inhibits growth of pathogenic bacteria on fresh top round beef steaks. All concentrations of the AMS exhibited some level of inhibition of pathogen growth; however, the pathogens utilized in this research behaved slightly differently to the low concentration. Thus, the medium concentration of the AMS was selected for future research as it is the lowest concentration with the most consistent inhibitory capability. Additional research is needed to elicit the mechanism by which the AMS inhibits growth of pathogenic bacteria. Research is also needed to validate the bactericidal or inhibitory properties of the solution. The AMS has great potential application in the meat industry. Some proposed applications to inhibit growth of pathogenic bacteria are to apply the solution topically as a spray, a dip, or an immersion, to include as an ingredient in product formulation, or to include as an ingredient in marinade solutions to add convenience and value to products. Additional research will be needed to determine the most practical and cost-effective application method for the industry.

Figure 2: Culture counts following different growth mediums.



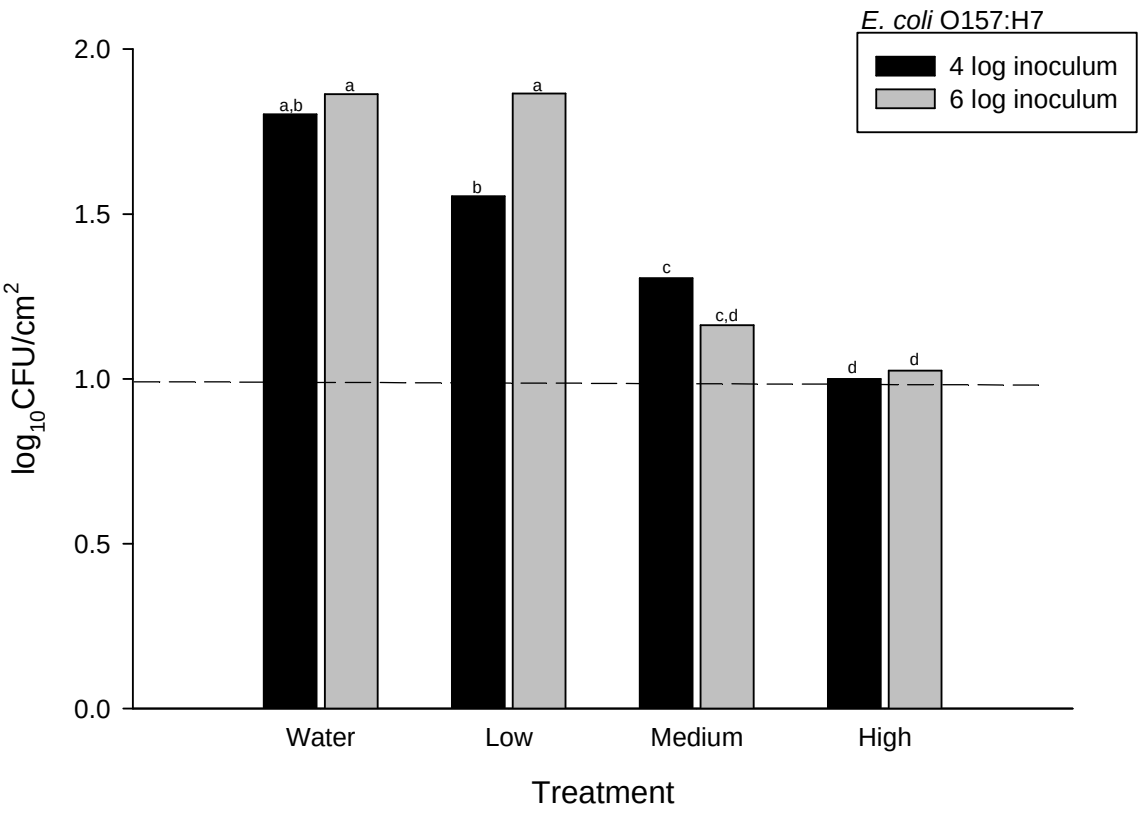
Data are presented in least squares means with pooled SEM. No differences were observed between cultures grown in broth or on plates (*P*=0.2792). Meat samples were inoculated with *E. coli* (EC) or *L. monocytogenes* (LM) and stored at 4°C until sampling at 0, 24, 48, or 72 hours. Broth indicates tryptic soy broth; plate indicates plate count agar.

Table 3: Effect of the AMS at high, medium, and low concentrations and of distilled water.

Antimicrobial Concentration	**log_{10}CFU lsmean**	**Pooled SEM**
Distilled Water	1.8177[a]	0.0340
Low (1:10)	1.5174[b]	0.0340
Medium (1:5)	1.1828[c]	0.0340
High (stock)	1.0079[c]	0.0340

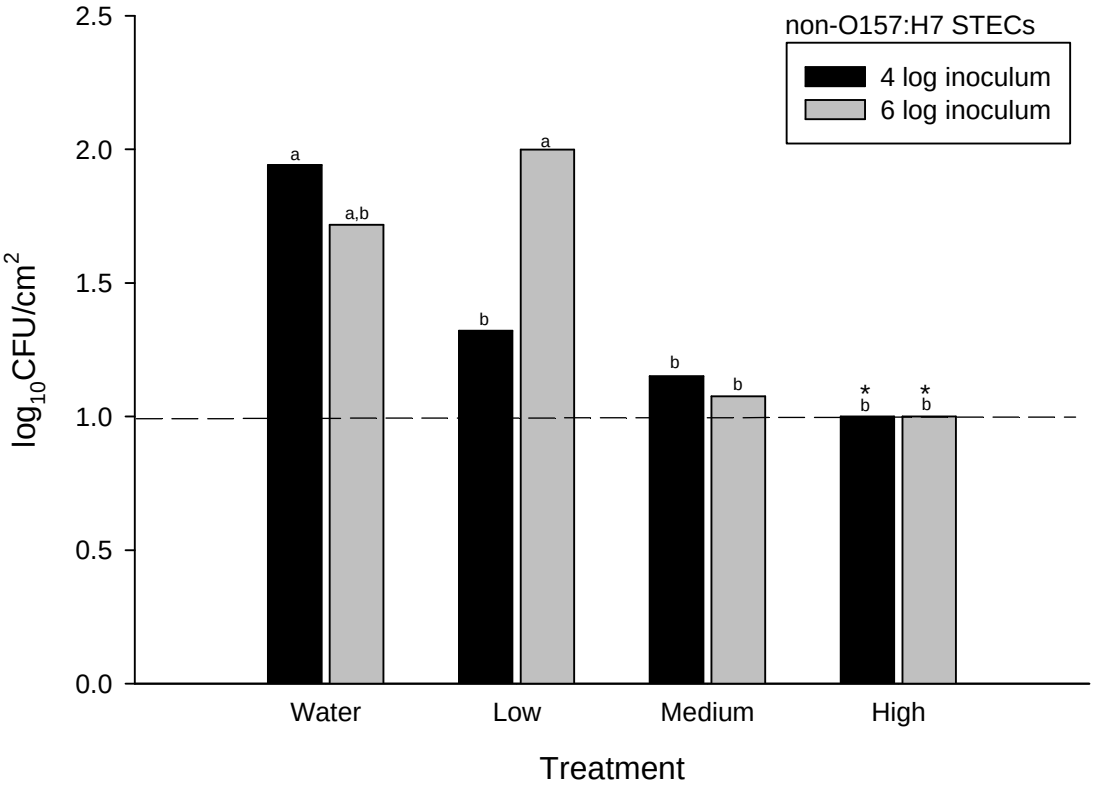
[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM (P<0.0017). AMS was prepared at Auburn University and diluted to high (no dilution), medium (1:5), or low (1:10) concentrations the morning of the experiment. Dilutions were prepared using distilled water which also served as the control treatment.

Figure 3: Effect of AMS against *Escherichia coli* O157:H7 on fresh beef top round.



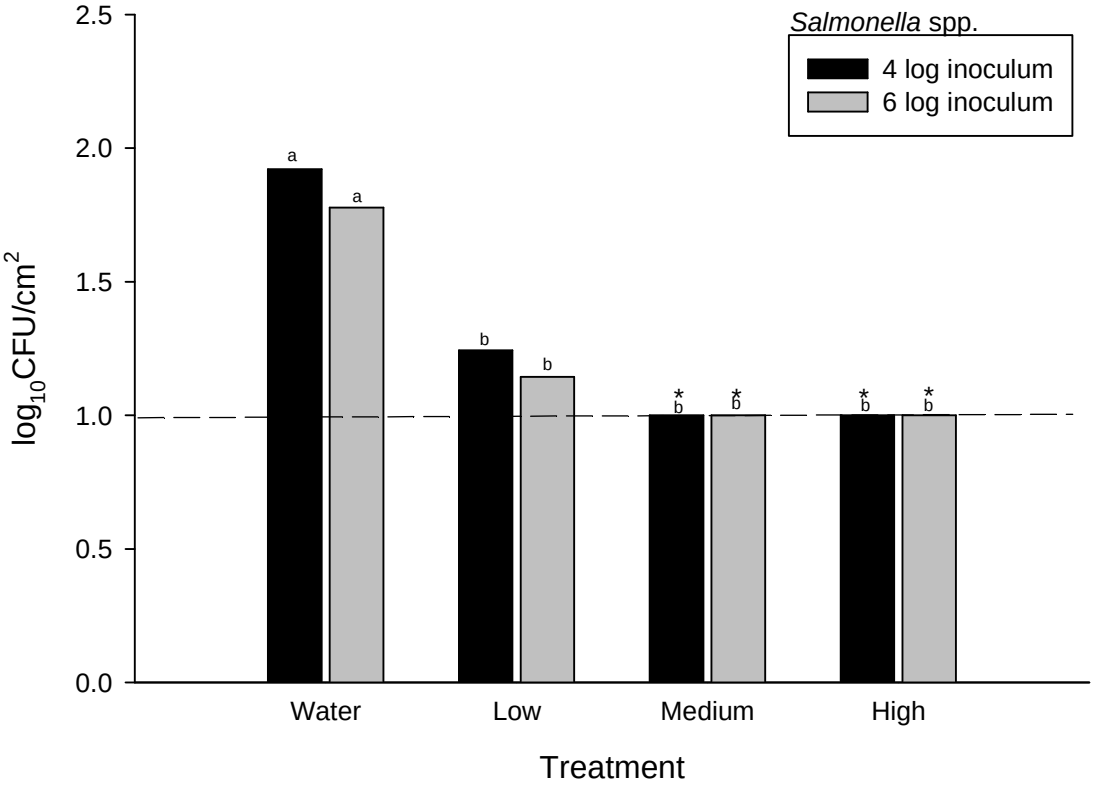
[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM of 0.0962 ($P<0.05$). The dashed line indicates the limit of detection which is 1 log, an * indicates the estimate is below the limit of detection. 4 and 6 log cultures of *E. coli* were prepared and inoculated onto meat samples. AMS was prepared at Auburn University and diluted to high (no dilution), medium (1:5), or low (1:10) concentrations the morning of the experiment. Dilutions were prepared using distilled water which also served as the control treatment.

Figure 4: Effect of AMS against non- O157:H7 STEC on fresh beef top round.



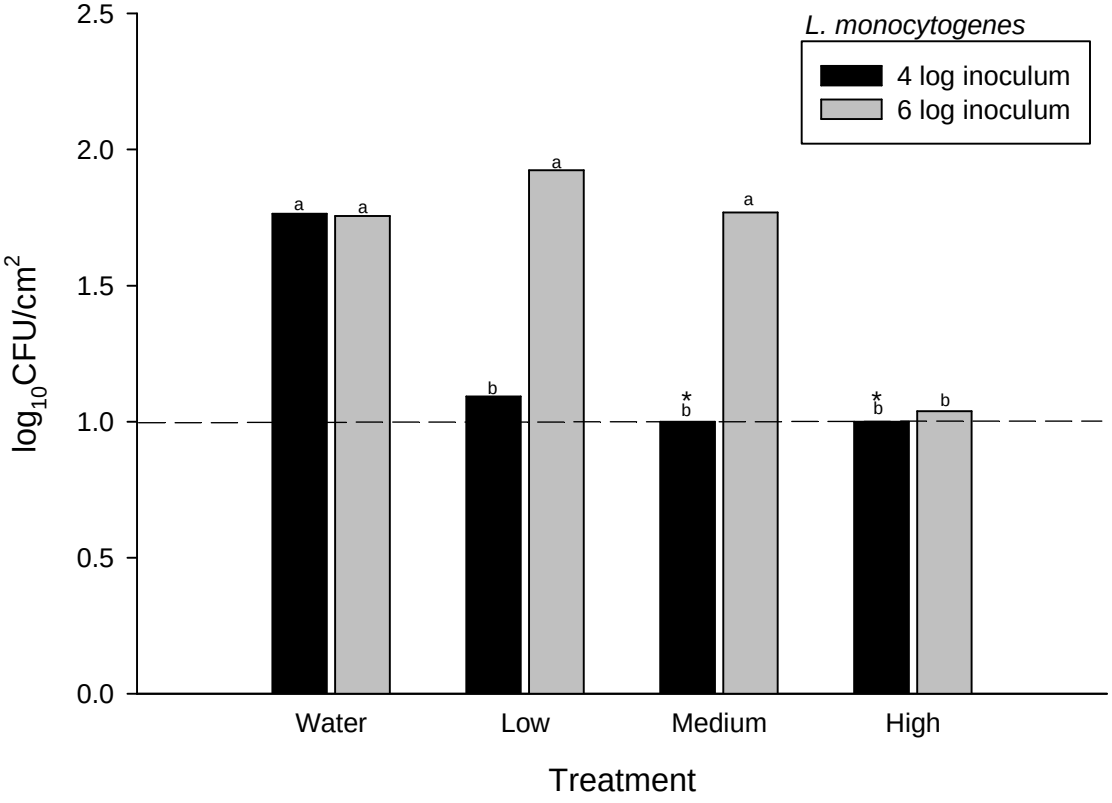
[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM of 0.0962 ($P<0.05$). The dashed line indicates the limit of detection which is 1 log, an * indicates the estimate is below the limit of detection. 4 and 6 log cultures of STECs were prepared and inoculated onto meat samples. AMS was prepared at Auburn University and diluted to high (no dilution), medium (1:5), or low (1:10) concentrations the morning of the experiment. Dilutions were prepared using distilled water which also served as the control treatment.

Figure 5: Effect of AMS against *Salmonella* spp. on fresh beef top round.



[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM of 0.0962 ($P<0.05$). The dashed line indicates the limit of detection which is 1 log, an * indicates the estimate is below the limit of detection. 4 and 6 log cultures of *Salmonella* were prepared and inoculated onto meat samples. AMS was prepared at Auburn University and diluted to high (no dilution), medium (1:5), or low (1:10) concentrations the morning of the experiment. Dilutions were prepared using distilled water which also served as the control treatment.

Figure 6: Effect of AMS against *Listeria monocytogenes* on fresh beef top round.



[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM of 0.0962 ($P<0.05$). The dashed line indicates the limit of detection which is 1 log, an * indicates the estimate is below the limit of detection. 4 and 6 log cultures of *L. monocytogenes* were prepared and inoculated onto meat samples. AMS was prepared at Auburn University and diluted to high (no dilution), medium (1:5), or low (1:10) concentrations the morning of the experiment. Dilutions were prepared using distilled water which also served as the control treatment.

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CHAPTER III: Evaluation of Three Retail Marinades Against Pathogens of Concern on Fresh Beef

Abstract

The presence of pathogenic bacteria on foods represents a concern for public health. Numerous strategies and technologies for reducing and preventing contamination by pathogenic bacteria have been evaluated and applied; however, one simplistic approach to controlling the growth of pathogenic bacteria of concern is to marinate meat products. Researchers have evaluated the effect of marinades against pathogens of concern in meat; however, there has been little evaluation of retail marinades. The objective of this study was to determine the effect of three retail marinades against growth of pathogenic *E. coli*, *Salmonella* spp., and *L. monocytogenes* on inoculated beef top round samples. Pathogens were individually cultured in sterile trypic soy broth (TSB) tubes before cells were harvested by centrifugation. The cell suspension was then used to inoculate the surface of fresh beef top round steaks. Marinades were chosen based on market and food trends in early 2014 and were acquired from the local Publix Super Market location in Auburn, AL. The marinades were ready to use at purchase and required no additional mixing or reconstitution. Marinades chosen were: 1) Ken's Steakhouse Marinade & Sauce, Balsamic & Roasted Onion, 2) KC Masterpiece 30 Minute Marinade, California Style Lemon & Cracked Pepper, and 3) KC Masterpiece 30 Minute Marinade, Classic Steakhouse. Distilled water served as the control treatment. A volume of 30 mL of the assigned treatment was applied to inoculated meat samples. Samples were placed in sterile stomacher bags and stored at 4°C until 0, 6, 24, or 48 hour sampling. All three marinades inhibited the growth of pathogenic bacteria on fresh beef top round steaks ($P<0.0001$). The lemon pepper marinade was slightly more inhibitory of the growth of pathogenic bacteria than the balsamic and roasted onion or classic

steakhouse marinades (P<0.05). The balsamic and roasted onion and classic steakhouse marinades did not differ in their inhibition of the growth of pathogenic bacteria (P<0.9585). All marinades were more inhibitory of pathogenic bacteria growth compared to the water control (P<0.0001). Marination of meat products has the potential to improve meat safety by inhibiting the growth of pathogenic bacteria, improve flavor, improve tenderness, and improve juiciness of the product.

Introduction

The food industry, particularly the meat industry, is under constant scrutiny in developed countries to produce safer food in the wake of outbreaks of foodborne illnesses. While most outbreaks of foodborne illness cause mild to moderate illness, sometimes serious illness results in death which is of particular concern in elderly and immunocompromised individuals. Meat, poultry, milk, and eggs are the primary foods of concern leading to human illness due to undercooking or cross contamination. While foodborne pathogens are subjected to physical, chemical, and nutritional stresses during processing (Yousef, 2003), there are still instances when pathogens survive and a person may become ill following consumption of those pathogens. *Escherichia coli* O157:H7, non-O157:H7 shiga toxin producing *E. coli* (STEC) serotypes, *Salmonella* spp. and *Listeria monocytogenes* are the main focus of companies, research scientists, and regulatory agencies in regards to outbreaks of foodborne illness linked to meat.

One value-adding food preparation step which has been found to provide an additional layer of food safety by inhibiting growth of pathogenic bacteria such as *E. coli*, *Salmonella* spp., and *L. monocytogenes* is marination. Marinades are typically a water-oil emersion containing a combination of sugar, salt, acids (acetic and citric acids), additives (Xanthan and guar gum), spices, sorbates, benzoates, and aroma enhancers (Bjorkroth, 2005). Commercial marination practices rely primarily on salt-water and phosphate formulations which increase tenderness, juiciness, and yield with current applications including injection technology, immersion, and vacuum tumbling (Alvarado & Mckee, 2007). Consumers rely on immersion for marination of meat at home by placing the meat in a suitable container and covering it with a marinade. A variety of spices and spice

extracts are utilized in the industry to cater to specific flavor profiles and taste preferences. Marinated meat products, particularly poultry products, represent a growing segment of the food industry on a global scale.

Studies indicate that marinade sauces prevent the growth of spoilage organisms based on a low pH, high salt concentration, sorbates and benzoates, and various spices (Bjorkroth, 2005). Marinades with paprika, garlic, coriander, salt (NaCl), sodium phosphates, and yogurt have been effective in reducing survival of *Campylobacter* cells (Birk & Knochel, 2009; Perko-Makela, Koljonen, Miettinen, & Hanninen, 2000). Teriyaki marinades, typically thick and highly acidic sauces with powdered onion, garlic, spices, and powdered soy sauce as the dominant ingredients, have shown bactericidal activity (Pathania, McKee, Bilgili, & Singh, 2010).

One study used three strains (Heidelberg, Typhimurium, and Senftenberg) to determine the effect of commercially available teriyaki and lemon pepper marinades on the survival of *Salmonella* (Pathania et al., 2010). Both teriyaki and lemon pepper marinades showed reduction of *Salmonella* spp. during the 32 hour storage period with greater reductions observed in marinades maintained at 4°C (Pathania et al., 2010). Other researchers have indicated that Gram positive bacteria are more sensitive to citrus essential oils *in vitro* than Gram negative bacteria (Fisher & Phillips, 2006). Additional reports indicate a higher activity of orange, lemon, grapefruit, and mandarin citrus oils and their derivatives *in vitro* (Dabbah, Edwards, & Moats, 1970).

