EVALUATION OF ANTIMICROBIAL INTERVENTIONS APPLIED DURING FURTHER PROCESSING OF RAW BEEF PRODUCTS TO REDUCE PATHOGEN CONTAMINATION

A Dissertation

by

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ABSTRACT

This project was designed to validate antimicrobial applications on intact beef subprimals, frozen beef, and moisture-enhanced beef for the reduction of Shiga toxin-producing *Escherichia coli* (STEC). In-plant validation trials were conducted to determine efficacy of interventions on Biotype I STEC surrogate microorganisms (ATCC: BAA-1427, BAA-1428, BAA-1429, BAA-1430, and BAA-1431). Two culture preparation and inoculation methods were evaluated. There was no difference ($P > 0.05$) between reductions of surrogate microorganisms on beef surfaces for inoculum preparation methods or for inoculation temperatures. In-plant evaluation of antimicrobial interventions for various beef cuts inoculated with surrogate *E. coli* and subsequently treated with solutions of lactic acid (3.5%), Citrilow™ (pH = 1.05), Beefxide® (2.0%), or peroxyacetic acid blends (PAA, 150 ppm-Inspexx™150 and 190 ppm-Inspexx™ 200) resulted in reductions ranging from 0.1 to 0.8 log10 CFU. Following in-plant experiments, a trial evaluating the efficacy of antimicrobial solutions prepared using water from different sources was conducted, and source did not affect ($P > 0.05$) reductions of *E. coli*. Additionally, warm (55˚C) lactic acid applied to frozen, inoculated beef trimmings at concentrations of 2.5% and 5.0% achieved reductions of 0.5 and 0.7 log10 CFU/g, respectively. Differences in reductions of *E. coli* achieved by lactic acid (2.5 and 5.0% treatment groups) applied to fresh and frozen beef surfaces were evaluated, and reductions achieved on frozen cuts treated with 5.0% lactic acid were greater ($P < 0.0001$) than reductions from either of the fresh treatment groups. Applying

lactic acid to frozen beef resulted in up to a 0.6 log10 CFU/cm2 greater reduction than when applied to fresh beef. Bottom sirloin tri-tips were marinated using cetylpyridinium chloride (CPC; 0.02%, 0.05%, or 0.10%; added as Cecure®) or sodium metasilicate (SMS, 0.2%, added as AvGard® XP) as antimicrobial treatments. There were no differences ($P > 0.05$) among treatments for reduction of surface or internalized surrogate *E. coli*. These data suggest that additional research should be conducted to determine the most suitable application of these ingredients for moisture-enhanced beef products. Overall, these results can be used by processors to fulfill the regulatory requirements for validating their food safety/HACCP programs.

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CHAPTER I

INTRODUCTION

Pathogenic microorganisms in foods are a threat to human health and have the potential to cause fatalities and severe illnesses. Foodborne pathogens cause approximately 9.4 million illnesses, 56,000 hospitalizations, and 1,400 deaths in the United States (U.S.) annually *(58)*. Consumption of beef products contaminated with Shiga-toxigenic *Escherichia coli* (STEC) have been associated with illnesses ranging from mild diarrhea to severe hemolytic uremic syndrome *(101)*. Ruminant animals are one of the primary reservoirs of STECs, and research has shown these pathogens can spread from the feces on the hide onto the carcass during the beef harvest process, resulting in contamination of some parts of the carcass *(8-10, 13, 38)*.

In 1992 and 1993, a foodborne illness outbreak resulting in several deaths and numerous hospitalizations was linked to the consumption of undercooked ground beef *(22)*. The associated beef products were later found to be contaminated with *Escherichia coli* (*E. coli*) O157:H7. In response, the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA) declared *E. coli* O157:H7 to be an adulterant in raw, ground beef products *(117)*. Multiple foodborne illness outbreaks involving 6 non-O157 STECs (O26, O45, O103, O111, O121, O145) led the FSIS to later classify these strains as adulterants in raw, ground and raw, non-intact beef products *(14, 41, 113)*. STECs pose a threat to consumers' health and can negatively impact the economy of the beef industry.

Meat processing establishments have designed and implemented control measures to assist in compliance with the mandated Hazard Analysis and Critical Control Points (HACCP) regulation by FSIS *(111)*. Antimicrobial interventions are used by the beef industry as a part of these HACCP/food safety systems to address specific pathogens of concern. Much of the early research for reducing pathogen contamination focused on the application of antimicrobial interventions to the carcasses during harvest and upon entering fabrication *(59)*. Several studies have reported on the efficacy of antimicrobial treatments applied to warm and chilled beef carcasses or trimmings to reduce the level of enteric pathogens *(17-21, 32, 33, 39, 40, 51, 65, 74, 123)*. The FSIS *(113)* declared the public health risks associated with raw beef products contaminated with *E. coli* O157:H7 and non-O157 STECs are not limited to ground beef, but also include non-intact beef products. Thus, further processors must have sufficient scientific data to support the use of antimicrobial interventions in their processes. Although the efficacy of many commonly applied antimicrobial interventions has been demonstrated in a laboratory setting, the results obtained in-plant vary, and unfortunately are sometimes less effective than in a laboratory trial. Therefore, it is important that in-plant validation studies be conducted to demonstrate efficacy of their food safety systems and antimicrobial interventions in full-scale applications.

Further processing is a complex system because there are multiple production processes, and varying procedures and equipment are used at different establishments. The application methods of antimicrobials in some operations differ from those in the published research making it difficult for processors to adequately support their

decisions, which creates issues during Food Safety Assessments (FSAs) and audits. Furthermore, a large percentage of further processing is conducted by small and very small establishments that do not have the technical staff and/or resources to conduct validation studies for the processing aids and interventions applied in their operations.

Therefore, the purpose of this study was to evaluate the efficacy of in-plant applications of commonly applied antimicrobial interventions during the production of raw intact, non-intact, and ground beef products and to explore the use of novel application methods and/or less frequently used antimicrobial interventions during the production of raw intact, non-intact, and ground beef products.

CHAPTER II

REVIEW OF LITERATURE

2.1 Foodborne Pathogens

Illnesses caused by foodborne pathogens have been documented for nearly 200 years. A pathogen is a microorganism, when contracted by humans, which can cause disease or illness. Some of the most common foodborne pathogens include *Campylobacter, Listeria, Salmonella*, Shiga toxin–producing *Escherichia coli*, *Shigella, Vibrio,* and *Yersinia.* The first case of illnesses linked to a specific organism was in 1888 when 57 people were sickened from meat contaminated with *Salmonella* Enteritidis *(67)*. *Escherichia coli* (*E. coli*) has been identified as a foodborne pathogen since a shipment of imported cheese was linked to almost 400 illnesses in 1971 *(62)*. In 1982 the first documented cases of illness caused by *E. coli* in meat products was linked to hamburger meat from a fast food restaurant in Oregon and Michigan *(100)*. Perhaps the most widely publicized case of *E. coli* in meat was in 1992 and 1993 when over 700 illnesses were associated with the consumption of undercooked hamburgers from a fast-food restaurant *(22)*. The strain of *E. coli* linked to these illnesses was identified as *E. coli* O157:H7, an enterohemmorhagic *E. coli* (EHEC) that can cause illnesses ranging from mild diarrhea to severe hemolytic uremic syndrome *(34, 62)*. *E. coli* O157:H7 as well as several other serotypes (O26, O45, O103, O111, O121, O145, and others) are known as Shiga-toxigenic *E. coli* (STEC). While the occurrence of foodborne STEC illnesses is more commonly attributed to meat and poultry products, several recent outbreaks have been

linked to fresh produce, sprouts, and other foods. However, the reduction of STEC remains one of the primary objectives of the food safety systems in beef harvest and processing establishments.