Marination has the potential to increase the shelf life of meat products while adding convenience and value for the consumer. Thus, the objective of this study was to determine the effect of three marinades available at retail stores against growth of

pathogenic *E. coli*, *Salmonella* spp., and *L. monocytogenes* on inoculated beef top round samples.

Materials and Methods

Culture Preparation

Five strains of *Escherichia coli* O157:H7, one strain of each of the big six STECs, five strains of *Salmonella* spp., and five strains of *Listeria monocytogenes* were used for this study as detailed in Table 2. All media was purchased from Neogen Corporation (Lansing, Michigan) unless otherwise stated. Cultured microorganisms were transferred individually to 9 mL sterile tryptic soy broth, vortexed (Labnet International, Inc., Edison, New Jersey), and incubated at 37°C for 24 hours (Jeio Tech, Inc., Des Plaines, Illinois). The overnight culture produced approximately 9 log CFU/mL culture suspensions. Equal parts of each strain were combined and vortexed to create the culture cocktail. Cells were harvested by centrifugation at 3650 rpm for 20 minutes at 37°C. The supernatant was discarded and the resulting pellet was gently washed before being resuspended in 0.1% peptone. The cell suspension was then diluted to a concentration of 4 or 6 log which was used to inoculate meat samples.

Sample Preparation

Fresh beef top round steaks were fabricated at the Lambert Powell Meats Laboratory at Auburn University without the use of antimicrobial solutions. Lean meat samples were cut to 100 cm^2 pieces. Each piece was individually inoculated with 1 mL of the assigned inoculum (either 4 or 6 log of *E. coli*, STECs, *Salmonella* spp., or *L.*

monocytogenes) which was then spread using a disposable L-shaped cell spreader (VWR International, LLC, Radnor, Pennsylvania). Thirty minutes of contact time was allowed for cell adhesion to the meat surface. After the allowed contact time, samples were treated with 30 mL of the assigned marinade. A control treatment of deionized distilled water (Barnstead Mega-Pure System Automatic Water Distillation Apparatus, Thermo Scientific, Waltham, MA) was also tested. Samples were then stored at 4°C in sterile stomacher bags (Nasco Whirl-Pak, Fort Atkinson, Wisconsin) for 0, 6, 24, or 48 hours.

A modified plating technique using 0.1% peptone was utilized (Podolak, 1995). One hundred mL of 0.1% peptone was added to each meat sample in stomacher bags prior to stomaching for 2 minutes at 300 rpm (400 Circular Seward Medical, London, England). Serial dilutions with 9 mL 0.1% peptone were created and dilutions were plated onto MacConkey Agar with Sorbitol (MSA; *E. coli*), XLT4 (*Salmonella* spp.), or Modified Oxford Medium (MOX; *L. monocytogenes*) plates. Plates were enumerated following 24 hours incubation at 37°C. Results are presented in $\log_{10}$CFU.

Marinade Selection

Retail marinades were chosen from commonly available marinades at the local Publix Super Market (Auburn, AL). Marinades chosen were: 1) Ken's Steakhouse Marinade & Sauce, Balsamic & Roasted Onion, 2) KC Masterpiece 30 Minute Marinade, California Style Lemon & Cracked Pepper, and 3) KC Masterpiece 30 Minute Marinade, Classic Steakhouse. All bottles of marinade were ready to use at purchase and required no additional mixing or reconstitution. A volume of 30 mL of the assigned marinade was applied to the surface of the inoculated meat sample and stored until the appropriate

sampling time at 0, 6, 24, or 48 hours. The pH values of the marinades were collected prior to application to the surface of the meat. pH values were 3.57 for the balsamic and roasted onion marinade, 2.85 for the lemon pepper marinade, and 3.67 for the classic steakhouse marinade.

Statistical Analysis

A completely randomized design was used to conduct these experiments. Each experiment was conducted in triplicate with two replications performed in separate weeks. All data were converted to $\log_{10}$CFU prior to performing statistical analysis. The independent variables were treatment (retail marinade), time, and pathogen/inoculum level and $\log_{10}$CFU was the dependent variable evaluated. Statistics were completed using the Proc Mixed procedure in SAS version 9.2 (SAS Institute, Inc., Cary, NC). All appropriate two and three way interactions were evaluated. In the event that no interactions were observed, main effects were evaluated. Least squares means were used to separate mean differences. There were no differences in replications and no treatment by replication interactions were included as no practical differences observed. Tukey pairwise comparisons were utilized due to potential unequal sample size resulting from the removal of data points due to contamination. Data are presented with pooled standard error.

Results and Discussion

An interaction of pathogen by marinade was observed (P=0.0002). The interaction of marinade and *E. coli* O157:H7 is presented in Figure 7, non-O157:H7

STECs in Figure 8, *Salmonella* spp. in Figure 9, and *Listeria monocytogenes* in Figure 10. The lemon pepper and classic steakhouse were slightly more inhibitory against *E. coli* while the balsamic and roasted onion and lemon pepper marinades were slightly more inhibitory against *Listeria monocytogenes*. Growth of *Salmonella* spp. was inhibited by balsamic and roasted onion, lemon pepper, and classic steakhouse marinades. This is in agreement with another study which found commercial teriyaki and lemon pepper marinades reduced *Salmonella* spp. during a 32 hour storage period (Pathania et al., 2010). The marinades were more effective when used to treat samples inoculated with 4 log cell culture as compared to the 6 log cell culture. It is hypothesized that this difference is due to the number of pathogenic cells present and may be overcome with the addition of or treatment with a larger volume of the marinade. The ingredients used for each marinade also contribute to an enhanced antimicrobial affect. Previous research indicates citrus oils, paprika, garlic, coriander, salt, and sodium phosphates have antimicrobial affects as well (Perko-Makela et al., 2000; Fisher & Phillips, 2006).

An interaction of marinade by time was observed (P=0.0414). This interaction is presented in Figure 11. The general trend from this interaction is that the growth is further inhibited with an increase in the duration of exposure to the marinade, consistent with previous research (Rhoades, Kargiotou, Katsanidis, & Koutsoumanis, 2013). The one exception to this trend is the 0 and 6 hour samples from the classic steakhouse marinade. The CFU count increased slightly from 0 (2.6927) to 6 (2.7333) hours with the classic steakhouse marinade before decreasing at the 24 (2.5019) hour sampling. Despite the slight numerical increase, the 0 and 6 hour samples did not differ (P>0.05) from one

another. However, both the 0 and 6 hour samples differed from the 24 hour samples ($P<0.05$).

The pathogens responded differently to the three marinades. Though the pH values of the marinades were similar, it is hypothesized that the varied response may be, in part, due to differences in pH. Though there are some exceptions, microorganisms generally do not grow below a pH of 4.6 (Chung & Goepfert 1970). The lemon pepper marinade had the lowest pH and was more inhibitory against growth of pathogenic bacteria of concern compared to the water ($P<0.0001$), balsamic and roasted onion ($P<0.0075$), or classic steakhouse ($P<0.0089$) marinades (Figure 11). The balsamic and roasted onion and classic steakhouse marinades were similar in the inhibition of growth of pathogenic bacteria of concern ($P=0.9585$) and had similar pH values. Additionally, the individual components of the marinades may contribute to an increased capacity to inhibit the growth of pathogenic bacteria. The lemon pepper marinade contained a greater amount of black pepper in the marinade formulation as well as oils of lemon, both of which have been implicated in the inhibition of the growth of pathogenic bacteria (Pathania et al., 2010).

Conclusions

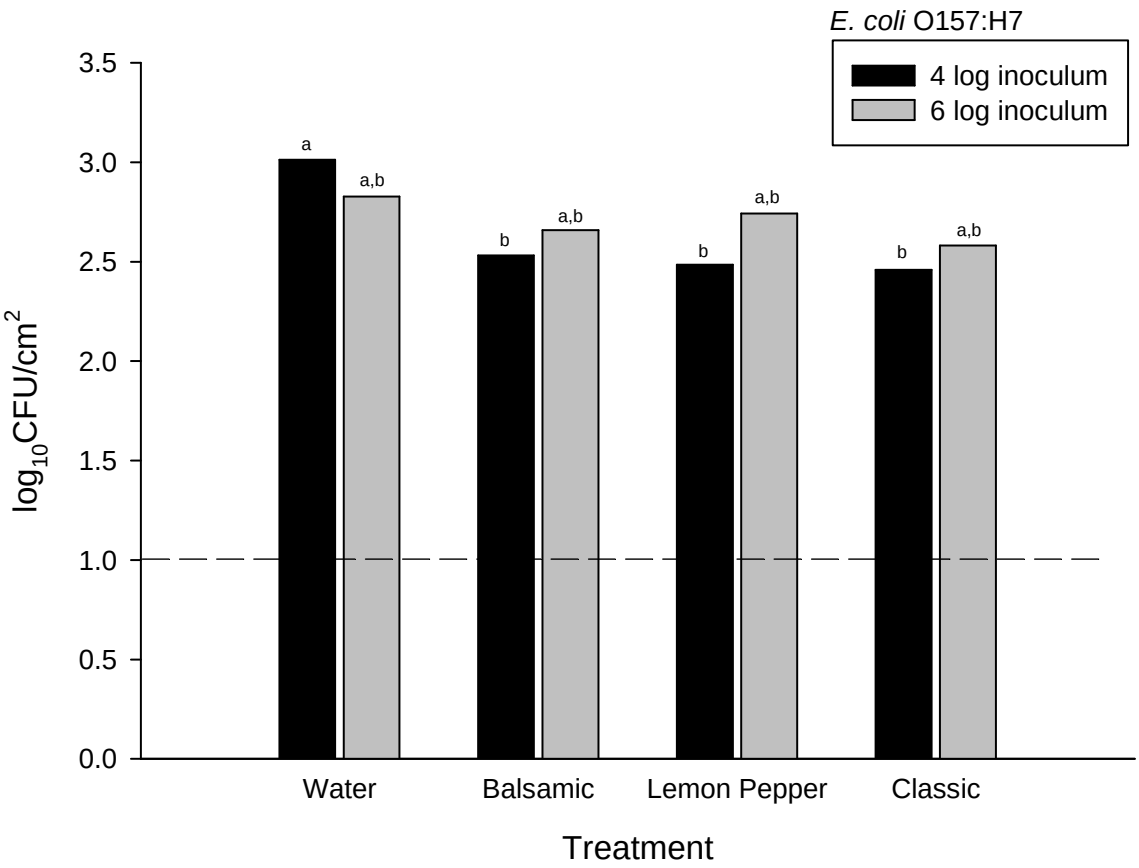
All three of the marinades chosen for this study inhibit growth of pathogenic bacteria inoculated onto the surface of meat samples. Those samples inoculated with 4 log concentration of pathogenic bacteria were inhibited to a greater extent than the samples inoculated with 6 log concentration. It is hypothesized that this difference is due to the number of cells present and may be overcome by using a greater volume of the

marinades. For our purposes, the 30 mL volume sufficiently covered the surface of the meat and mimicked the type of marination which consumers may perform in their home.

During the course of this research, the balsamic & roasted onion marinade was discontinued in the area. The manufacturer was not able to locate the marinade within 250 miles of Auburn nor could they guarantee enough of the marinade from other locations to meet our needs. As a result, the balsamic and roasted onion marinade was removed from future research due to the lack of availability in the area and the similar pH and performance of the classic steakhouse marinade.

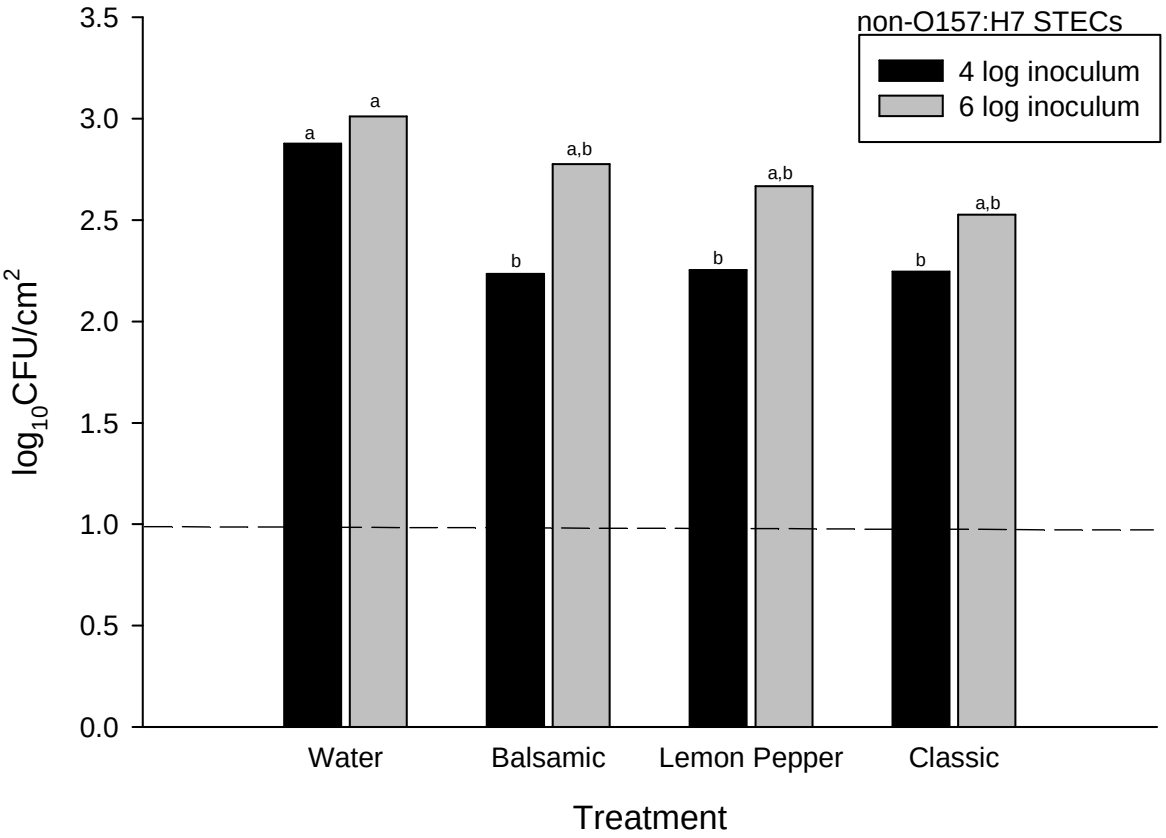
Marination offers additional value and convenience to the consumer. Marination of meat products enhances tenderness, juiciness, and flavor of the product while also creating a small amount of protection against overcooking. The flavor profile of meat, especially poultry, can be changed with marination to accommodate the taste preferences of the consumer. Marination of meat products also inhibits growth of pathogenic bacteria of concern on fresh top round beef steaks and may offer an improved level of food safety.

Figure 7: Effect of retail marinades against *Escherichia coli* O157:H7 on fresh beef top round.



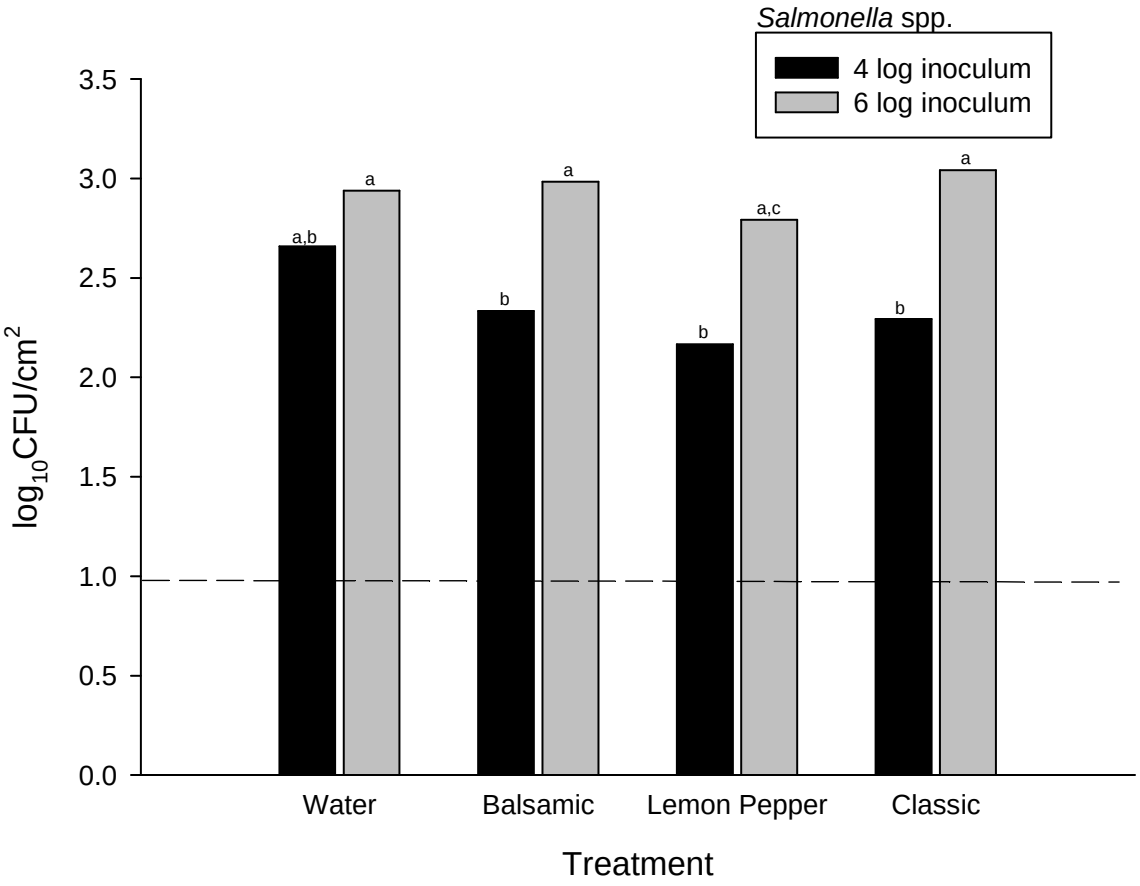
[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM of 0.1002 ($P<0.05$). The dashed line indicates the limit of detection which is 1 log. 4 and 6 log cultures were prepared and inoculated onto meat samples. Marinades were purchased from the Auburn, AL, Publix Super Market. Distilled water served as the control treatment.

Figure 8: Effect of retail marinades against non-O157:H7 STEC on fresh beef top round.