2.2 *Escherichia coli*

In 1885, *E. coli* was first isolated by the bacteriologist Theodor Escherich from infant stools while studying the intestinal bacteria and their relation to pathologic conditions in infants. *Escherichia coli* is a predominant part of intestinal microbiome for various species, which includes cattle, sheep, horses, wild game, dogs, cats, and humans *(1, 62)*. Ruminant animals tend to be the most common reservoirs for *E. coli (9, 43)*. The genus *Escherichia* is part of the family Enterobacteriaceae and includes 6 species: *E. hermanii, E. fergusonii, E. vulneris, E. blattae, E. albertii*, and *E. coli (1)*. Additional members of the Enterobacteriaceae family include other enteric pathogens such as *Salmonella* spp*., Shigella* spp*.,* and *Yersinia* spp*.,* all of which can be human pathogens. Although most strains of *E. coli* have been described as harmless microorganisms, some serotypes can be an opportunistic pathogen in immunocompromised patients *(1)*. Many pathogenic strains *E. coli* can be responsible for urinary tract infections, while some pathogenic strains are attributed to foodborne illness.

Escherichia coli is a non-spore forming, gram-negative, facultative anaerobic, mesophilic bacteria. The cell has a rod shape and flagella, if present, are in a peritrichious arrangement. Most strains ferment glucose with production of acid and gas, and lactose is fermented with production of both acid and gas by most strains. *E. coli*

grows at temperatures ranging from 7-10ºC and up to 50ºC. The optimum temperature for growth is 37ºC. These bacteria do not have a marked heat resistance, with a D-value at 60ºC of 0.1 min, but can survive refrigeration and freezing temperatures for prolonged periods. The optimal pH for growth is 7.0 but has been shown to grow at a pH as low as 4.4 if all other conditions are in optimal ranges. Under optimum conditions, the minimum a^w for growth is 0.95 *(1, 62)*. The time from exposure to onset of symptoms ranges from 1 to 14 days *(25)*.

Kauffmann established the basis for the serological studies of the lipopolysaccharide somatic-O antigen, capsular-K antigen and flagellar-H antigenic reaction of the *coli* group *(71)*. Kauffmann described the relationship of the K antigen with the strain and, the O antigen classification group and its necrotizing hemolytic and toxicity virulence factors. Serogroups are defined by O antigens and then subdivided into serotypes based on H antigens. Strains of each category of pathogenic *E. coli* tend to fall within certain O:H serotypes. This method plays an important role in the detection of pathogens and for epidemiological studies *(1, 71, 84)*. In 1988, 171 O serogroups and 56 H types were recognized *(66, 77, 89)*.

2.3 Pathogenic *E. coli*

Some *E. coli* help to maintain gastrointestinal functions and are not harmful to humans *(26)*. These are known as generic *E. coli*, or biotype-I *E. coli*, while other strains are major causes of different syndromes of diarrheal disease and are called pathogenic *E. coli (69, 77)*. Some of these pathogenic *E. coli* share some virulence characteristics such as plasmids encoding for critical virulence factors, the particular interaction with the

intestinal mucosa, and the production of enterotoxins or cytotoxins. *(77)*.

There are 6 recognized virulence groups for *E. coli*: enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAggEC), diffuse-adherent (DAEC) and enterohemorrhagic (EHEC) *(66, 69, 77, 89)*. The EPEC group is recognized as the main cause of infantile diarrhea. These strains do not develop the heat-labile and heat-stable enterotoxins found in ETEC. EPEC do not exhibit the invasiveness of EIEC. They do produce a toxin almost identical to Toxin 1 from *Shigella dysenteriae* which causes diarrhea by destruction of microvilli without further invasion *(77)*. Common symptoms of EPEC infection include fever, malaise, vomiting, and diarrhea with an elevated amount of mucus but without blood *(77)*. EIEC infection frequently causes watery to bloody diarrhea (hemorrhagic colitis) and vomiting. The EHEC group is very similar to EPEC due to the fact they possess common genes, the type of attachment, and epithelial effacement lesions. EHEC are a subset of a group called vero cytotoxin-producing *E. coli* (VTEC). These strains produce toxins that are toxic to vero (African green monkey kidney) tissue cultured cells, giving them the name vero cytotoxins *(35, 36, 75)*. Some literature has described the expression of toxins similar to *Shigella dysenteriae* (Shiga toxin). These toxins were different from any previously described *E. coli* toxins and are named Stx_1 and Stx_2 *(62)*. Common nomenclature of these bacteria can be either VTEC or STEC (Shiga toxin-producing *E. coli*). Virulence genes of the pathogenic island on the chromosome of STEC include the *eae* gene that encodes the intimin protein which is essential for attachment/effacement (A/E), observed through the attachment of the bacteria to the epithelial cells in the lining of the intestines.

Another virulence gene that STEC can carry is the EHEC-hemolysin (*hlyA*) gene. The presence of these 2 virulence factors in combination with the production of the Shiga toxin, potentially results in these highly virulent strains of STEC *(11, 64)*. The host receptor for these toxins is globotriasylceramide (Gb3). Human renal tissue contains large amounts of Gb3 and thus it is highly sensitive to the Stx toxins, which can lead to hemolytic uremic syndrome (HUS) symptoms such as hemolytic anemia, thrombocytopenia, and acute renal failure. Often, death of renal cells occurs due to ribosome destruction, which inhibits protein synthesis *(62, 92)*. In the elderly, Thrombotic thrombocytopenic purpura (TTP) is seen when a combination of HUS, fever and neurologic dysfunctions are present. Left untreated, TTP has an approximate mortality rate of 95%; though, survival rates of 80-90% can be seen with early diagnosis *(68)*. Other STEC strains have been related with hemorrhagic colitis and HUS; however, *E. coli* O157:H7, the most widely known member of the group, is currently recognized as the most common cause of STEC-associated human illness *(34)*.

2.4 Sources and Epidemiology

A STEC infection can have different clinical manifestations. These differences are related to the patient, the dose of the pathogen, and the infecting strain. Some strains, such as *E. coli* O157:H7 could cause infection with as few as 100 cells *(84)*. The highest risk groups for infection are children under 5 years, elderly, and immunocompromised individuals *(52)*. STEC infections are transmitted via 3 primary routes: directly from animals (farm animals, domestic pets, deer, dogs, wild birds), by person-to-person contact such as day care centers and nursing homes, and from contaminated foods and

water *(62)*. Ground beef has been associated with several outbreaks in humans *(92)*. Although beef products are the most common cause of illnesses related to STEC, many outbreaks also have been linked to a wide variety of food items. Raw milk also has been related to outbreaks and HUS syndrome in humans and STEC has been isolated from healthy dairy cattle *(119)*. Hazelnuts, fresh produce, cheese, juice, yogurt, dried salami, raw milk, mayonnaise, and raw cookie dough are examples of food matrixes that have been involved in STEC related illnesses *(24, 42, 63, 97)*. Because more foodborne outbreaks of STEC syndromes have been linked to beef than any other single food source, cattle have typically been considered the primary reservoir for STEC in United States *(22)*. STEC also has been found in other ruminants in several countries *(34)*. STEC can be found in cattle gastrointestinal tract and excreted in feces and been isolated from fecal samples collected from healthy calves or cattle in the U.S., Canada, United Kingdom, Germany, and Spain *(62)*.