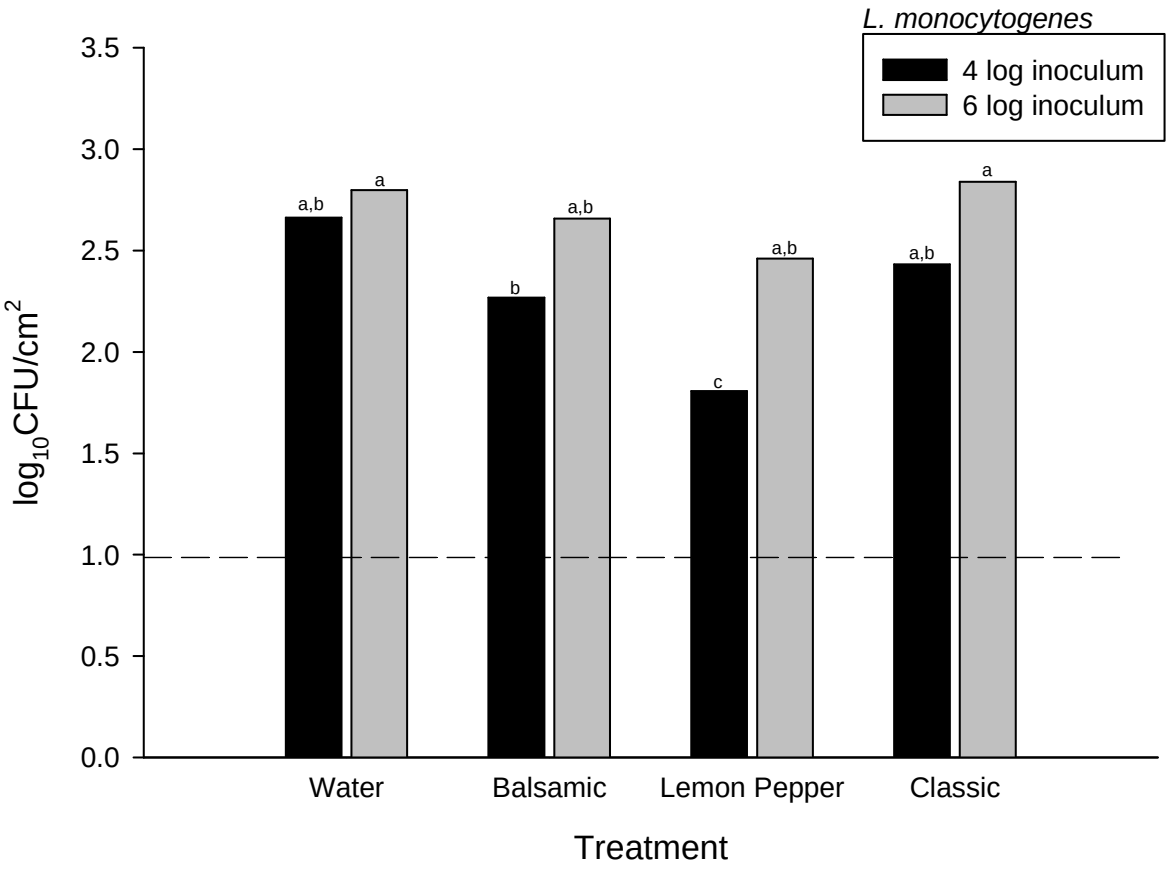
[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM of 0.0988 ($P<0.05$). The dashed line indicates the limit of detection which is 1 log. 4 and 6 log cultures were prepared and inoculated onto meat samples. Marinades were purchased from the Auburn, AL, Publix Super Market. Distilled water served as the control treatment.

Figure 9: Effect of retail marinades against *Salmonella* spp. on fresh beef top round.



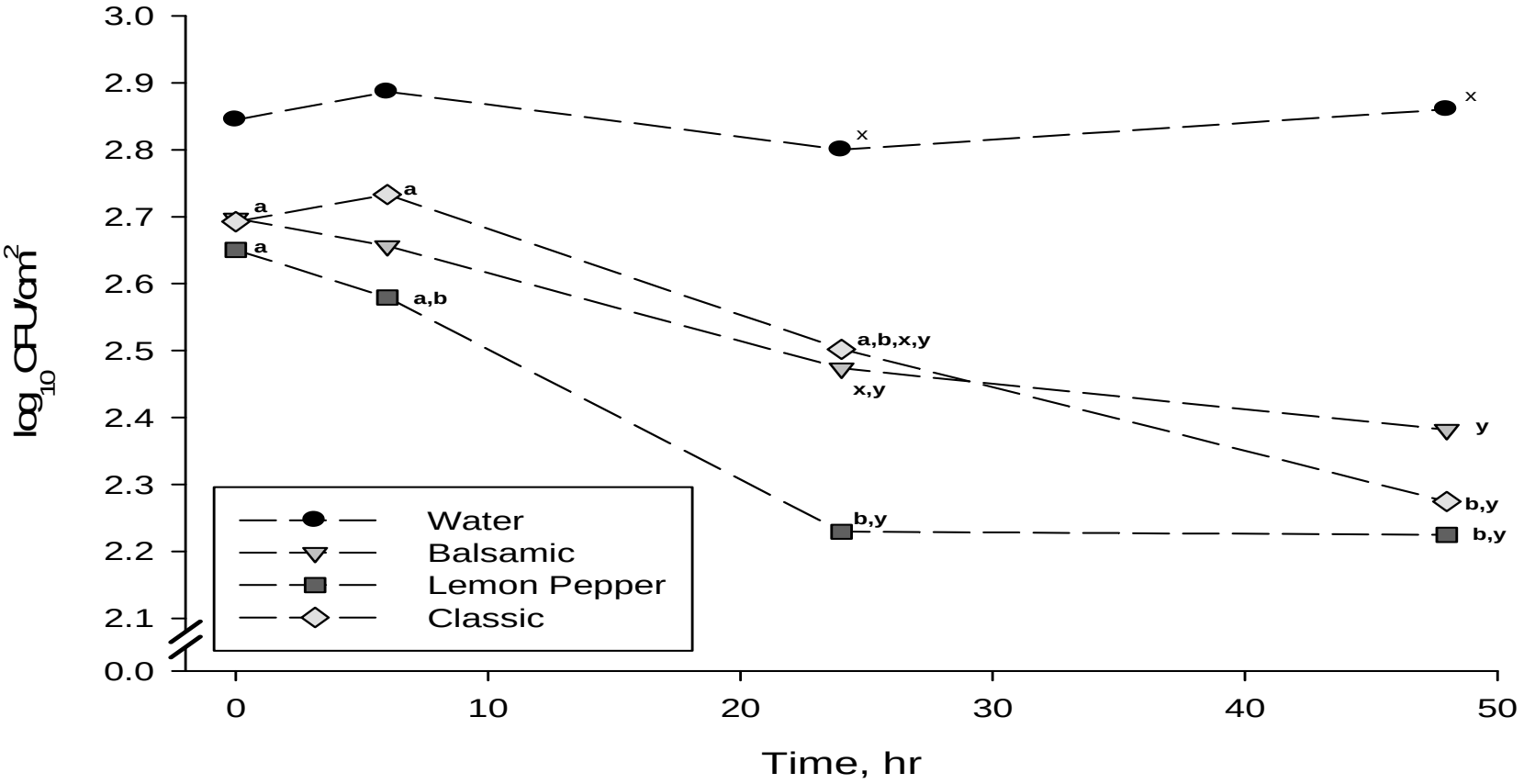
[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM of 0.0988 ($P<0.05$). The dashed line indicates the limit of detection which is 1 log. 4 and 6 log cultures were prepared and inoculated onto meat samples. Marinades were purchased from the Auburn, AL, Publix Super Market. Distilled water served as the control treatment.

Figure 10: Effect of retail marinades against *Listeria monocytogenes* on fresh beef top round.



[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM of 0.0988 ($P<0.05$). The dashed line indicates the limit of detection which is 1 log. 4 and 6 log cultures were prepared and inoculated onto meat samples. Marinades were purchased from the Auburn, AL, Publix Super Market. Distilled water served as the control treatment.

Figure 11: Interaction of retail marinades and time against pathogenic *Escherichia coli* O157:H7, non-O157:H7 STECs, *Salmonella* spp., and *Listeria monocytogenes* on fresh beef top round.



[a,b]Indicates differences within marinade, [x,y]indicates differences within time with pooled SEM of 0.0988 ($P<0.05$). Meat samples were inoculated, treated with marinades purchased from the Auburn, AL, Publix (retail) location, and stored for 0, 6, 24, or 48 hours. Distilled water served as the control.

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CHAPTER IV: Evaluation of a Novel Antimicrobial Solution (AMS) in Combination with Retail Marinades Against Pathogens of Concern on Fresh Beef

Abstract

The meat industry is under constant scrutiny to produce safer food in the wake of outbreaks of foodborne illnesses. Marination and antimicrobial solutions have the potential to increase the shelf life of meat products while adding convenience and value for the consumer. Our objective was to evaluate the combined effects of retail marinades and a novel antimicrobial solution (AMS). Fresh beef top round steaks, prepared without antimicrobial solution, and the AMS were prepared at Auburn University. KC Masterpiece 30 Minute Marinade, California Style Lemon & Cracked Pepper, and KC Masterpiece 30 Minute Marinade, Classic Steakhouse were purchased from the local Publix Supermarket location based on early 2014 market and food trends. A completely randomized design was used. Each experiment was conducted in triplicate with two replications (in separate weeks). The samples inoculated with 4 log of culture prior to treatment had less growth of pathogenic bacteria compared to the samples inoculated with 6 log of culture ($P<0.05$) in *E. coli* and STEC samples. In *Salmonella* and *Listeria monocytogenes* samples, both the lemon pepper and classic steakhouse marinade solutions inhibited the growth of pathogenic bacteria ($P<0.05$). In all samples, the lemon pepper and classic steakhouse marinade solutions were more inhibitory of the growth of pathogenic bacteria compared to water ($P<0.0001$). The lemon pepper and classic steakhouse marinade solutions did not differ ($P=0.1391$) in their ability to inhibit the growth of pathogenic bacteria. An interaction of treatment by time was observed when water, marinades, and marinade solutions were compared ($P=0.0004$). Both the classic steakhouse marinade solution and the lemon pepper marinade solution were more inhibitory against pathogenic bacteria then either water or the marinades alone ($P<0.0001$). Based on this comparison, the AMS combined with the marinade is more effective against pathogenic bacteria than either used singly ($P<0.05$).

Introduction

In developed countries, the meat industry is under constant scrutiny to produce safer food in the wake of outbreaks of foodborne illnesses. While most foodborne outbreaks cause mild to moderate illness, sometimes serious illness results in death which is of particular concern in elderly and immunocompromised individuals. Although the United States has the safest food supply in the world, consumers continue to demand safer products free from contamination by pathogenic bacteria including *Escherichia coli* O157:H7, non-O157:H7 shiga toxin producing *E. coli* (STEC) serotypes, *Salmonella* spp. and *Listeria monocytogenes.* While foodborne pathogens are subjected to physical, chemical, and nutritional stresses during processing (Yousef, 2003), there are still instances when pathogens survive and a person may become ill following consumption. Meat, poultry, milk, and eggs are the primary foods of concern leading to human illness due to undercooking or cross contamination. The meat industry is particularly involved in research and development of strategies to produce safer products.

Escherichia coli (*E. coli*) is part of the normal microflora of the intestinal tract of most warm-blooded mammals, including humans (Marth, 1998). The "big six" non-O157:H7 shiga toxin producing *E. coli* (STECs) serotypes associated with human illness are O26, O45, O103, O111, O121, and O145 and account for approximately 70% of the non-O157:H7 infections (Brooks et al., 2005). *Salmonella* spp. are commonly present in the environment and in the intestinal tract of warm-blooded animals. Not all serovars of *Salmonella* are pathogenic; however, they remain an important human pathogen associated with meat (Bryan, 1995). *Listeria monocytogenes* is ubiquitous in the environment (Marth, 1998). As few as 10 to 1000 cells may be required as an infectious dose of these pathogens. Individuals with compromised immune systems, including newborns, the elderly, and pregnant women are most susceptible to foodborne

illness (Marth, 1998; Morey, Bratcher, Singh, & McKee, 2012). Symptoms commonly associated with foodborne illnesses include fever, abdominal cramps, diarrhea, headaches, nausea, vomiting, and a general feeling of exhaustion.

Topical treatments are effective against pathogenic *E. coli*, *Salmonella* spp., and *L. monocytogenes* (Avery, 1997; Gao, Zhu, & Zhang, 2013; Guo, Jin, Wang, Scullen, & Sommers, 2014; Huffman, 2002; Mani-Lopez, 2012; Mattick & Hirsch, 1947; Podolak, 1995a, 1995b; Ruckman, Rocabayera, Borzelleca, & Sandusky, 2004; Theron, 2007; Yoder et al., 2012). Topical treatments including lactic acid, acetic acid, and other organic acids which are "Generally Recognized As Safe" (GRAS) are commonly used in the meat industry to decontaminate carcasses without negative quality affects (Huffman, 2002; Theron, 2007). Lactic and acetic acids elicit bactericidal and bacteriostatic affects by reducing the pH of the substrate to a lower level than the intracellular pH, thus disrupting the cell membrane (Chung, 1991). Post-processing spray and dip applications have effectively reduced *L. monocytogenes* (Podolak, 1995a, 1995b; Theron, 2007), *E. coli* O157:H7 and *Salmonella* Typhimurium (Mani-Lopez, 2012; Podolak, 1995a, 1995b; Yoder et al., 2012). Though lactic and acetic acids have inhibitory effects on pathogen growth, emerging antimicrobial compounds may offer additional inhibitory capabilities when used in combination with organic acids.

Lauric arginate ester (LAE) is GRAS, verified nontoxic, and metabolized to naturally occurring amino acids following consumption (Ruckman et al., 2004). It is believed that the antimicrobial action of LAE originates from its ability to cause membrane disruption and disturbance of vital cell functions (Rodriguez, Seguer, Rocabayera, & Manresa, 2004). LAE has been used in combination with chitosan in food packaging film applications as well (Guo, Jin, Scullen, & Sommers, 2013; Guo et al., 2014). Chitosan is a natural polymer obtained by

deacetylation of chitin, the primary component of crustacean shells. Chitosan is verified nontoxic, biodegradable, and biocompatible (Guo et al., 2014). Although the antimicrobial mechanism of chitosan remains unclear, it is thought to involve disruption of the cell membrane as a result of the association between positively charged amino groups of chitosan and negatively charged anions on the surface of bacteria (Friedman & Juneja, 2010).

Nisin is a well known GRAS bacteriocin produced by certain strains of *Lactobacillus lactis* subspecies *lactis*. The effects of nisin have been well studied and it has been found to be effective against a wide range of Gram positive bacteria, including *L. monocytogenes* and spore formers, as well as Gram negative bacteria (Delves-Broughton, Blackburn, Evans, & Hugenholtz, 1996; Henning, 1986). Studies on the application of nisin have been conducted for decades on dairy and processed foods (Delves-Broughton et al., 1996) with recent focus on the application to fresh meat and poultry (Avery, 1997; Gonzalez-Fandos & Dominguez, 2006).

One value-adding food preparation step which has been found to provide an additional layer of food safety by inhibiting growth of pathogenic bacteria such as *E. coli, Salmonella* spp., and *L. monocytogenes* is marination. Marinades are typically a water-oil emersion containing a combination of sugar, salt, acids (acetic and citric acids), additives (Xanthan and guar gum), spices, sorbates, benzoates, and aroma enhancers (Bjorkroth, 2005). Commercial marination practices rely primarily on salt-water and phosphate formulations which increase tenderness, juiciness, and yield (Alvarado & Mckee, 2007). At home, consumers rely on immersion to marinate meat. Studies indicate that marinade sauces prevent the growth of spoilage organisms based on a low pH, high salt concentration, sorbates and benzoates, and various spices (Bjorkroth, 2005). Previous research, including research from our lab, has been conducted to evaluate the effect of retail marinades of various compositions against pathogens of concern on

meat (Birk & Knochel, 2009; Dabbah, Edwards, & Moats, 1970; Fisher & Phillips, 2006; Pathania, McKee, Bilgili, & Singh, 2010; Perko-Makela, Koljonen, Miettinen, & Hanninen, 2000).

Detection and enumeration of food associated pathogenic bacteria is complicated by emerging strains which are not routinely encountered and may have an unclear transmission route (Mead et al., 1999). Molecular approaches offer the capacity for near-time or real-time detection of bacteria and are rapid, sensitive, and specific for target pathogens or the virulence determinants of that pathogen (Feng, 2001; Rijpens & Herman, 2002; Smith, O'Connor, Glennon, & Maher, 2000). Polymerase chain reaction (PCR) is a technique which is useful to amplify target DNA from strains of pathogenic bacteria. Pulse field gel electrophoresis (PFGE) can then be used to separate the DNA fragments based on size.

Marination has the potential to increase the shelf life of meat products while adding convenience and value for the consumer. The known inhibitory effect of lactic acid, acetic acid, LAE, chitosan, and nisin used singularly suggests that a combination would produce improved inhibitory effects. A novel antimicrobial solution (AMS) containing acetic acid, lactic acid, levulinic acid, LAE, and chitosan has been developed with application to meats. Previous research in our laboratory has evaluated the effects of this AMS against pathogens of concern on fresh beef top round steaks as well as evaluated the effects of retail marinades against pathogens of concern on fresh beef top round steaks. Based on previous results, we sought to determine the effects of the combined AMS and retail marinades. Thus, the first objective of this study was to determine the effectiveness of the retail marinade combined with the AMS at inhibiting pathogen growth on inoculated meat samples. The medium concentration (1:5 dilution) of the AMS was chosen due to the consistent inhibition of pathogen growth (previous research in our laboratory)

and the marinade served as the diluent for this study. The second objective was: 1) to determine if pathogens survive following application of the AMS, the lemon pepper marinade, or the lemon pepper marinade + AMS, and 2) to determine what genetic markers are present which may allow pathogens to survive. Only the lemon pepper was selected for the second portion of the study because previous research in our laboratory shows it is the most inhibitory retail marinade.

Materials and Methods

Culture Preparation

Five strains of *Escherichia coli* O157:H7, one strain of each of the big six STECs, five strains of *Salmonella* spp., and five strains of *Listeria monocytogenes* were used for this study as detailed in Table 2. All media was purchased from Neogen Corporation (Lansing, Michigan) unless otherwise stated. Cultured microorganisms were transferred individually to 9 mL sterile tryptic soy broth, vortexed (Labnet International, Inc., Edison, New Jersey), and incubated at 37°C for 24 hours (Jeio Tech, Inc., Des Plaines, Illinois). The overnight culture produced approximately 9 log CFU/mL culture suspensions. Equal parts of each strain were combined and vortexed to create the culture cocktail. Cells were harvested by centrifugation at 3650 rpm for 20 minutes at 37°C. The supernatant was discarded and the resulting pellet was gently washed before being resuspended in 0.1% peptone. The cell suspension was then diluted to a concentration of 4 or 6 log which was used to inoculate meat samples.

AMS Preparation

Food grade LAE (CytoGuard LA 2X; A&B Ingredients, 24 Spielman Road, Fairfield, NJ), levulinic acid (natural, 99%, FG; Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO),

chitosan (low molecular weight; Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO), acetic acid (natural, ≥99.5%, FG; Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO), and lactic acid (natural, ≥85% FG; Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO) were obtained. Compounds were weighed and mixed into MilliQ water (Milli-Q Integral Water Purification System, Darmstadt, Germany) at Auburn University. The AMS was allowed to mix overnight on a stir plate (VWR International, LLC, Radnor, Pennsylvania) and refrigerated at 4°C prior to use.

Marinade Selection

Retail marinades were chosen from commonly available marinades at the local Publix Super Market (Auburn, AL). Marinades chosen were: 1) KC Masterpiece 30 Minute Marinade, California Style Lemon & Cracked Pepper, and 2) KC Masterpiece 30 Minute Marinade, Classic Steakhouse. All bottles of marinade were ready to use at purchase and required no additional mixing or reconstitution. On the day of use, each of the marinades were used as the solvent to create a 1:5 dilution of the AMS. The resulting treatments were a lemon pepper marinade + AMS and a classic steakhouse marinade + AMS hereafter referred to as lemon pepper marinade solution and classic steakhouse marinade solution. Deionized distilled water (Barnstead Mega-Pure System Automatic Water Distillation Apparatus, Thermo Scientific, Waltham, MA) served as the control treatment.

A volume of 30 mL of the assigned marinade solution, or water control, was applied to the surface of the inoculated meat sample and stored until the appropriate sampling time at 0, 6, 24, or 48 hours. The pH values of the treatments were collected prior to application to the surface of the meat. The pH of the distilled water was 6.72, the lemon pepper marinade solution was 2.94, and the classic steakhouse marinade solution was 3.39.

Sample Preparation

Fresh beef top round steaks were fabricated at the Lambert Powell Meats Laboratory at Auburn University without the use of antimicrobial solutions. Lean meat samples were cut to 100 cm^2 pieces. Each piece was individually inoculated with 1 mL of the assigned inoculum (either 4 or 6 log of *E. coli*, STECs, *Salmonella* spp., or *L. monocytogenes*) which was then spread using a disposable L-shaped cell spreader (VWR International, LLC, Radnor, Pennsylvania). Thirty minutes of contact time was allowed for cell adhesion to the meat surface. After the allowed contact time, samples were treated with 30 mL of the assigned treatment. Samples were then stored at 4°C in sterile stomacher bags (Nasco Whirl-Pak, Fort Atkinson, Wisconsin) for 0, 6, 24, or 48 hours.