Current research has established that STEC is transferred via fecal contamination from the hide, hooves, and other parts of the animal onto the carcass during harvest *(4-6, 8-10, 12, 13, 38, 91)*. Bosilevac et al. *(12)* sampled 1,995 hides and 1,995 carcasses in 7 U.S. harvest facilities finding prevalence of enumerable levels of *E. coli* O157:H7 and *Salmonella* of 12 and 36% in hides of stunned animals, and of 2 and 8% for carcasses on the pre-evisceration process. Elder et al. *(38)* investigated cattle at 4 different Midwestern U.S. harvest facilities and found 72% of lots with at least 1 positive fecal sample for *E. coli* O157:H7 and 38% of the same lots with at least 1 positive hide sample for this pathogen. However Arthur et al. *(6)* found even when 75% of the hides

sampled were positive for *E. coli* O157:H7, none of the post-chill carcasses tested positive for the pathogen. These results suggest levels of STEC on the carcass can be controlled using effective food safety interventions at different locations in the harvest process. Major components that effect carcass contamination are: amount of hide contamination, use of proper sanitary dressing procedures, and the efficacy of the food safety interventions during processing *(120)*.

2.5 Regulatory Food Safety

In 2015, the Centers for Disease Control and Prevention (CDC) data for culture-confirmed bacterial infections reported there were 1,259 cases, 306 hospitalizations, and 4 deaths attributed to foodborne STEC *(58)*. However, due to underdiagnoses and underreporting, the CDC's estimates for 2016 expect that foodborne STEC is responsible for over 175,000 illnesses, 2,400 hospitalizations, and 20 deaths each year *(102)*. *Escherichia coli* O157:H7 is estimated to cause 63,153 illnesses followed by 112,752 cases of non-O157 STEC strains (O26, O45, O103, O111, O121, O145) every year in the U.S. *(102)*. However, because the detection and isolation of these strains is costly and time consuming, it is possible that the number of foodborne illnesses caused by non-O157 STEC may truly be higher *(14, 60)*. The annual healthcare expenses related to STEC foodborne illnesses are estimated to be $478 million (Economic Research Service - USDA, 2009).

Regulatory measures have been taken by FSIS to protect public health against *E. coli* O157:H7 since 1982, when it was first recognized as a human pathogen *(100)*. In August 1994, FSIS declared *E. coli* O157:H7 an adulterant in raw, ground beef and

products intended for production of raw, ground beef *(117)*. On July 25, 1996, FSIS issued the final rule, named Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) Systems *(111)*. This rule addresses foodborne illness associated with meat and poultry products by focusing more attention on the prevention and reduction of microbial pathogens on raw products that can cause illness.

The HACCP rule require meat and poultry establishments under Federal inspection take responsibility for, among other things, reducing the contamination of meat and poultry products with disease-causing (pathogenic) microorganisms by implementing a system, known as HACCP, of preventive controls designed to improve the safety of their products. The HACCP requirements that establishments must meet are set out in 9 CFR Part 417. These requirements are based on the 7 HACCP principles recommended by the National Advisory Committee on Microbiological Criteria for Food (NACMCF) in 1992 *(88)*. One of the principles identified by the NACMCF was "Verification" describing that HACCP systems should be systematically verified. In the NACMCF explanation of the verification principle, which FSIS follows, an establishment is responsible for the following 3 processes encompassing the verification principle: Validation, Verification, and Reassessment *(115)*. FSIS stated in the HACCP Final Rule that validation data for any HACCP system must include practical data or information reflecting an establishment's actual experience in implementing the HACCP system.

In January, 1999, FSIS also declared that any non-intact (injected, mechanically tenderized, blade- or needle-tenderized, etc.) beef containing *E. coli* O157:H7 would be