A modified plating technique using 0.1% peptone was utilized (Podolak, 1995a). One hundred mL of 0.1% peptone was added to each meat sample in stomacher bags prior to stomaching for 2 minutes at 300 rpm (400 Circular Seward Medical, London, England). Serial dilutions with 9 mL 0.1% peptone were created and dilutions were plated onto MacConkey Agar with Sorbitol (MSA; *E. coli*), XLT4 (*Salmonella* spp.), or Modified Oxford Medium (MOX; *L. monocytogenes*) plates. Plates were enumerated following 24 hours incubation at 37°C. Results are presented in log_{10}CFU.

PCR Sample Preparation

Fresh beef top round steaks were fabricated at the Lambert Powell Meats Laboratory at Auburn University without the use of antimicrobial solutions. Lean meat samples were cut to 100 cm^2 pieces. Each piece was individually inoculated with 1 mL of the assigned inoculum (2

to 9 log of *E. coli*, STECs, *Salmonella* spp., or *L. monocytogenes*) which was then spread using a disposable L-shaped cell spreader (VWR International, LLC, Radnor, Pennsylvania). Thirty minutes of contact time was allowed for cell adhesion to the meat surface. After the allowed contact time, samples were treated with 30 mL of water, lemon pepper marinade, lemon pepper marinade solution, or stock concentration of the AMS. Only the lemon pepper was selected for the second portion of the study because previous research in our laboratory shows it is the most inhibitory retail marinade. Samples were immediately diluted with 100 mL of 0.1% peptone prior to stomaching for 2 minutes at 300 rpm (400 Circular Seward Medical, London, England).

A 20 mL volume of the suspension was then collected into a conical tube, centrifuged at 3650 rpm for 20 minutes at 37°C, the supernatant was discarded and the resulting pellet was resuspended in 5 mL 0.1% peptone. DNA was extracted utilizing the PrepMan Ultra Sample Preparation Reagent (Life Technologies, Grand Island, NY) followed by ten minutes of boiling at 100°C. The PCR program ran 35 cycles (pre-denaturing at 94°C for 5 min; 94°C for 15 sec, 58°C for 30 sec, and 72°C for 30 sec; post extension 72°C for 10 min). The resulting PCR amplicons were then determined by 2% agarose gel electrophoresis and imaged.

Statistical Analysis

A completely randomized design was used to conduct these experiments. Each experiment was conducted in triplicate with two replications performed in separate weeks. All data were converted to $\log_{10}$CFU prior to performing statistical analysis. The independent variables were treatment (combined retail marinade and AMS), time, and pathogen/inoculum level and $\log_{10}$CFU was the dependent variable evaluated. Statistics were completed using the Proc Mixed procedure in SAS version 9.2 (SAS Institute, Inc., Cary, NC). All appropriate two

and three way interactions were evaluated. In the event that no interactions were observed, main effects were evaluated. Least squares means were used to separate mean differences. There were no differences in replications and no treatment by replication interactions were included as no practical differences observed. Tukey pairwise comparisons were utilized due to potential unequal sample size resulting from the removal of data points due to contamination. Data are presented with pooled standard error.

Results and Discussion

An interaction of pathogen by marinade solution was observed (P=0.0002). The interaction of marinade solution and *E. coli* O157:H7 is presented in Figure 12, non-O157:H7 STECs in Figure 13, *Salmonella* spp. in Figure 14, and *Listeria monocytogenes* in Figure 15. The samples inoculated with 4 log of culture prior to treatment had less growth of pathogenic bacteria compared to the samples inoculated with 6 log of culture (P<0.05) in the *E. coli* and STEC samples. In the *Salmonella* and *Listeria monocytogenes* samples, both the lemon pepper and classic steakhouse marinade solutions inhibited the growth of pathogenic bacteria (P<0.05). In all samples, the lemon pepper and classic steakhouse marinade solutions were more inhibitory of the growth of pathogenic bacteria compared to water (P<0.0001). The lemon pepper and classic steakhouse marinade solutions did not differ (P=0.1391) in their ability to inhibit the growth of pathogenic bacteria. These results are consistent with previous research which evaluated antimicrobial properties of commercial marinades and individual components of the marinades (Perko-Makela et al., 2000; Pathania et al., 2010).

The marinade solutions were effective against pathogenic bacteria of concern on fresh beef top round steaks (P<0.05). A comparison of the effectiveness of the marinades and the

marinade solutions was also evaluated and is presented in Figure 16. Previous research in two phases in our laboratory (Chapters 2 and 3) evaluated the AMS and the marinades, respectively. Addition of the AMS to the retail marinades did not change the pH of the marinade solution. The pH values were as follows: water = 6.72, lemon pepper marinade = 2.85, classic steakhouse marinade = 3.67, lemon pepper marinade solution = 2.94, and classic steakhouse marinade solution = 3.39. All of the marinade and marinade solution pH values were below 4.6 which generally the limit for growth of microorganisms (Chung & Goepfert, 1970). Although the pH difference when the AMS was added was minor, the antimicrobial effect was pronounced (Figure 16). An interaction of treatment by time was observed (P=0.0004; Table 4). The water control samples had the highest $\log_{10}$CFU estimates at the 0 and 48 hour samplings though it did not differ from the 0 hour sampling of the classic steakhouse and lemon pepper marinades (P>0.51). The $\log_{10}$CFU estimates for the 48 hour sampling of classic steakhouse and lemon pepper marinades were intermediate to the estimates for the marinade solutions. Both the classic steakhouse marinade solution and the lemon pepper marinade solution were more inhibitory against pathogenic bacteria than either water or the marinades alone (P<0.0001). Based on this comparison, the AMS combined with the marinade is more effective against pathogenic bacteria than either used singly (P<0.05).

PCR amplicons were successfully separated on an agarose gel. Imaging of the gels revealed that the sensitivity of the technique is likely below the optimum level as determined by a lack of bands present in the samples inoculated with lower levels of cell culture. Bands were visualized in samples inoculated with 5 -9 log of *E. coli* (Figure 17) and *Salmonella* spp. Interestingly, no bands were visualized in samples inoculated with *Listeria monocytogenes*. Based on these findings, the technique to isolate DNA from samples should be further refined. In

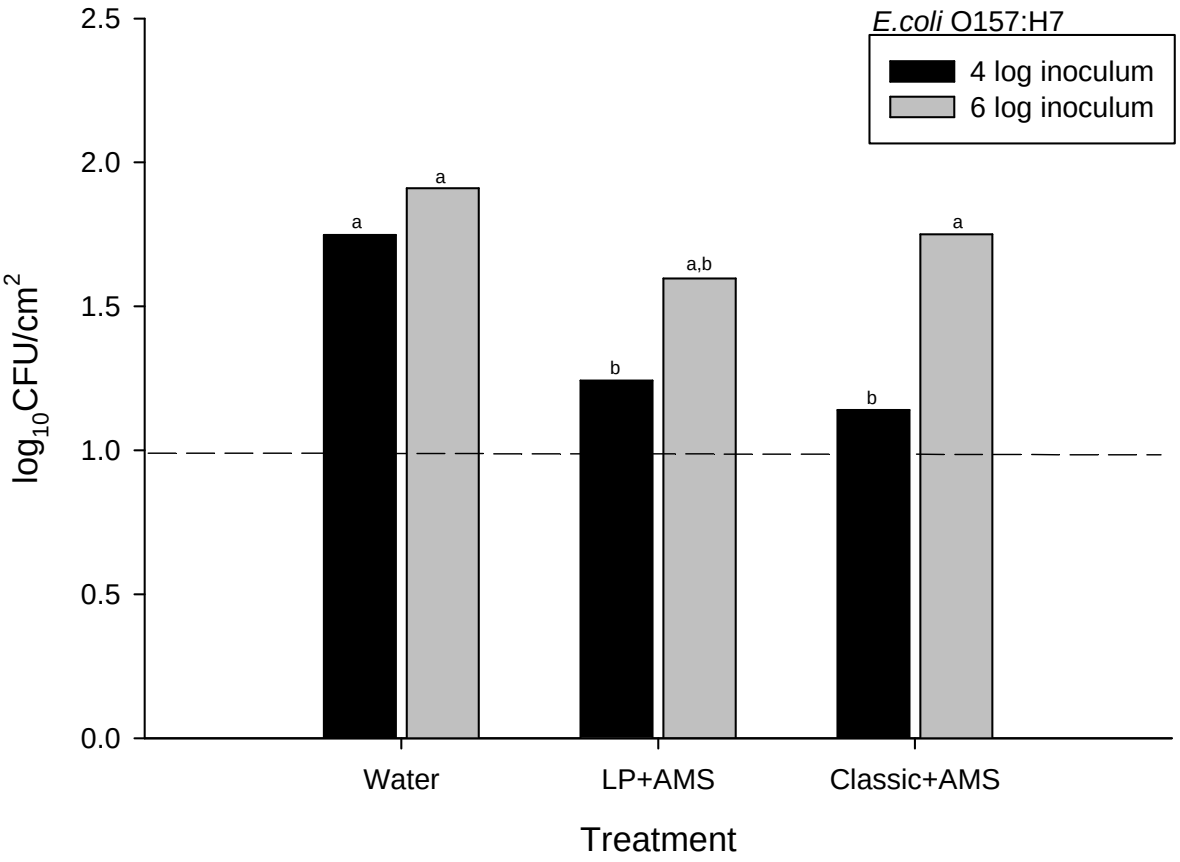
the current study, the addition of marinade and AMS created a viscous solution with many small particles in the sample bag following stomaching. Some of these issues may be corrected by using sample bags with filters or adding additional centrifugation and washing steps to remove foreign material from the cell pellet. Additional primers may also be explored as well as evaluation of a variety of pathogenic strains of bacteria.

Conclusions

The marinade solutions (commercially available marinade + antimicrobial solution) inhibit the growth of pathogenic bacteria of concern inoculated onto the surface of fresh beef top round steaks prepared without antimicrobial solution. This suggests that the novel AMS may be mixed with commercial marinade products to improve the safety of meat by inhibiting the growth of pathogenic bacteria. This has potential for further processing of meat in which a marinade is used to increase juiciness and product yield. The AMS utilized herein may be mixed with existing marinades and applied to products through industrial marination practices such as immersion, tumbling, and injection. Additional research will determine any organoleptic qualities which may be affected by the inclusion of the AMS as a marinade ingredient.

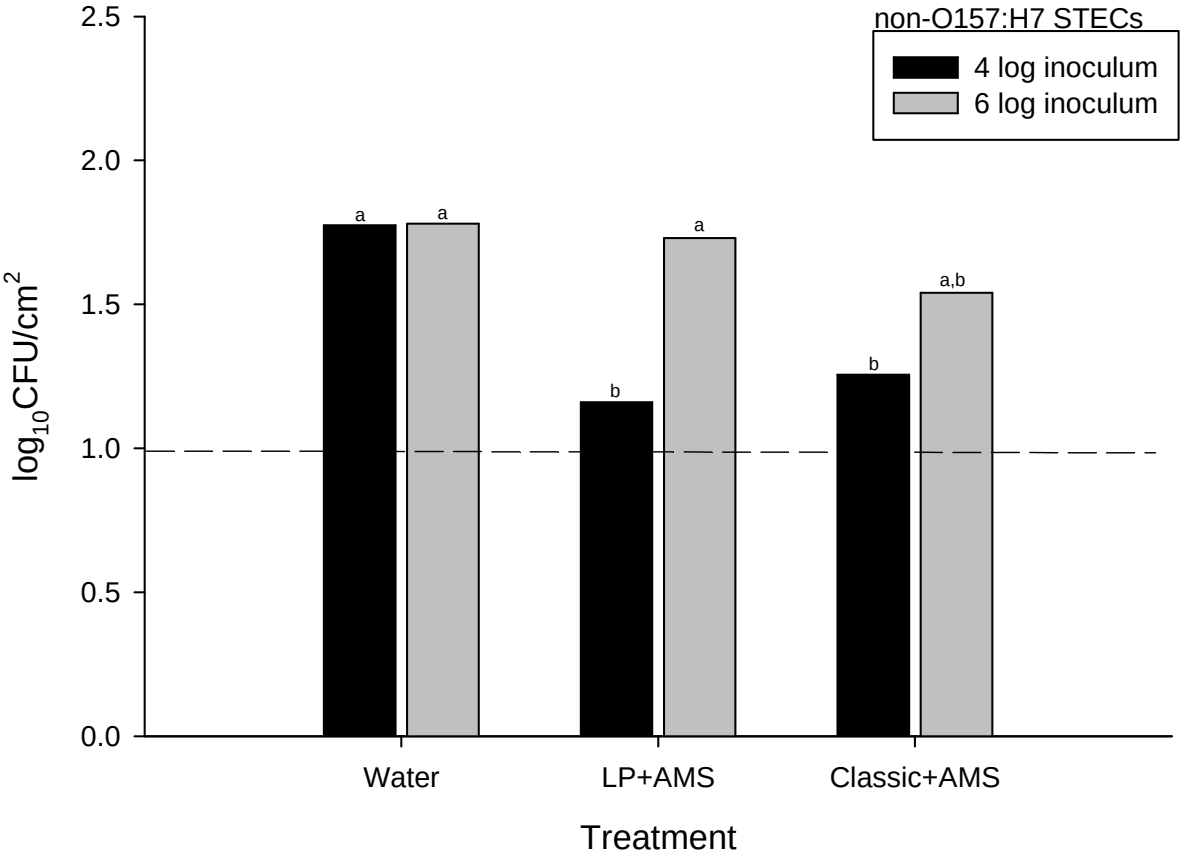
The technique to isolate DNA from samples treated with marinade and/or AMS must be refined. Additional primers as well as sample bags should be evaluated. The PCR protocol may also be adjusted to include additional cycles which may create more copies of the target DNA. Further research is needed to refine the technique and select appropriate products for each step of the protocol.

Figure 12: Effect of retail marinades combined with AMS against *Escherichia coli* O157:H7 on fresh beef top round.



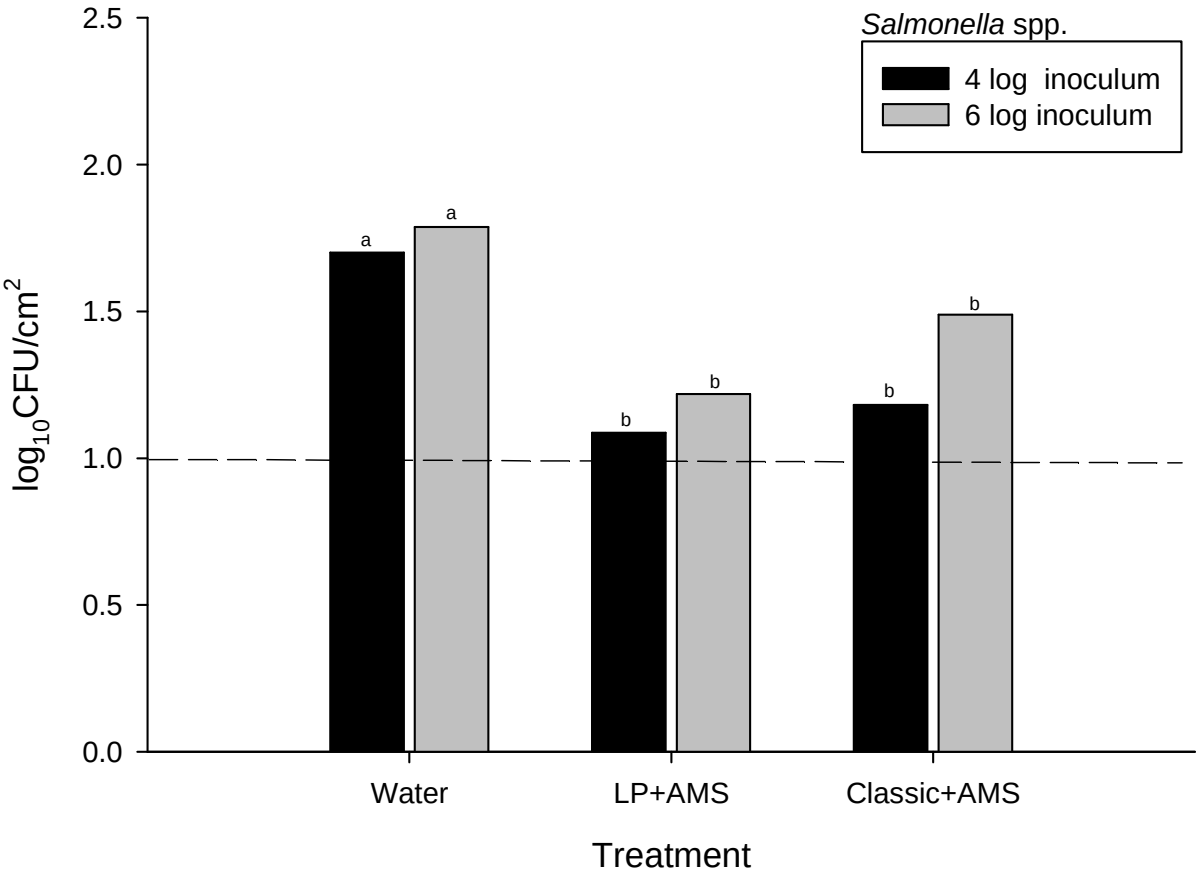
[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM of 0.0712 ($P<0.05$). The dashed line indicates the limit of detection which is 1 log. 4 and 6 log cultures were prepared and inoculated onto meat samples. Marinades were purchased from the Auburn, AL, Publix Super Market and were used to dilute the AMS (1:5 dilution) which was prepared at Auburn University. Distilled water served as the control treatment.

Figure 13: Effect of retail marinades combined with AMS against non-O157:H7 STEC on fresh beef top round.



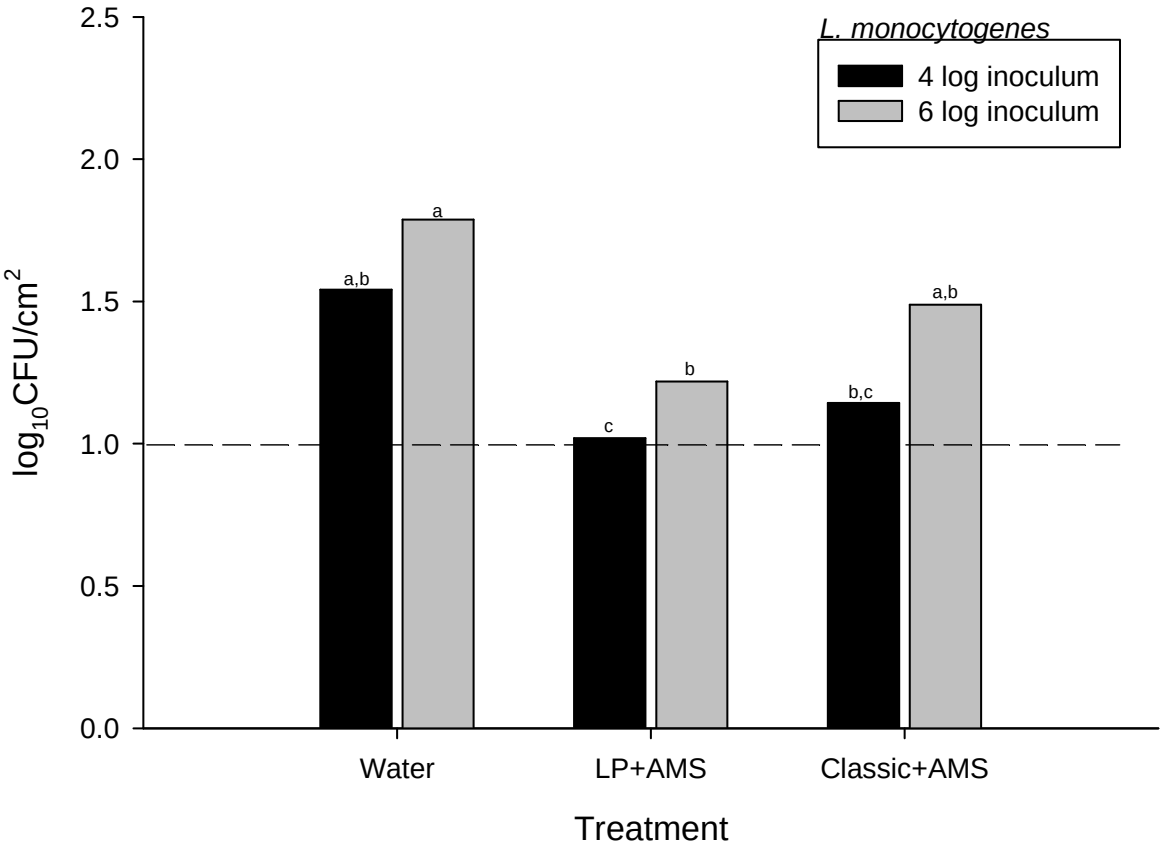
[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM of 0.0712 ($P<0.05$). The dashed line indicates the limit of detection which is 1 log. 4 and 6 log cultures were prepared and inoculated onto meat samples. Marinades were purchased from the Auburn, AL, Publix Super Market and were used to dilute the AMS (1:5 dilution) which was prepared at Auburn University. Distilled water served as the control treatment.

Figure 14: Effect of retail marinades combined with AMS against *Salmonella* spp. on fresh beef top round.



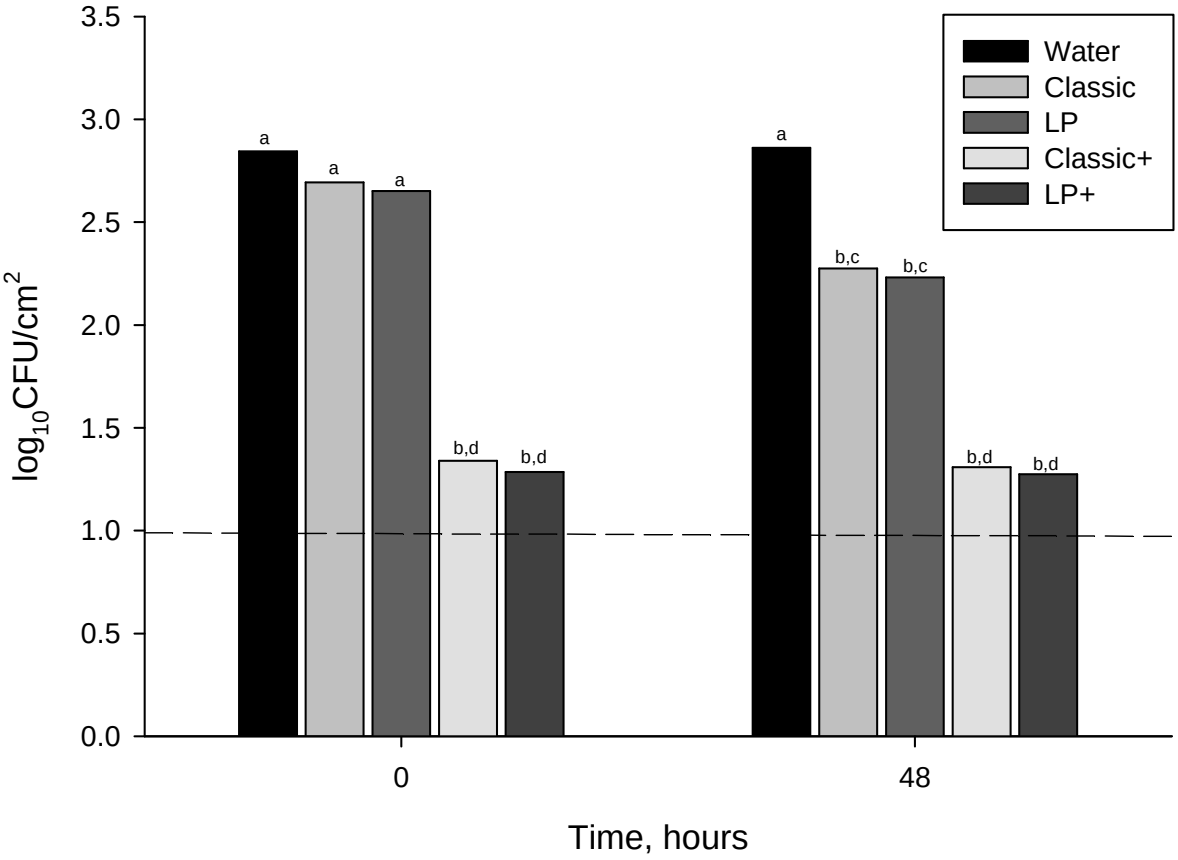
[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM of 0.0712 (P<0.05). The dashed line indicates the limit of detection which is 1 log. 4 and 6 log cultures were prepared and inoculated onto meat samples. Marinades were purchased from the Auburn, AL, Publix Super Market and were used to dilute the AMS (1:5 dilution) which was prepared at Auburn University. Distilled water served as the control treatment.

Figure 15: Effect of retail marinades combined with AMS against *Listeria monocytogenes* on fresh beef top round.



[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM of 0.0712 ($P<0.05$). The dashed line indicates the limit of detection which is 1 log. 4 and 6 log cultures were prepared and inoculated onto meat samples. Marinades were purchased from the Auburn, AL, Publix Super Market and were used to dilute the AMS (1:5 dilution) which was prepared at Auburn University. Distilled water served as the control treatment.

Figure 16: Effect of retail marinades and retail marinades combined with AMS on fresh beef top round.



[a,b,c]Data with differing superscripts indicate differences between the least squares means (P<0.05). The dashed line indicates the limit of detection which is 1 log. 4 and 6 log cultures were prepared and inoculated onto meat samples. Marinades were purchased from the Auburn, AL, Publix Super Market and were used to dilute the AMS (1:5 dilution) which was prepared at Auburn University. Distilled water served as the control treatment.

Table 4: Interaction of treatment and time against pathogens of concern on fresh beef top round.

Treatment	Time	log_{10}CFU	Pooled SEM
Water	48	2.8605[a]	0.0699
Water	0	2.8446[a]	0.0699
Classic	0	2.6927[a]	0.0699
Lemon Pepper	0	2.6503[a]	0.0699
Classic	48	2.2744[b,c]	0.0699
Lemon Pepper	48	2.2305[b,c]	0.0699
Classic Solution	0	1.3383[b,d]	0.0699
Classic Solution	48	1.3078[b,d]	0.0699
Lemon Pepper Solution	0	1.2846[b,d]	0.0699
Lemon Pepper Solution	48	1.2748[b,d]	0.0699

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM ($P<0.05$). Superscripts were not assigned to the inoculation level because they are significantly different ($P<0.0001$) and this difference is easily visualized. Meat samples were inoculated, allowed 30 minutes of contact time, then treated with 30 mL of distilled water (control), marinade, or marinade solution. Marinades were purchased from the Auburn, AL, Publix Super Market. AMS was prepared at Auburn University and diluted (1:5) in the retail marinades to prepare the classic marinade solution and lemon pepper marinade solution.

Figure 17: Agarose gel image of *Escherichia coli* samples.

Lane 1 of the gel is the ladder. Lanes 2, 3, 4, and 5 are 9 log *E. coli* treated with water, lemon pepper marinade, lemon pepper marinade solution, and antimicrobial solution, respectively. Lanes 6, 7, 8, and 9 are 8 log *E. coli* treated with water, lemon pepper marinade, lemon pepper marinade solution, and antimicrobial solution, respectively. Lanes 10, 11, 12, and 13 are 7 log *E. coli* treated with water, lemon pepper marinade, lemon pepper marinade solution, and antimicrobial solution, respectively. Lanes 14, 15, 16, and 17 are 6 log *E. coli* treated with water, lemon pepper marinade, lemon pepper marinade solution, and antimicrobial solution, respectively. Lanes 17, 18, and 19 are 5 log *E. coli* treated with water, lemon pepper marinade, lemon pepper marinade solution, respectively. Lane 20 is empty.

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CHAPTER V: Sensory and Objective Color Evaluation of Beef Top Round Steaks Marinated in a Retail Marinade Combined with a Novel Antimicrobial Solution (AMS)

Abstract

The meat industry relies on multiple techniques to determine the shelf life of meat products including color and sensory evaluation. Fresh beef top round steaks were fabricated to one inch thickness without the use of antimicrobial solutions. Three steaks were placed in a Ziplock bag along with 500 mL of the assigned treatment: water, water + AMS, lemon pepper marinade + AMS, or classic steakhouse marinade + AMS for 0, 6, 24, or 48 hours. The combination treatments are hereafter referred to as water+ solution, lemon pepper solution, and classic steakhouse solution, respectively. Each steak was scanned three times with the MiniScan XE, Plus colorimeter, grilled to an internal temperature of 71°C, cut into 1 cm^2 pieces, and labeled for the sensory panel. Each sample was evaluated twice in the sensory panel with a randomized order of presentation. Statistics were completed using the Proc Mixed procedure in SAS version 9.2 (SAS Institute, Inc., Cary, North Carolina). The fixed effects were time and treatment. Tukey pairwise comparisons were utilized due to potential unequal sample sizes resulting from removal of outlying data points. An interaction of time and treatment was observed for initial juiciness ($P<0.0001$), sustained juiciness ($P<0.0001$), initial tenderness ($P<0.0001$), sustained tenderness ($P=0.0008$), flavor intensity ($P=0.0039$), and off-flavor intensity ($P<0.0009$). An effect of treatment ($P<0.0001$) and time ($P<0.0014$) were observed for aroma intensity. Steaks marinated in lemon pepper marinade solution, classic steakhouse marinade solution, or water+ solution received more favorable ratings compared to steaks marinated in water alone ($P<0.05$). The lemon pepper marinade solution treatment was rated as the most juicy and most tender, followed by the classic steakhouse marinade solution, and the water+ solution. Water was rated as the least juicy and tender. Flavor intensity increased with increasing marination time ($P=0.0002$) such that steaks marinated for 48 hours received higher

flavor intensity ratings than those marinated for 24, 6, and 0 hours. All combination treatments, lemon pepper marinade solution, classic steakhouse marinade solution, and water+ solution, were rated as having greater aroma intensity compared to water ($P<0.05$). Marination times of 6, 24, and 48 hours generated a more intense aroma than did 0 hours of marination ($P<0.05$). As marination time increased, off-flavor intensity also increased ($P<0.0001$). Panelists identified samples marinated in the water+ solution as having the most intense off-flavor. All panelists described the off-flavors associated with these samples as being vinegar-like and metallic. Off-flavor descriptors associated with samples marinated in water were bloody and livery. Sour and sweet off-flavors were associated with the steaks marinated in lemon pepper marinade solution while metallic and sour off-flavors were associated with steaks marinated in the classic steakhouse marinade solution.

An interaction of treatment by time was observed for L* color values ($P=0.0013$). The steaks marinated in water were the most white in color, followed by lemon pepper marinade solution, water+ solution, and finally classic steakhouse marinade solution which was the darkest ($P<0.0001$). A trend of increasing marination time leading to darker color was observed ($P=0.0708$). An interaction of treatment by time was observed for a* color values ($P=0.0225$). Steaks marinated in water+ solution were more red in color, followed by classic steakhouse marinade solution, lemon pepper marinade solution, and finally water. Increasing marination time resulted in lower a* values, indicating more green color ($P<0.0001$). No interaction of treatment by time was observed for b* color values ($P=0.2401$). Time was not significant ($P=0.0718$) although treatment was ($P<0.0001$). Steaks marinated in classic steakhouse marinade solution had higher b* values than the other marinade solutions ($P=0.0001$). Sensory properties of marinated steaks were improved in those steaks marinated in combination with the

antimicrobial solution. Meat color is altered during marination and is reflective of the marinade selected.

Introduction

The meat industry relies on several techniques to determine the shelf life of meat products. Two common techniques are color and sensory evaluation. Sensory evaluation is a set of techniques developed to accurately measure human responses to foods while minimizing potential bias effects (Lawless, 2010). Sensory can be defined as a scientific method to evoke, measure, analyze, and interpret responses to products as perceived by the senses (Lawless, 2010). Sensory evaluation is important to the meat industry because it helps relate consumer perceptions to the quality of the meat product. The validity of the sensory panel is dependent upon the control of various factors within the testing environment (AMSA, 1995). The specific parameters measured during a sensory evaluation panel are selected by the individual organizing the panel and are designed to answer a specific research question about the product. Trained sensory panels are used to identity and quantify specific parameters.

In situations where cookery method or treatment may create variation in color, red filtered lights are necessary to provide uniform and adequate lighting (AMSA, 1995). Panelists are provided a standardized amount of each sample and an evaluation form with a numerical scale for responses. Samples are held in a warmer to maintain the appropriate temperature for tasting and are presented to panelists in a randomized design (AMSA, 1995). The number of samples presented during each panel should be managed to prevent panelist fatigue but also should be a function of product characteristics, experience of the panelists, and number of attributes to be measured per sample (AMSA, 1995).

Panelists are recruited and trained prior to the sensory panel. Objectives of training are to: 1) familiarize the individual with test procedures, 2) improve an individual's ability to recognize and identify sensory attributes, and 3) improve an individual's sensitivity and memory,

permitting precise and consistent sensory judgments (AMSA, 1995). Numerous decisions must be made prior to the panel including if samples are to be swallowed, the rinsing process should be standardized, and the temperature of the water provided should be standardized (AMSA, 1995). Unsalted crackers may be provided for panelists when considerable aftertaste is present in the samples; however, caution should be exerted as the mouth feel may be impacted (AMSA, 1995). During training, panelists are provided reference samples along with a corresponding score for each parameter. Panelists provide a numerical response corresponding with the provided scale; these responses are then analyzed statistically.

Appearance and color of a product are the first attributes consumers evaluate when selecting a product for purchase because the consumer links the visual appearance with the expected quality of the product (Carpenter, Cornforth, & Whittier, 2001). Product appearance is easily altered through addition, deletion, or modification of ingredients in the product formulation. Consumers also attribute texture and juiciness of products to product quality and eating satisfaction. Texture of foods is dependent upon product formulation while juiciness is a sensory measurement of water-holding capacity of the product (Morey, Bratcher, Singh, & McKee, 2012). Juiciness is affected by the fat content of the product, higher fat content is associated with a more juicy product (Mittal & Barbut, 1993), as well as the pH of the additives (Morey et al., 2012). Extended storage periods have been found to decrease the level of juiciness in products (Morey et al., 2012) The specific desired texture and level of juiciness are dependent upon the type of product.

Consumers rely on the eye to evaluate color while researchers use instrumental colorimeters to objectively evaluate color. The AMSA Meat Color Measurement Guidelines are a comprehensive review of meat color measurements (Hunt, 2012). Briefly, instrumental color is

expressed in three dimensional terms using the CIE L* a* b* scale (Hunt, 2012). The center of the color scale is neutral gray. Positive a* values represent red and negative a* values represent green. Similarly, positive b* values represent yellow and negative b* values represent blue. The scale for L* is somewhat different in that a value of 100 represents white while a value of 0 represents black. The L* scale is used to determine the darkness or lightness of the sample.

Meat color, as well as the expectation of the color, is adjusted when the meat is marinated. It is expected that the meat color will be changed to reflect the color properties of the marinade. Thus, dark marinades, such as soy sauce based marinades, will alter the color of the meat such that it is darker, more like the marinade. If a lighter colored marinade is chosen, Italian dressing for example, the meat will appear lighter on the surface. Consumers evaluate the visual appeal of these color changes while a colorimeter objectively detects differences.

The objectives of this study were multifaceted. The first objective was to identify and quantify any organoleptic (sensory) attributes which may be perceived by consumers when the AMS is incorporated into a retail marinade. The second objective was to objectively evaluate color differences in steaks marinated 0, 6, 24, or 48 hours using a colorimeter. Three steaks were prepared for each time and treatment combination and the colorimeter was used to measure color on each of the three steaks.

Materials and Methods

AMS Preparation

Food grade LAE (CytoGuard LA 2X; A&B Ingredients, 24 Spielman Road, Fairfield, NJ), levulinic acid (natural, 99%, FG; Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO), chitosan (low molecular weight; Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO), acetic acid

(natural, ≥99.5%, FG; Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO), and lactic acid (natural, ≥85% FG; Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO) were obtained. Compounds were weighed and mixed into MilliQ water (Milli-Q Integral Water Purification System, Darmstadt, Germany) at Auburn University. The AMS was allowed to mix overnight on a stir plate (VWR International, LLC, Radnor, Pennsylvania) and refrigerated at 4°C prior to use.

Marinade Selection

Retail marinades were chosen from commonly available marinades at the local Publix Super Market (Auburn, AL). Marinades chosen were: 1) KC Masterpiece 30 Minute Marinade, California Style Lemon & Cracked Pepper and 2) KC Masterpiece 30 Minute Marinade, Classic Steakhouse. All bottles of marinade were ready to use at purchase and required no additional mixing or reconstitution. On the day of use, each of the marinades were used as the diluent to create a 1:5 dilution of the AMS, generating the "solution" treatments. Deionized distilled water (Barnstead Mega-Pure System Automatic Water Distillation Apparatus, Thermo Scientific, Waltham, MA) was the diluent for the water + AMS solution and the control. Samples were marinated in the assigned treatment for 0, 6, 24, or 48 hours.

Sample Preparation

Fresh beef top round steaks were fabricated at the Lambert Powell Meats Laboratory at Auburn University without the use of antimicrobial solutions. Lean meat samples were cut to one inch thick pieces. Three pieces of fresh beef top round steaks were placed in a Ziplock bag along with 500 mL of the assigned treatment. Treatments consisted of water, water + AMS, lemon pepper marinade + AMS, or classic steakhouse marinade + AMS. The combination treatments

are hereafter referred to as water+ solution, lemon pepper marinade solution, and classic steakhouse marinade solution, respectively.

Cookery and Sensory Evaluation

After marination, steaks were grilled on clam shell grills (Calphalon 5 in 1 Removable Plate Grill Model HE400CG, Atlanta, GA) to an internal temperature of 71°C as determined by a thermocouple inserted into the center of each steak. Steaks were removed from the grill and allowed 3-5 minutes to rest after which the steaks were cut into 1 cm^2 pieces, placed in clear plastic cups with lids, and held in a warmer (LabLine, Inc., Chicago, IL) until the panel began. Each sample was evaluated twice in the sensory panel (one panel with four samples in duplicate, generating eight samples per panel session). Order of presentation of samples was randomized for each sensory booth.

Sensory booths were prepared with room temperature water, a waste cup, unsalted crackers, a napkin, an evaluation form (Appendix O), and a writing utensil. Red lighting was selected for the sensory booths to eliminate potential bias due to color variation from marination and cooking (AMSA, 1995). Twelve trained panelists were instructed to cleanse the palette with a cracker and water prior to tasting the first sample (order was randomized for each booth). Parameters panelists evaluated were initial juiciness, sustained juiciness, initial tenderness, sustained tenderness, flavor intensity, aroma intensity, off flavor intensity. Panelists were also asked to describe the off flavor, if noted. Panelists were then instructed to taste the first sample, chew ten times, and dispel the sample into the waste cup. The palette was then cleansed again as previously described and the process repeated for the next sample.

Color Measurement

The colorimeter was calibrated prior to each use using black glass and white tile placed inside a Ziplock bag. The calibration was performed in the Ziplock bags because the steak samples were also scanned while in the bags. Each steak (three steaks per treatment and time combination) was scanned three times with the MiniScan XE Plus (MiniScan by HunterLab, Reston, VA) colorimeter and the average of the three scans was recorded. This was repeated for each of the three steaks within the treatment and time combination. Color measurements (L*, a*, and b*) were taken immediately prior to steaks being placed on the grill for cooking.

Statistical Analysis

A completely randomized design was used to conduct these experiments. Each sensory experiment was conducted in duplicate and each color experiment was conducted in triplicate. The independent variables were treatment (marinade solution) and time. Statistics were completed using the Proc Mixed procedure in SAS version 9.2 (SAS Institute, Inc., Cary, NC). Appropriate two way interactions of treatment and time were evaluated. In the event that no interactions were observed, main effects were evaluated. Least squares means were used to separate mean differences. There were no differences in replications and no treatment by replication interactions were included as no practical differences observed. Tukey pairwise comparisons were utilized due to potential unequal sample size resulting from the removal of outlying data points. Data are presented with pooled standard error.

Results and Discussion

Sensory Evaluation

An interaction of time and treatment was observed for initial juiciness ($P<0.0001$; Table 5), sustained juiciness ($P<0.0001$; Table 6), initial tenderness ($P<0.0001$; Table 7), sustained tenderness ($P=0.0008$; Table 8), flavor intensity ($P=0.0039$; Table 9), and off-flavor intensity ($P=0.0009$; Table 12). The effect of treatment ($P<0.0001$) and the effect of time ($P=0.0014$) on aroma intensity are presented in Tables 10 and 11, respectively. Higher numerical values indicate a juicier sample. Those samples treated with water+ or marinade solutions received higher overall scores for initial juiciness ($P=0.0004$; Table 5). The lemon pepper marinade solution treatment was rated as the most juicy, followed by the classic steakhouse marinade solution, and the water+ solution. Water was rated as the least juicy. Similar results were collected for sustained juiciness as well (Table 6). Higher numerical values indicate increased tenderness (Tables 6 and 7). As with initial and sustained juiciness, initial and sustained tenderness was highest in samples treated with lemon pepper marinade solution, followed by classic steakhouse marinade solution, water+ solution, and finally water ($P=0.0008$; Tables 6 and 7).

An interaction of treatment by time was observed to affect flavor intensity ($P=0.0039$; Table 9). Flavor intensity increased with increasing marination time ($P=0.0002$) such that steaks marinated for 48 hours received higher flavor intensity ratings than those marinated for 24, 6, and 0 hours. Samples marinated in lemon pepper marinade solution received higher flavor intensity ratings compared to classic steakhouse marinade solution, water+ solution, or water ($P<0.0001$). Both the lemon pepper marinade solution and the classic steakhouse marinade solution were rated as having more flavor intensity compared to both water+ solution and water ($P<0.05$). These results were consistent with our expectations for flavor intensity.

No interaction of treatment by time was observed for aroma intensity ($P=0.1528$); however, both time ($P=0.0014$) and treatment ($P<0.0001$) effects were observed. The effect of

treatment on aroma intensity is presented in Table 10. All combination treatments, lemon pepper marinade solution, classic steakhouse marinade solution, and water+ solution, were rated as having greater aroma intensity compared to water ($P<0.05$). The effects of time are presented in Table 11. Marination times of 6, 24, and 48 hours generated a more intense aroma than did water alone ($P<0.05$). Marination times of 6, 24, and 48 hours did not differ from one another ($P>0.05$). Marination for 0 hours generated less aroma intensity that 24 and 48 hour marination ($P<0.05$) yet was comparable to 6 hours marination ($P>0.05$).

An interaction of time by treatment was observed with off-flavor intensity ($P=0.0009$; Table 12). As marination time increased, off-flavor intensity also increased ($P<0.0001$). Interestingly, samples marinated for 0 hours were slightly higher, numerically, in off-flavor intensity compared to samples marinated for 6 hours though this difference was not statistically significant ($P>0.05$). Panelists identified samples marinated in the water+ solution as having the most intense off-flavor. All twelve panelists described the off-flavors associated with these samples as being vinegar-like and metallic. Off-flavor descriptors associated with samples marinated in water were bloody and livery. Sour and sweet off-flavors were associated with the steaks marinated in lemon pepper marinade solution while metallic and sour off-flavors were associated with steaks marinated in the classic steakhouse marinade solution. Personal taste preferences may have influenced the off-flavors associated with each marinade type. For example, those panelists who prefer lemon pepper flavors may have noted more off-flavors associated with other marinades.

Color Measurement

An interaction of treatment by time was observed for L* color values (P=0.0013; Table 13). L* values are used to denote white to black on the color scale with the center being gray. Thus, higher values indicate a steak which is more white in color. As presented in Table 13, the steaks marinated in water were the most white in color, followed by lemon pepper marinade solution, water+ solution, and finally classic steakhouse marinade solution which was the darkest (P<0.0001). A trend of increasing marination time leading to darker color was also observed for marination time (P=0.0708) although it was not significant. These results were expected given the marinades selected. The water+ solution was slightly surprising; however, in earlier meat microbiology studies in our laboratory (Chapter 2) we observed the AMS affected color of the meat samples. This affect was not measured during that time but is clearly defined in the current study.

An interaction of treatment by time was also observed for a* color values (P=0.0225; Table 14). a* values are used to determine green and red color in meat such that positive values indicate red color while negative values indicate green values. As presented in Table 14, steaks marinated in water+ solution were more red in color, followed by classic steakhouse marinade solution, lemon pepper marinade solution, and finally water. Increasing marination time resulted in lower a* values, indicating less red color (P<0.0001). Objective a* values indicate a less red color while visual evaluation indicates a more gray color. This is important to note as the steaks remained visually appealing to the panelists.

No interaction of treatment by time was observed for b* color values (P=0.2401). Time was not significant (P=0.0718) although treatment was (P<0.0001; Table 15). b* values are used to indicate yellow and blue such that positive values indicate yellow color while negative values indicate blue color. As presented in Table 15, steaks marinated in classic steakhouse marinade

solution had higher b* values than the other marinades (P=0.0001). Steaks marinated in lemon pepper marinade solution and water+ solution did not differ from one another (P>0.05). Water marinated steaks had the lowest b* color values, indicating less yellow color. As with the a* values, it is important to note that the steaks remained visually appealing to panelists with the exception of the steaks marinated in water. The color of the steaks changed to be more reflective of the marinade while steaks marinated in water appeared to lose color and became gray in color.

Conclusions

Marination of meat increases juiciness, tenderness, and flavor while also increasing shelf life and improving safety. Specific taste preferences can be catered to in marinade selection. Marination of meat alters the color of the meat and is reflective of the marinade chosen. For example, the classic steakhouse marinade chosen for this study is very dark in color, thus the meat color became darker during marination as the flavor and color of the marinade was absorbed into the meat. Conversely, the lemon pepper marinade chosen was yellow in color and meat marinated in this marinade became lighter, with more yellow or gray hues, over time. The marinade solutions utilized in this study are more effective against pathogenic bacteria than the marinade alone. Thus, the novel AMS investigated herein increases inhibitory capacity of marinades while also positively affecting meat sensory characteristics. Steaks marinated in water alone received lower ratings for all sensory parameters evaluated, suggesting the AMS improves juiciness, tenderness, flavor, and aroma. Although the AMS improves sensory characteristics, it should not be used alone or in combination with water due to the intense flavor of the solution itself. Marinades help to balance the intense vinegar-like flavor of the antimicrobial solution.

Table 5: Interaction of treatment and time on sensory parameters: initial juiciness.

Treatment	Time	Estimate	Std. Error
Lemon Pepper	0	6.9091[a]	0.2941
Water	24	6.3077[a]	0.2706
Classic Steakhouse	6	6.2000[a]	0.3085
Lemon Pepper	48	6.1563[a]	0.2439
Lemon Pepper	6	6.0000[a]	0.2941
Water+	24	5.9375[ab]	0.2439
Classic Steakhouse	48	5.4545[b]	0.2941
Classic Steakhouse	24	5.4091[b]	0.2941
Water+	6	5.3636[b]	0.2941
Water+	48	5.3125[b]	0.2439
Classic Steakhouse	0	5.0000[b]	0.2941
Water	48	4.7500[c]	0.2816
Water+	0	4.7500[c]	0.2816
Water	6	4.5833[c]	0.2816
Water	0	4.5714[c]	0.2607
Lemon Pepper	24	4.5500[c]	0.3085

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM ($P<0.05$). Antimicrobial solution was prepared at Auburn University and diluted (1:5) in the marinades or distilled water (Water+). Distilled water alone served as the control.

Table 6: Interaction of treatment and time on sensory parameters: sustained juiciness.

Treatment	Time	Estimate	Std. Error
Lemon Pepper	0	6.5455[a]	0.3477
Water	24	6.3077[a]	0.3199
Lemon Pepper	48	6.0625[a]	0.2883
Lemon Pepper	6	5.9091[a]	0.3477
Water+	24	5.8750[a]	0.2883
Classic Steakhouse	6	5.7000[a]	0.3647
Classic Steakhouse	24	5.3636[ab]	0.3477
Water+	6	5.1818[ab]	0.3477
Classic Steakhouse	48	5.0455[ab]	0.3477
Water+	48	5.0000[b]	0.2883
Classic Steakhouse	0	4.9091[b]	0.3477
Water	0	4.7857[b]	0.3082
Lemon Pepper	24	4.6000[b]	0.3647
Water+	0	4.5833[b]	0.3329
Water	48	4.4167[b]	0.3329
Water	6	4.1667[b]	0.3329

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM ($P<0.05$). Antimicrobial solution was prepared at Auburn University and diluted (1:5) in the marinades or distilled water (Water+). Distilled water alone served as the control.

Table 7: Interaction of treatment and time on sensory parameters: initial tenderness.

Treatment	Time	Estimate	Std. Error
Classic Steakhouse	6	6.4000[a]	0.3313
Water	24	6.3846[a]	0.2906
Lemon Pepper	48	6.3125[a]	0.2619
Lemon Pepper	0	6.2727[a]	0.3159
Lemon Pepper	6	6.0000[a]	0.3159
Water+	24	5.8750[a]	0.2619
Classic Steakhouse	0	5.6364[a]	0.3159
Water+	48	5.5000[a]	0.2619
Classic Steakhouse	24	5.4545[a]	0.3159
Water	0	5.3571[a]	0.2800
Classic Steakhouse	48	5.1818[a]	0.3159
Water+	6	5.0909[a]	0.3159
Lemon Pepper	24	5.0000[a]	0.3313
Water	48	4.9167[a]	0.3025
Water+	0	4.9167[a]	0.3025
Water	6	4.5000[b]	0.3025

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM ($P<0.05$). Antimicrobial solution was prepared at Auburn University and diluted (1:5) in the marinades or distilled water (Water+). Distilled water alone served as the control.

Table 8: Interaction of treatment and time on sensory parameters: sustained tenderness.

Treatment	Time	Estimate	Std. Error
Water	24	6.3846[a]	0.3261
Classic Steakhouse	6	6.3000[a]	0.3718
Lemon Pepper	0	6.1818[a]	0.3545
Lemon Pepper	48	6.1250[a]	0.2939
Lemon Pepper	6	6.0909[a]	0.3545
Classic Steakhouse	0	5.9091[a]	0.3545
Water+	24	5.6875[a]	0.2939
Water	0	5.6429[a]	0.3142
Classic Steakhouse	24	5.6364[a]	0.3545
Water+	06	5.5455[a]	0.3545
Water+	48	5.4375[a]	0.2939
Classic Steakhouse	48	5.3636[a]	0.3545
Lemon Pepper	24	5.3000[a]	0.3718
Water	0	4.8333[a]	0.3394
Water	48	4.6667[b]	0.3394
Water	6	4.5000[b]	0.3394

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM ($P<0.05$). Antimicrobial solution was prepared at Auburn University and diluted (1:5) in the marinades or distilled water (Water+). Distilled water alone served as the control.

Table 9: Interaction of treatment and time on sensory parameters: flavor intensity.

Treatment	Time	Estimate	Std. Error
Lemon Pepper	48	6.4375[a]	0.3720
Classic Steakhouse	24	6.0000[a]	0.4487
Classic Steakhouse	48	5.8182[a]	0.4487
Lemon Pepper	6	5.5455[a]	0.4487
Lemon Pepper	24	5.0000[a]	0.4706
Water+	24	5.0000[a]	0.3720
Water+	48	4.4375[b]	0.3720
Lemon Pepper	0	4.3636[b]	0.4487
Water+	6	4.3636[b]	0.4487
Classic Steakhouse	6	4.1000[b]	0.4706
Classic Steakhouse	0	3.7273[b]	0.4487
Water	0	3.4286[b]	0.3977
Water	6	3.4167[b]	0.4296
Water	24	3.3077[b]	0.4127
Water+	0	3.1667[b]	0.4296
Water	48	2.8333[b]	0.4296

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM (P<0.05). Antimicrobial solution was prepared at Auburn University and diluted (1:5) in the marinades or distilled water (Water+). Distilled water alone served as the control.

Table 10: Effect of treatment on sensory parameters: aroma intensity.

Treatment	Estimate	Std. Error
Lemon Pepper	4.9159[a]	0.2372
Classic Steakhouse	4.8023[a]	0.2468
Water+	4.1065[a]	0.2211
Water	3.0156[b]	0.2269

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM ($P<0.05$). Antimicrobial solution was prepared at Auburn University and diluted (1:5) in the marinades or distilled water (Water+). Distilled water alone served as the control.

Table 11: Effect of time on sensory parameters: aroma intensity.

Time	Estimate	Std. Error
24	4.5889[a]	0.2323
48	4.5814[a]	0.2211
6	4.2284[a,c]	0.2443
0	3.4416[b,c]	0.2345

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM (P<0.05). Antimicrobial solution was prepared at Auburn University and diluted (1:5) in the marinades or distilled water (Water+). Distilled water alone served as the control.

Table 12: Interaction of treatment and time on sensory parameters: off-flavor intensity.

Treatment	Time	Estimate	Std. Error
Lemon Pepper	48	3.5625[a]	0.3617
Water+	24	3.3750[a]	0.3617
Water+	48	2.7500[a]	0.3617
Water	24	2.3077[a]	0.4013
Classic Steakhouse	48	2.1818[a]	0.4363
Water	0	2.0000[a]	0.3867
Classic Steakhouse	24	1.8182[a]	0.4363
Lemon Pepper	6	1.6364[a]	0.4363
Lemon Pepper	0	1.5455[b]	0.4363
Water	48	1.5000[b]	0.4177
Water+	6	1.3636[b]	0.4363
Classic Steakhouse	0	1.3636[b]	0.4363
Water	6	1.3333[b]	0.4177
Classic Steakhouse	6	1.2000[b]	0.4575
Water+	0	1.1667[b]	0.4177
Lemon Pepper	24	1.1000[b]	0.4575

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM ($P<0.05$). Antimicrobial solution was prepared at Auburn University and diluted (1:5) in the marinades or distilled water (Water+). Distilled water alone served as the control.

Table 13: Interaction of treatment and time on color parameters: L*.

Treatment	Time	Estimate	Std. Error
Water	48	68.7367[a]	1.9059
Water	24	61.6433[a]	1.9059
Water	6	61.6300[a]	1.9059
Water	0	52.7200[b]	1.9059
Lemon Pepper	24	50.6067[b]	1.9059
Lemon Pepper	48	49.1300[b]	1.9059
Lemon Pepper	6	48.7767[b]	1.9059
Lemon Pepper	0	44.9633[b]	1.9059
Water+	0	44.4167[b]	1.9059
Water+	24	40.5600[b]	1.9059
Water+	6	40.4933[b]	1.9059
Water+	48	38.7800[b]	1.9059
Classic Steakhouse	0	36.2900[b]	1.9059
Classic Steakhouse	48	36.2300[b]	1.9059
Classic Steakhouse	24	36.1667[b]	1.9059
Classic Steakhouse	6	36.0600[b]	1.9059

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM ($P<0.05$). Antimicrobial solution was prepared at Auburn University and diluted (1:5) in the marinades or distilled water (Water+). Distilled water alone served as the control.

Table 14: Interaction of treatment and time on color parameters: a*.

Treatment	Time	Estimate	Std. Error
Water+	6	17.2733[a]	1.0482
Classic Steakhouse	0	16.6033[a]	1.0482
Water+	0	14.3467[a]	1.0482
Classic Steakhouse	6	13.3067[a]	1.0482
Lemon Pepper	0	11.7267[b]	1.0482
Classic Steakhouse	48	11.6833[b]	1.0482
Water+	24	11.5767[b]	1.0482
Classic Steakhouse	24	11.2833[b]	1.0482
Water+	48	10.9700[b]	1.0482
Lemon Pepper	6	8.4700[c]	1.0482
Water	0	8.3700[c]	1.0482
Water	24	8.0567[c]	1.0482
Water	6	6.7700[c]	1.0482
Lemon Pepper	48	5.4300[c]	1.0482
Water	48	5.3100[c]	1.0482
Lemon Pepper	24	5.1433[c]	1.0482

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM (P<0.05). Antimicrobial solution was prepared at Auburn University and diluted (1:5) in the marinades or distilled water (Water+). Distilled water alone served as the control.

Table 15: Effect of treatment on color parameters: b*.

Treatment	Estimate	Std. Error
Classic Steakhouse	24.2317[a]	0.6862
Lemon Pepper	19.0408[b]	0.6862
Water+	17.7983[b]	0.6862
Water	15.2342[b]	0.6862

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM (P<0.05). Antimicrobial solution was prepared at Auburn University and diluted (1:5) in the marinades or distilled water (Water+). Distilled water alone served as the control.

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CHAPTER VI: Summary and Future Research

Summary

Pathogenic bacteria represent a public health concern when present on meat and meat products. Numerous strategies and technologies for reducing and preventing contamination by pathogenic bacteria have been evaluated and applied. Hurdle technologies are perhaps a more effective strategy to inhibit bacterial growth. Hurdle technology is the use of multiple hurdles against bacterial growth, utilized at sub-inhibitory levels, which inhibit bacterial growth when combined. Marination and antimicrobial solutions are two examples of hurdle technologies. This research evaluated the combined hurdles of marinade and antimicrobial solution (AMS) against pathogens of concern on fresh beef.

Multiple antimicrobial agents and solutions currently exist and many more are being developed. Common examples of antimicrobial solutions or compounds include lactic acid, acetic acid, chitosan, nisin, and lauric arginate ester. Many of these antimicrobials are being used in direct application to meat as well as in active packaging. Lactic and acetic acids solutions of 1-2% are commonly sprayed onto carcasses following harvest as a decontaminant. Often, a ten to fifteen minute contact time is allowed before the carcass is rinsed again and the organic acid solution is washed off. Active packaging is being developed to slowly release the antimicrobial agent during the storage period. While the results have been favorable for both types of application, these are limited to use in the industry and little research has been conducted with an application for consumers to utilize in their homes. This research was designed with the consumer in mind and the design was to mimic the way a consumer may marinate meat in their home.

A novel antimicrobial solution (AMS) has been developed by researchers at USDA Agriculture Research Service using GRAS ingredients with potential antimicrobial properties.

The AMS yielded favorable findings in previous research conducted on fruit rinds and vegetable stem scars. The next step in evaluating the AMS was to determine if it is effective in meat. The collaborative research team designed this research to simultaneously evaluate the efficacy of AMS in meat as well as evaluate a potential use for consumers. While AMS has great potential for application within the meat industry as a topical spray or dip, our ultimate goal is for retail marinade companies to include AMS in their marinade products which are then purchased directly by consumers.

During our research, three concentrations of AMS were evaluated and all were found to inhibit growth of pathogenic bacteria inoculated onto meat samples. The low concentration AMS was found to be less inhibitory than the high and medium concentrations, but was more inhibitory than the water control. The medium concentration functions the same as the high concentration, but it is a less expensive alternative to the high concentration because it can be diluted into the marinade. The AMS should be combined with a flavorful marinade as some off flavors have been associated with the AMS. Our research indicates that both lemon pepper marinade and classic steakhouse marinade are suitable for use with AMS. Many other retail marinade flavors are available and should also be evaluated with AMS.

Sensory attributes were improved with inclusion of AMS in the marinade solution. Steaks marinated in a solution with AMS received higher ratings for tenderness, juiciness, flavor, and aroma. The lemon pepper marinade was rated as the most tender, juicy, and flavorful. It is likely that lower pH marinades, like the lemon pepper marinade, will yield a more tender, juicy, and flavorful marinated product due to more absorption of the marinade. The lower pH will increase the rate of muscle breakdown, thereby increasing the rate of marinade absorption. Off flavors were also observed during the sensory evaluation of marinated meat products. While these off

flavors may be associated with the inclusion of AMS, it is likely that they are an effect of the marinade. Off flavors associated with the lemon pepper marinade were sweet and sour while those associated with the classic steakhouse marinade were metallic and sour. The off flavors described are characteristic of each marinade alone. An additional sensory evaluation of steaks marinated in the AMS alone would provide additional information on any effects of AMS on off flavors. Meat color is altered with marination. The color of the steaks became more reflective of the marinade used such that lemon pepper marinated steaks were lighter and more yellow in color compared to classic steakhouse marinated steaks which were darker and more red in color. Again, the alterations in color were consistent with the marinade chosen. Marination of steaks in AMS alone would provide additional information on any color alterations due to AMS.

This AMS has great potential for a consumer application. Very few other antimicrobial agents or solutions have a consumer-friendly application as most are an industry or commercial application. Our consumer-based approach suggests inclusion of AMS in retail marinades. This would allow consumers to continue using products familiar to them while improving food safety and sensory characteristics including tenderness, juiciness, and flavor. Retail marinade companies could mix AMS into existing marinades or develop new marinade options. This is perhaps the simplest application of AMS to meat marination as retail marinades are ready-to-use at purchase. The manufacturer could mix the AMS in-house and dilute it into the marinade batches. Packaging would not required alterations, though the label may need to include some additional ingredients. The unique aspect of AMS is that all ingredients are GRAS and are currently used in the meat and dairy industries. Additionally, common or household names can be used on the label rather than scientific names. For example, acetic acid is commonly known as vinegar.

Other applications may include use of AMS in further processing facilities. This is more of an industry and commercial application though consumers may be able to purchase items at the retail level. This application would require some additional consideration and evaluation for application. The AMS, as currently formulated, requires at least 24 hours of mixing for all ingredients to go into solution and form the proper consistency when 1 liter is prepared. For a further processing application, several gallons would need to be mixed which would require considerable time. Perhaps a reformulation of AMS would create a better application in further processing scenarios. An initial investigation into reformulation may involve removing chitosan from the formulation. The other ingredients go into solution quickly and would require much less time for mixing. Additionally, an investigation into a dry packet of the ingredients may be another potential application. In this way the AMS would perform much like a spice pre-mix which is added to liquid (water) and mixed. The marinated product could then be packaged, frozen, and sold in retail stores.

Additionally, AMS could be utilized in retail meat counters where products offered include pre-marinated or pre-seasoned ready-to-cook meat items. This is another application which is consumer-based and would allow the consumer to make selections based on taste preferences. For this scenario, a dry application of AMS would be beneficial as it could be included in a spice mix or packet. The dry mix AMS could then be included in roast seasoning packets and other seasoning options available to consumers at retail stores.

AMS offers benefits above other antimicrobials because it can be a consumer-based product and application. AMS also improves sensory characteristics and would improve meat quality and consistency for consumers. Additional research will determine any differences in efficacy between various antimicrobials, AMS included. This research demonstrates the efficacy

of AMS, retail marinades, and retail marinades + AMS against pathogens of concern on fresh beef. The research also demonstrates the enhanced sensory characteristics of marinated steaks achieved when AMS is included in the marinade.

Future Research

The first step in future research is to determine if the AMS inhibits growth of pathogenic bacteria or if it exhibits bactericidal activity against pathogens. The mechanism by which the AMS elicits an effect against pathogenic bacteria is currently unknown and future research should provide a better understanding of the mechanism(s) involved. Such research may focus on decreased a_w, decreased pH, and disruption of the cell membrane.

Research is also needed to determine the effects of the AMS used in these studies in processed and ready-to-eat meats. The current study focused on application to fresh beef steaks though additional research is needed to determine inhibitory properties against pathogens of concern in fresh poultry, pork, and in processed meats including but not limited to deli meats, hotdogs, and frankfurters. Research in fresh meats may be conducted in a similar manner as detailed in the current research. However, a different approach may be applied for research in processed meats. Potential research topics include: 1) incorporating the AMS as an ingredient in processed meat batters, 2) evaluation of topical application techniques to processed meats including sprays, dips, and immersions, 3) evaluation of sensory and quality properties of processed meats which may be impacted by inclusion of the AMS as an ingredient in the meat batter, and 4) evaluation of the AMS as a soak for processed meat casings. Though the previously mentioned areas of research are limited, the potential topics represent a broad field of opportunities.

Additional research is needed to determine if a lower concentration of the AMS can be incorporated into the marinades while maintaining the inhibitory properties observed with inclusion of the medium concentration described herein. Other retail marinade options should also be evaluated for their inhibitory effects against pathogens of concern as well as the inhibitory effects of the combination of the marinade and the antimicrobial solution. Some potential marinades to evaluate include other flavors in the KC Masterpiece and Ken's Steakhouse marinade lines such as teriyaki, honey teriyaki, Santa Fe picante, buffalo, Caribbean jerk, southwestern chipotles and lime, Tuscan garlic and herb, Hawaiian spicy mango, and Napa garlic balsamic, to name a few. The pH of some of these marinades is likely to be similar, thus producing similar inhibitory effects.

Another interesting avenue for future research is to evaluate the effects of each component of the AMS against pathogens of concern. Though previous research indicates the inhibitory effects of each ingredient separately or in combination with other compounds, it would be interesting and valuable to determine the effects of each component in one laboratory utilizing the same equipment, environment, technique, and the same pathogenic strains of bacteria. This would allow for a more level comparison of the AMS components and may identify some components which can be removed from the AMS without negatively affecting the inhibition of growth of pathogenic bacteria. Additional reformulations of the AMS may be identified; some potential reformulations may include use of other organic acids or sodium metasilicate.

As research on the inhibitory effects of marinades and antimicrobial solutions against pathogens of concern gains additional attention, more novel antimicrobial solutions are likely to be developed. Future research should evaluate natural antimicrobial compounds which will meet clean and green labeling demands. This will create a unique application for the meat industry

while meeting consumer demands for clean labels and natural, minimally processed products. Research should also elicit and analyze differences in spray, packaging, and immersion applications as different antimicrobial solutions may perform differently when various application techniques are employed. Previous research indicates that antimicrobial compounds may be incorporated into active packaging products, yet various antimicrobial compounds may behave differently in these applications. Any differences in use of antimicrobials as processing aides and as ingredients in product formulation or marination should also be evaluated.

Other applications for antimicrobial solutions could also be evaluated. Some new avenues for application include seafood and pet products. Some research has indicated inhibitory effects of antimicrobial compounds in seafood, though that research focused on frozen shrimp. Pet products, including pet food and treats, represent unchartered territory for application of this AMS or other options. Though limited compared to products in the human food supply, recalls of pet products have occurred in recent years. Pet companies may be able to capitalize on the application of the AMS as an ingredient in their products or as a topical, spray-type application. New sensory data will be required to determine the palatability for pets and overall acceptance of an antimicrobial in products.

The possibilities for research related to this AMS are limitless. Many untapped markets exist which may benefit from use of an AMS or active packaging. The directions of future research discussed herein represent only a small portion of the existing opportunities. As more is learned about how antimicrobial solutions inhibit pathogen growth, concentrations at which antimicrobials are effective in various mediums, and how formulation of antimicrobial solutions affects capacity to inhibit growth of pathogenic bacteria, new research directions will be

identified and pursued. Meat safety and microbiology is a growing, dynamic, and exciting field for young and established researchers alike and will continue to gain interest and attention.

APPENDICES

Appendix A. Phase 1: Survivability

1) Day 0 – Pull pathogens from the freezer and thaw.
   a) Inoculate sterile TSB broth (This is now the "stock" culture.)
   b) Incubate at 37°C for 24 hours
2) Day 1 – Transfer from stock culture to broth. ("Tube")
   a) 100 µL from stock into 9 mL sterile TSB
   b) Incubate at 37°C for 24 hours
3) Day 2 – From tube:
   a) 100 µL onto PCA plate (x 10 plates to recover 9 mL of cell suspension)
   b) 100 µL into sterile TSB tube (x 1 tube)
   c) Incubate at 37°C for 24 hours
4) Day 3 – Harvest Cells (for determination of number of cells harvested)
   a) From broth tubes:
      i) Pour culture from tube into sterile conical tube.
      ii) Centrifuge at 3650 RPM for 20 minutes at 37°C.
      iii) Discard supernatant.
      iv) Gently wash resulting pellet with 0.1% peptone (used about 3 mL).
      v) Discard wash supernatant.
      vi) Resuspend cells in 9 mL 0.1% peptone.
      vii) Serial dilutions in 9 mL 0.1% peptone.
      viii) Plate, incubate at 37°C for 24 hours, then enumerate plates.
      ix) Refrigerate inoculum for later use.
   b) From plates (x 10 for 10 plates):
      i) Pipette 1 mL 0.1% peptone onto plate.
      ii) Use hockey stick/cell spreader to scrape cells off plate. (Work quickly but carefully. Circular scraping motions seem most effective.)
      iii) Pipette the cell suspension from the surface of the plate (tilt the plate to one side to allow the suspension to gather) into a conical tube. The suspension from all plates can be combined into one conical tube.
      iv) Add 0.1% peptone to reach a total volume of 9 mL.
      v) Centrifuge at 3650 RPM for 20 minutes at 37°C.
      vi) Discard supernatant.
      vii) Gently wash resulting pellet with 0.1% peptone (used about 3 mL).
      viii) Discard wash supernatant.
      ix) Resuspend cells in 9 mL 0.1% peptone.
      x) Serial dilutions in 9 mL 0.1% peptone.
      xi) Plate, incubate at 37°C for 24 hours, then enumerate plates.
      xii) Refrigerate inoculum for later use.
5) Day 4 – Count cells plated on Day 3 to determine the number of cells harvested.
   a) Dilute inoculums to the same concentration.
   b) Inoculate meat samples.
      i) Fresh top round beef steaks cut to 100 cm^2.
      ii) Pipette 100 µL of the inoculum onto the surface of the meat and spread with cell spreader.
      iii) Allow 30 minutes of contact/adhesion time.

iv) Sample 0 hour samples.
    (1) Place meat sample in sterile stomacher bags.
    (2) Add 100 mL of 0.1% peptone to the stomacher bag.
    (3) Stomach at 300 RPM for 2 minutes.
    (4) Create serial dilutions in 9 mL 0.1%peptone.
    (5) Plate 100 µL onto PCA plates for enumeration. (Surface plating followed by spreading with a cell spreader.)
    (6) Incubate at 37°C for 24 hours, then enumerate plates.
v) For 24. 48, and 72 hour samples:
    (1) Place in sterile stomacher bags and close.
    (2) Refrigerate at 4°C until appropriate sampling time.
    (3) Add 100 mL of 0.1% peptone to the stomacher bag.
    (4) Stomach at 300 RPM for 2 minutes.
    (5) Create serial dilutions in 9 mL 0.1%peptone.
    (6) Plate 100 µL onto PCA plates for enumeration. (Surface plating followed by spreading with a cell spreader.)
    (7) Incubate at 37°C for 24 hours, then enumerate plates.

6) Day 5:
    a) Count 0 hour samples.
    b) Sample 24 hour samples.
7) Day 6:
    a) Count 24 hour samples.
    b) Sample 48 hour samples.
8) Day 7:
    a) Count 48 hour samples.
    b) Sample 72 hour samples.
    c) Transfer cultures for replicates 2 and 3.
9) Day 8:
    a) Count 72 hour samples.
    b) Transfer cultures for replicates 2 and 3.
10) Day 9 – Begins replicates 2 and 3 following the steps described above.

Notes:

- Always do replicate 1 alone. Can do replicates 2 and 3 together but do not overlap with replicate 1 counting. This allows you to eliminate some unnecessary plates and dilutions which are not required. Do at least 5 dilutions to make sure you catch your count.
- Expect 9 log CFU/mL from broth cultures.
- Expect 10 to 11 log CFU/mL from plate cultures. (Due to decreased suspension volume recovered from the plate. The agar absorbs some of the liquid added to harvest the cells.)
- Go with the higher dilution factor if two are countable.
- Can overlap replicates 2 and 3 but need to be organized.
- Make media, then peptone tubes, then bulk peptone. Otherwise, will run out of glassware.

Appendix B. Phase 2: Antimicrobial Solution Baseline

1) Day 1:
   a) Transfer EC, STEC, and Sal for 4 & 6 log inoculums.
2) Day 2:
   a) Prepare antimicrobial solutions.
   b) Transfer EC, STEC, and Sal for 4 & 6 log inoculums.
   c) Transfer LM for 4 & 6 log inoculums.
3) Day 3:
   a) Dilute antimicrobial solutions.
   b) Transfer EC, STEC, and Sal for 4 & 6 log inoculums.
   c) Cut meat pieces and place in styrofoam trays.
   d) Harvest EC, STEC, and Sal cells.
   e) Inoculate, treat, bag, sample/store following the schedule included below.

Pathogen	Inoculate	Treat	24, 48 hour	6 hour
4EC	6:30	7:00	7:00	1:00
4ES	7:00	7:30	7:30	1:30
4Sal	7:30	8:00	8:00	2:00
6EC	8:00	8:30	8:30	2:30
6ES	8:30	9:00	9:00	3:00
6Sal	9:00	9:30	9:30	3:30
4EC	8:30	9:00		
4ES	9:00	9:30		
4Sal	9:30	10:00		
6EC	10:00	10:30		
6ES	10:30	11:00		
6Sal	11:00	11:30	0 hour samples	

4) Day 4 :
   a) Cut meat pieces and place in styrofoam trays.
   b) Harvest LM cells.
   c) Count plates from Day 3.
   d) Inoculate, treat, bag, sample/store following the schedule included below.

Pathogen	Inoculate	Treat	24, 48 hour	6 hour
4EC			7:00	
4ES			7:30	
4Sal			8:00	
6EC			8:30	
6ES			9:00	
6Sal			9:30	
4LM	9:30	10:00	10:00	4:00
6LM	10:00	10:30	10:30	4:30

4LM	10:30	11:00	
6LM	11:00	11:30	0 hour samples

5) Day 5:
   a) Count plates from Day 4.
   b) Plating following the schedule below.

Pathogen	24, 48 hour
4EC	7:00
4ES	7:30
4Sal	8:00
6EC	8:30
6ES	9:00
6Sal	9:30
4LM	10:00
6LM	10:30

6) Day 6:
   a) Count plates from Day 5.
   b) Plating following the schedule below.

Pathogen	24, 48 hour
4LM	10:00
6LM	10:30

7) Day 7:
   a) Count plates from Day 6.
   b) Clean.

*2 replicates following the schedule outlined herein.

Appendix C. Phase 3: Marinade Baseline

1) Day 1:
   a) Transfer EC, STEC, and Sal for 4 & 6 log inoculums.
2) Day 2:
   a) Prepare antimicrobial solutions.
   b) Transfer EC, STEC, and Sal for 4 & 6 log inoculums.
   c) Transfer LM for 4 & 6 log inoculums.
3) Day 3:
   a) Dilute antimicrobial solutions.
   b) Transfer EC, STEC, and Sal for 4 & 6 log inoculums.
   c) Cut meat pieces and place in styrofoam trays.
   d) Harvest EC, STEC, and Sal cells.
   e) Inoculate, treat, bag, sample/store following the schedule included below.

Pathogen	Inoculate	Treat	24, 48 hour	6 hour
4Sal	6:30	7:00	7:00	1:00
4EC	7:00	7:30	7:30	1:30
4ES	7:30	8:00	8:00	2:00
6Sal	8:00	8:30	8:30	2:30
6EC	8:30	9:00	9:00	3:00
6ES	9:00	9:30	9:30	3:30
4Sal	8:15	8:45		
4EC	8:45	9:15		
4ES	9:30	10:00		
6Sal	10:00	10:30		
6EC	10:15	10:45		
6ES	10:15	10:45	0 hour samples	

4) Day 4 :
   a) Cut meat pieces and place in styrofoam trays.
   b) Harvest LM cells.
   c) Count plates from Day 3.
   d) Inoculate, treat, bag, sample/store following the schedule included below.

Pathogen	Inoculate	Treat	24, 48 hour	6 hour
4Sal			7:00	
4EC			7:30	
4ES			8:00	
6Sal			8:30	
6EC			9:00	
6ES			9:30	
4LM	6:30	7:00	7:00	1:00
6LM	7:00	7:30	7:30	1:30

4LM	7:45	8:15	0 hour samples
6LM	7:45	8:15	

5) Day 5:
   a) Count plates from Day 4.
   b) Plating following the schedule below.

Pathogen	24, 48 hour
4Sal	7:00
4EC	7:30
4ES	8:00
6Sal	8:30
6EC	9:00
6ES	9:30
4LM	7:00
6LM	7:30

6) Day 6:
   a) Count plates from Day 5.
   b) Plating following the schedule below.

Pathogen	24, 48 hour
4LM	7:00
6LM	7:30

7) Day 7:
   a) Count plates from Day 6.
   b) Clean.

Notes:
- *2 replicates following the schedule outlined herein.
- Marinades:
  - X = Ken's Steakhouse Balsamic & Roasted Onion
  - Y = KC Masterpiece Lemon & Cracked Pepper
  - Z = KC Masterpiece Classic Steakhouse

Appendix D. Phase 4: Marinade + Antimicrobial

8) Day 1:
   a) Transfer EC, STEC, and Sal for 4 & 6 log inoculums.
9) Day 2:
   a) Prepare antimicrobial solutions.
   b) Transfer EC, STEC, and Sal for 4 & 6 log inoculums.
   c) Transfer LM for 4 & 6 log inoculums.
10) Day 3:
   a) Dilute antimicrobial solutions.
   b) Transfer EC, STEC, and Sal for 4 & 6 log inoculums.
   c) Cut meat pieces and place in styrofoam trays.
   d) Harvest EC, STEC, and Sal cells.
   e) Inoculate, treat, bag, sample/store following the schedule included below.

Pathogen	Inoculate	Treat	24, 48 hour	6 hour
4EC	6:45	7:15	7:15	1:15
4ES	7:00	7:30	7:30	1:30
4Sal	7:15	7:45	7:45	1:45
6EC	7:30	8:00	8:00	2:00
6ES	7:45	8:15	8:15	2:15
6Sal	8:00	8:30	8:30	2:30
4EC	9:00	9:30		
4ES	9:15	9:45		
4Sal	9:30	10:00		
6EC	9:45	10:15		
6ES	10:00	10:30		
6Sal	10:15	10:45	0 hour samples	

11) Day 4 :
   a) Cut meat pieces and place in styrofoam trays.
   b) Harvest LM cells.
   c) Count plates from Day 3.
   d) Inoculate, treat, bag, sample/store following the schedule included below.

Pathogen	Inoculate	Treat	24, 48 hour	6 hour
4EC			7:15	
4ES			7:30	
4Sal			7:45	
6EC			8:00	
6ES			8:15	
6Sal			8:30	
4LM	6:45	7:15	7:15	1:15
6LM	7:00	7:30	7:30	1:30

4LM	7:15	7:45	0 hour samples
6LM	7:15	7:45	

12) Day 5:
   a) Count plates from Day 4.
   b) Plating following the schedule below.

Pathogen	24, 48 hour
4EC	7:15
4ES	7:30
4Sal	7:45
6EC	8:00
6ES	8:15
6Sal	8:30
4LM	7:15
6LM	7:30

13) Day 6:
   a) Count plates from Day 5.
   b) Plating following the schedule below.

Pathogen	24, 48 hour
4LM	7:15
6LM	7:30

14) Day 7:
   a) Count plates from Day 6.
   b) Clean.

Notes:
- *2 replicates following the schedule outlined herein.
- Marinades:
  - X = Removed from this portion of research due to unavailability from manufacturer and performance comparable to the classic steakhouse marinade.
  - Y = KC Masterpiece Lemon & Cracked Pepper
  - Z = KC Masterpiece Classic Steakhouse

Appendix E. Colorimeter Settings

Set Up = #94
Standard = WORKING
Display = ABSOLUTE
Average = 2 SAMPLES
ILL/OBS = D65/10*

Appendix F. Antimicrobial Solution Preparation

1) If the levulinic acid gets solid, place bottle in a 50°C water bath for 1-2 hours until completely thawed.
2) Add 10 mL acetic acid, 10 mL lactic acid, and 10 mL levulinic acid to 250 mL of MilliQ water.
3) Bring the total volume of the acid solution to 500 mL using MilliQ water.
4) Add 100 mL of LAE to the acid solution.
5) Mix solution with stir bar for 1 hour.
6) Add 10 g of chitosan to the solution (Add VERY slowly or it will clump together and not go into solution) and stir on stir plate overnight (4-12 hours) until completely dissolved.
7) Store solution at 4°C for up to 1 year. Mix well before use.

*Set the stir bar to the highest speed it will go WITHOUT creating bubbles in the solution.

Appendix G. Sample Preparation, DNA Extraction, PCR Protocol, Gel Preparation, Electrophoresis and Imaging

Day 1: Sample Preparation and DNA Extraction

1) Harvest cells.
    a) Centrifuge at 3650 RPM for 20 min at 37°C.
    b) Discard supernatant
        i) Wash cell pellet with 0.1% peptone (~3mL)
        ii) Discard supernatant
    c) Resuspend cells in 9 mL 0.1% peptone
    d) Create serial dilutions in 9 mL 0.1% peptone tubes
2) Cut meat slices to 100 cm^2 and place in styrofoam trays
3) Inoculate meat samples with 1 mL of assigned
    a) Spread inoculum with cell spreader
    b) Allow 30 min contact time
4) Treat with 30 mL of assigned treatment (Water, LP, AMS, or AMS+LP)
5) Put in sterile stomacher bag
    a) Add 100 mL 0.1% peptone
    b) Stomach 2 min at 300 RPM
6) Collect 20 mL of cell suspension into conical tube
7) Centrifuge conical tube at 3650 RPM for 20 min at 37°C
8) Discard supernatant
    a) Resuspend pellet in 5 mL 0.1% peptone

DNA Extraction

1) Shake the PrepMan Ultra Sample Preparation Reagent well, then let the reagent settle until all bubbles have disappeared
2) Using 100 µL per reaction and a sterile pipette, transfer the appropriate quantity of PrepMan Ultra Sample Preparation Reagent into a 50 mL sterile conical tube or other sterile container
3) Label the tubes and pipette 1 mL of culture broth containing bacteria into a new 2 mL or other appropriate microcentrifuge screw-cap tube
4) Spin the tubes in the microcentrifuge at the highest speed for 2 min
5) Aspirate and discard the supernatant using a disposable transfer pipette
    a) Use a new pipette for each sample. Do not decant the sample.
    b) Remove as much of the supernatant as possible without disturbing the pellet
6) Using a 1 mL pipette, aseptically add 100 µL of the PrepMan Ultra Sample Preparation Reagent into each tube
7) Tightly cap tubes, then vigorously vortex the sample
8) Place the microcentrifuge screw-cap tubes in a heat block set to 100 °C for 10 min
9) While the samples are heating, label a second set of 2 mL or other appropriate microcentrifuge screw-cap tubes
10) Remove the sample tubes from the heat block and allow them to cool to room temperature for 2 min
11) Spin the tubes in the microcentrifuge at the highest speed for 2 min

12) Transfer 50 µL of the supernatant from the spun tubes into a second set of labeled microcentrifuge screw-cap tubes and discard remaining supernatnat. Use 5 µL of supernatant per assay reaction
    a) Refrigerate or freeze sample when not in use

Day 2: PCR Procedure

1. Add the following reagents to 0.1 mL PCR tubes:

PCR Template	2.5 uL
Forward Primer (10 uM)	1.25 uL
Reverse Primer (10 uM)	1.25 uL
PCR Master Mix (2x)	12.5 uL
MilliQ Water	7.5 uL
Total Volume	**25 uL**

2. Place the tubes in the thermal cycler, set the "Touch Down" PCR program as follows:

Pre-denaturing	94°C 5 min
Cycling (35 cycles)	94°C 15 sec 58°C 30 sec 72°C 30 sec
Post Extension	72°C 10 min

3. After run, determine the PCR amplicons by 2% agarose gel electrophoresis.

Gel Preparation:

Introduction: Agarose gel electrophoresis is the most common method to separate and analyze DNA. The purpose of the gel may be to visualize the DNA, to quantify it, or to isolate a particular band. DNA is negatively charged due to the sugar-phosphate structure. In an electric field, DNA will be driven toward the positive pole; this is the motive power of DNA in electrophoresis. Migrating speed of linear double stranded DNA in agarose gels is dependent upon the size of the DNA. DNA can be visualized in the gel with the addition of ethidium bromide, Gelred, or other suitable alternative. They bind DNA strongly, absorb UV light and transmit energy as visible light. Light intensity corresponds to quantity of DNA which may be used for DNA measurement.

Material:
DNA Sample
SeaKem LE Agarose
1x TAE Buffer (pH=8.0)
Gelred dye
6x Loading Buffer
Ready Ladder 100 bp DNA
VWR Horizontal Electrophoresis Systems
VWR Electrophoresis Power Supply
Microwave Oven

Gel imaging system
Pipette and Sterile Tips

Procedure:

1. Weigh 2 g of agarose. Transfer into a 500 mL flask.
2. Add 100 mL of 1x TAE buffer to the flask and mix by hand shaking.
3. Place the flask in the microwave and heat at high power for 3 min. Pause the microwave and shake the flask at every one minute (wear an oven mitt). Keep the solution boiling for 1 min to melt the agarose completely.
4. Remove the flask from the microwave. The solution should now be clear; if not, reboil. Cover the flask with foil to reduce evaporation and leave at room temperature for 10 min to allow it to cool to approximately 60°C.
5. Assemble the gel mold, insert the comb, and place on a level table.
6. Add 5 µL of Gelred dye to gel solution and mix well. Leave the solution for 3 min to allow bubbles to settle.
7. Pour the agarose solution into the mold. The liquid level should be above the 1/3 position of the comb. Drive any bubbles to the lower corner of the gel by pipette action.
8. Leave the mold at room temperature for 20 min to allow the gel to solidify.
9. Add about 500 mL of the TAE buffer to the electrophoresis chamber. Gently remove the gel from the mold, remove the comb, and place the gel in the chamber, so that the wells are closet to the negative pole. Make sure that the buffer covers the gel.
10. Pipette 10 µL of the 6x loading dye onto Parafilm in an approximate 2 µL dots.
11. Pipette 10 µL of the sample into the loading buffer dots, mix thoroughly by pipette action. (The dye color will change from an iodine brown color to a deep blue color.)
12. Carefully load 10 µL of the mixed sample and loading buffer into the gel wells, skipping the first well.
13. Load 10 µL of the DNA ladder into the first well.
14. Place the lid of the chamber on the apparatus. (Black to the left, red to the right.) Turn on the power supply and set the voltage (150) and timer (.6-.9).
15. After the run, turn off the power supply, remove the gel, and thoroughly rinse the gel with water.
16. Clean the UV imaging apparatus with EtOH. Place the gel on the surface.
17. Take a picture. Adjustments can be made within the imaging system.

Appendix H. Sample Numbering for PCR and Gel Electrophoresis

No.	log_{10}	Path.	Treat.	No.	log_{10}	Path.	Treat.	No.	log_{10}	Path.	Treat.
1	9	EC	Water	33	9	Sal	Water	65	9	LM	Water
2	9	EC	Mar	34	9	Sal	Mar	66	9	LM	Mar
3	9	EC	Mar+Anti	35	9	Sal	Mar+Anti	67	9	LM	Mar+Anti
4	9	EC	Anti	36	9	Sal	Anti	68	9	LM	Anti
5	8	EC	Water	37	8	Sal	Water	69	8	LM	Water
6	8	EC	Mar	38	8	Sal	Mar	70	8	LM	Mar
7	8	EC	Mar+Anti	39	8	Sal	Mar+Anti	71	8	LM	Mar+Anti
8	8	EC	Anti	40	8	Sal	Anti	72	8	LM	Anti
9	7	EC	Water	41	7	Sal	Water	73	7	LM	Water
10	7	EC	Mar	42	7	Sal	Mar	74	7	LM	Mar
11	7	EC	Mar+Anti	43	7	Sal	Mar+Anti	75	7	LM	Mar+Anti
12	7	EC	Anti	44	7	Sal	Anti	76	7	LM	Anti
13	6	EC	Water	45	6	Sal	Water	77	6	LM	Water
14	6	EC	Mar	46	6	Sal	Mar	78	6	LM	Mar
15	6	EC	Mar+Anti	47	6	Sal	Mar+Anti	79	6	LM	Mar+Anti
16	6	EC	Anti	48	6	Sal	Anti	80	6	LM	Anti
17	5	EC	Water	49	5	Sal	Water	81	5	LM	Water
18	5	EC	Mar	50	5	Sal	Mar	82	5	LM	Mar
19	5	EC	Mar+Anti	51	5	Sal	Mar+Anti	83	5	LM	Mar+Anti
20	5	EC	Anti	52	5	Sal	Anti	84	5	LM	Anti
21	4	EC	Water	53	4	Sal	Water	85	4	LM	Water
22	4	EC	Mar	54	4	Sal	Mar	86	4	LM	Mar
23	4	EC	Mar+Anti	55	4	Sal	Mar+Anti	87	4	LM	Mar+Anti
24	4	EC	Anti	56	4	Sal	Anti	88	4	LM	Anti
25	3	EC	Water	57	3	Sal	Water	89	3	LM	Water
26	3	EC	Mar	58	3	Sal	Mar	90	3	LM	Mar
27	3	EC	Mar+Anti	59	3	Sal	Mar+Anti	91	3	LM	Mar+Anti
28	3	EC	Anti	60	3	Sal	Anti	92	3	LM	Anti
29	2	EC	Water	61	2	Sal	Water	93	2	LM	Water
30	2	EC	Mar	62	2	Sal	Mar	94	2	LM	Mar
31	2	EC	Mar+Anti	63	2	Sal	Mar+Anti	95	2	LM	Mar+Anti
32	2	EC	Anti	64	2	Sal	Anti	96	2	LM	Anti

Primers:

- EC1 86593652
- EC2 86593653
- Sal F 125222061
- Sal R 125222062
- LM F 125222063
- LM R 12522206

Appendix I. Table of Culture counts following different growth mediums.

Pathogen	Growth Medium	Storage Time	N	Mean CFU	Standard Error	*P*-value
E. coli	Broth	0	9	5.795[a]	0.2417	0.31
E. coli	Plate	0	9	5.907[a]	0.2417	0.31
E. coli	Broth	24	9	5.761[a]	0.2417	0.31
E. coli	Plate	24	9	5.916[a]	0.2417	0.31
E. coli	Broth	48	9	5.846[a]	0.2417	0.31
E. coli	Plate	48	9	5.913[a]	0.2417	0.31
E. coli	Broth	72	9	5.925[a]	0.2417	0.31
E. coli	Plate	72	9	5.769[a]	0.2417	0.31
L. monocytogenes	Broth	0	9	5.628[a]	0.2417	0.31
L. monocytogenes	Plate	0	9	5.618[a]	0.2417	0.31
L. monocytogenes	Broth	24	9	5.667[a]	0.2417	0.31
L. monocytogenes	Plate	24	9	5.886[a]	0.2417	0.31
L. monocytogenes	Broth	48	9	5.725[a]	0.2417	0.31
L. monocytogenes	Plate	48	9	5.732[a]	0.2417	0.31
L. monocytogenes	Broth	72	9	5.669[a]	0.2417	0.31
L. monocytogenes	Plate	72	9	5.669[a]	0.2417	0.31

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM ($P<0.05$). Meat samples were inoculated and stored at 4°C until sampling at 0, 24, 48, or 72 hours. Broth growth medium indicates tryptic soy broth; plate growth medium indicates plate count agar.

Appendix J. Table of Effects of a novel antimicrobial solution against *Escherichia coli* O175:H7 strains on fresh beef top round.

Inoculum	Treatment	log_{10}CFU	Std. Error
6 log	Low	1.8649[a]	0.0962
6 log	Water	1.8640[a]	0.0962
4 log	Water	1.8020[a]	0.0962
4 log	Low	1.5541[a]	0.0962
4 log	Medium	1.3046[b]	0.0962
6 log	Medium	1.1627[b]	0.0962
6 log	High	1.0246[b]	0.0962
4 log	High	1.0000*[b]	0.0962

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM ($P<0.05$). *****Indicates the estimate is below the limit of detection which is 1 log. 4 and 6 log cultures of *E. coli* (EC), STECs (ES), *Salmonella* (Sal), and *L. monocytogenes* (LM) were prepared and inoculated onto meat samples. Antimicrobial solution was prepared at Auburn University and diluted to high (no dilution), medium (1:5), or low (1:10) concentrations the morning of the experiment. Dilutions were prepared using distilled water which also served as the control treatment.

Appendix K. Table of Effects of a novel antimicrobial solution against non-O175:H7 STEC strains on fresh beef top round.

Inoculum	Treatment	log_{10}CFU	Std. Error
6 log	Low	1.9981[a]	0.0962
4 log	Water	1.9412[a]	0.0962
6 log	Water	1.7170[a]	0.0962
4 log	Low	1.3201[b]	0.0962
4 log	Medium	1.1512[b]	0.0962
6 log	Medium	1.0752[b]	0.0962
4 log	High	1.0000*[b]	0.0962
6 log	High	1.0000*[b]	0.0962

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM ($P<0.05$). *****Indicates the estimate is below the limit of detection which is 1 log. 4 and 6 log cultures of *E. coli* (EC), STECs (ES), *Salmonella* (Sal), and *L. monocytogenes* (LM) were prepared and inoculated onto meat samples. Antimicrobial solution was prepared at Auburn University and diluted to high (no dilution), medium (1:5), or low (1:10) concentrations the morning of the experiment. Dilutions were prepared using distilled water which also served as the control treatment.

Appendix L. Table of Effects of a novel antimicrobial solution against *Salmonella* spp. on fresh beef top round.

Inoculum	Treatment	log_{10}CFU	Std. Error
4 log	Water	1.9213[a]	0.0962
6 log	Water	1.7768[a]	0.0962
4 log	Low	1.2423[b]	0.0962
6 log	Low	1.1438[b]	0.0962
4 log	Medium	1.0000*[b]	0.0962
6 log	Medium	1.0000*[b]	0.0962
4 log	High	1.0000*[b]	0.0962
6 log	High	1.0000*[b]	0.0962

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM ($P<0.05$). *****Indicates the estimate is below the limit of detection which is 1 log. 4 and 6 log cultures of *E. coli* (EC), STECs (ES), *Salmonella* (Sal), and *L. monocytogenes* (LM) were prepared and inoculated onto meat samples. Antimicrobial solution was prepared at Auburn University and diluted to high (no dilution), medium (1:5), or low (1:10) concentrations the morning of the experiment. Dilutions were prepared using distilled water which also served as the control treatment.

Appendix M. Table of Effects of a novel antimicrobial solution against *Listeria monocytogenes* on fresh beef top round.

Inoculum	Treatment	log_{10}CFU	Std. Error
6 log	Low	1.9230[a]	0.0962
6 log	Medium	1.7690[a]	0.0962
4 log	Water	1.7637[a]	0.0962
6 log	Water	1.7558[a]	0.0962
4 log	Low	1.0927[b]	0.0962
6 log	High	1.0383[b]	0.0962
4 log	Medium	1.0000*[b]	0.0962
4 log	High	1.0000*[b]	0.0962

[a,b,c]Data with differing superscripts indicate differences between the least squares means with pooled SEM ($P<0.05$). *****Indicates the estimate is below the limit of detection which is 1 log. 4 and 6 log cultures of *E. coli* (EC), STECs (ES), *Salmonella* (Sal), and *L. monocytogenes* (LM) were prepared and inoculated onto meat samples. Antimicrobial solution was prepared at Auburn University and diluted to high (no dilution), medium (1:5), or low (1:10) concentrations the morning of the experiment. Dilutions were prepared using distilled water which also served as the control treatment.

Appendix N. Sample Sensory Form

Project: Fisher Dissertation Research Date: August/September 2014

Beef Trained Sensory Evaluation Form

Sample Number	Initial Juiciness	Sustained Juiciness	Initial Tenderness	Sustained Tenderness	Flavor Intensity	Aroma Intensity	Off Flavor Intensity	Off Flavor Descriptor

Juiciness	Tenderness	Flavor Intensity	Aroma Intensity	Off Flavor Intensity	Off Flavor Descriptor
8 = Extremely juicy	8 = Extremely tender	8 = Extremely intense	8 = Extremely intense	8 = Extremely off flavor	8 = Metallic
7 = Very juicy	7 = Very tender	7 = Very intense	7 = Very intense	7 = Intense off flavor	7 = Salty
6 = Moderately juicy	6 = Moderately tender	6 = Moderately intense	6 = Moderately intense	6 = Very off flavor	6 = Livery
5 = Slightly juicy	5 = Slightly tender	5 = Slightly intense	5 = Slightly intense	5 = Moderate off flavor	5 = Sour
4 = Slightly dry	4 = Slightly tough	4 = Slightly bland	4 = Slightly bland	4 = Modest off flavor	4 = Sweet
3 = Moderately dry	3 = Moderately tough	3 = Moderately bland	3 = Moderately bland	3 = Small off flavor	3 = Vinegar
2 = Very dry	2 = Very tough	2 = Very bland	2 = Very bland	2 = Slight off flavor	2 = Bloody
1 = Extremely dry	1 = Extremely tough	1 = Extremely bland	1 = Extremely bland	1 = No	1 = Other, Explain

Appendix O. ANOVA Table Chapter 2: Antimicrobial Solution

Phase 2 Antimicrobial Solution ANOVA Table

Source	**DF**	**SS**	**MS**	**F value**	**Pr > F**
Model	127	153.6845	1.2101	5.45	<0.0001
Error	640	142.1064	0.2220		
Total	767	295.7909			

Source	**DF**	**Type 1 SS**	**MS**	**F value**	**Pr > F**
Pathogen	7	12.7267	1.8181	8.19	<0.0001
AMS	3	74.4623	24.8208	111.78	<0.0001
Pathogen*AMS	21	22.6097	1.0767	4.85	<0.0001
Time	3	0.4487	0.1624	0.73	0.5334
Pathogen*Time	21	11.8775	0.5656	2.55	0.0002
AMS*Time	9	2.0369	0.2263	1.02	0.4228
Pathogen*AMS*Time	63	29.4841	0.4680	2.11	<0.0001

DF = degrees of freedom
SS = Sum of Squares
MS = Mean Square
AMS = The term used to identify the novel antimicrobial solution developed by researchers at USDA and evaluated in these studies.

Model includes Pathogen, AMS, and Time. Inoculum is included in the model as part of the pathogen.

Appendix P. ANOVA Table Chapter 3: Marinades available at Retail Stores

Phase 3 Three marinades available at retail stores ANOVA Table

Source	**DF**	**SS**	**MS**	**F value**	**Pr > F**
Model	127	103.7278	0.8168	3.95	<0.0001
Error	631	130.3565	0.2066		
Total	758	234.0843			

Source	**DF**	**Type 1 SS**	**MS**	**F value**	**Pr > F**
Pathogen	7	32.7292	4.6756	22.63	<0.0001
Marinade	3	18.4431	6.1477	29.76	<0.0001
Pathogen*Marinade	21	12.4835	0.5945	2.88	<0.0001
Time	3	12.2699	4.0900	19.80	<0.0001
Pathogen*Time	21	12.2684	0.5842	2.83	<0.0001
Marinade*Time	9	4.7881	0.5320	2.58	0.0065
Pathogen*Marinade*Time	63	10.7457	0.1706	0.83	0.8281

DF = degrees of freedom
SS = Sum of Squares
MS = Mean Square

Model includes Pathogen, AMS, and Time. Inoculum is included in the model as part of the pathogen.

Appendix Q. ANOVA Table Chapter 4: Retail Marinade+AMS

Phase 4 Combined marinades available at retail stores and AMS ANOVA Table

Source	DF	SS	MS	F value	Pr > F
Model	95	56.5597	0.5954	4.97	<0.0001
Error	480	57.4753	0.1197		
Total	575	114.0350			

Source	DF	Type 1 SS	MS	F value	Pr > F
Pathogen	7	15.6710	2.2387	18.70	<0.0001
Marinade+	2	26.0652	13.0326	108.84	<0.0001
Pathogen*Marinade+	14	5.0690	0.3621	3.02	0.0002
Time	3	0.3864	0.1288	1.08	0.3590
Pathogen*Time	21	3.7440	0.1783	1.49	0.0756
Marinade+*Time	6	0.9688	0.1615	1.35	0.2340
Pathogen*Marinade+*Time	42	4.6553	0.1108	0.93	0.6069

DF = degrees of freedom
SS = Sum of Squares
MS = Mean Square
AMS = AMS = The term used to identify the novel antimicrobial solution developed by researchers at USDA and evaluated in these studies
Marinade+ = The marinade served as the diluent to dilute the AMS to the medium (1:5 dilution) concentration.

Model includes Pathogen, AMS, and Time. Inoculum is included in the model as part of the pathogen.