considered adulterated *(112)*. FSIS announced that 6 additional serotypes of *E. coli* (O26, O45, O103, O111, O121, and O145) are considered adulterants in raw, ground beef and raw non-intact beef in September 2011 and May 2012, respectively *(113)*. This announcement meant that beef processing establishments should re-assess their HACCP plans to identify methods to control, as well as perform additional verification procedures for the presence of STEC. This followed a petition filed by food safety advocate groups who argued that all pathogenic STEC posed threats equal to *E. coli* O157:H7. In May 2015, FSIS began requiring the labeling of raw or partially cooked needle- or blade- tenderized beef products *(114)*. Including beef products injected with marinade or solution, these labels should bear a descriptive designation that clearly indicates the product has been mechanically tenderized. As mentioned in the final rule, the affected products must include the descriptive designation 'mechanically tenderized,' 'blade tenderized,' or 'needle tenderized' and an accurate description of the beef component. Even though vacuum-tumbled or enzyme-formed beef products are processed in a way that they can introduce pathogens below the product surface, FSIS has concluded there are not sufficient data to understand whether the risk that pathogens may be introduced into product as a result of vacuum tumbling or enzyme formed beef product is similar to that associated with needle- and blade-tenderized beef *(114)*.

Recalled product associated with a positive test for STEC can have a meaningful impact on the profitability of beef processors. From 2000 to 2012, a total of 172 recalls by FSIS-regulated establishments took place due to STEC adulteration, and were associated mostly with beef products *(103)*. An estimated 84,000,000 lb of beef, pork,

and bison products were recalled during this same period *(103)*. As another example, the median ground beef recall involved an estimated 98,000 lb of product with a retail value of nearly $235,000 *(107)*. Furthermore, a food safety recall that receives public attention is believed to have an effect on the short-term demand of beef, potentially resulting in as much as $97,000,000 in lost revenue to the beef industry over a 2-week period after the recall *(107)*. These examples help to further demonstrate the importance of the implementation of validated food safety interventions in beef processing.

Antimicrobial interventions are used by the beef industry as a part of these HACCP/food safety systems to address specific pathogens of concern. Early research on the reduction of pathogen contamination was focused on the antimicrobial intervention application to carcasses during harvest and upon entering fabrication *(59)*. Several studies have reported on the efficacy of antimicrobial treatments applied to warm and chilled beef carcasses or trimmings to reduce the level of enteric pathogens *(18-21, 32, 33, 39, 47, 53, 65, 123)*. Although the efficacy of many commonly applied antimicrobial interventions has been demonstrated in a laboratory setting, the results obtained in-plant vary, and unfortunately are sometimes less desirable than expected. Further processing is a complex system because there are multiple production processes used, and varying procedures and equipment are used at different establishments. Further processors must have sufficient scientific data to support the use of antimicrobial interventions in their processes. The application methods in some operations differ from those in the published research making it difficult for them to support their decisions, which creates issues during Food Safety Assessments (FSAs) and audits. Furthermore, a large percentage of

further processing is conducted by small and very small establishments that do not have the technical staff and/or resources to conduct validation studies for the processing aids and interventions applied in their operations. Therefore, it is important that in-plant validation studies are conducted to demonstrate efficacy of food safety systems and antimicrobial interventions in full-scale applications.

2.6 Non-intact and Raw, Ground Beef

As referenced in the above section, FSIS defines non-intact beef as injected, mechanically tenderized, and/or blade- or needle-tenderized. Studies *(56, 78, 99)* have reported that piercing of the surface using needles or blades may allow microorganisms an opportunity to become internalized. Internalization of surface pathogens is the primary concern for non-intact beef products. Furthermore, grinding of beef provides an opportunity for any microorganisms on the surface of a cut to become dispersed throughout a much larger area within a ground beef lot. For these reasons, the current antimicrobial interventions in use by further processors are aimed at reducing surface contamination of carcasses and subprimals before mechanical tenderization takes place.

2.7 Surrogate *E. coli*

Surrogates are microorganisms that can display similar or identical properties of one or more pathogens. Surrogates can be useful in research to help validate pathogen intervention strategies in a laboratory or production setting without exposing the equipment, facilities, or consumers to the pathogens. Therefore, effective surrogate microorganisms should be non-pathogenic, have well defined characteristics, be easily enumerated, have similar tolerances/susceptibilities to the target pathogen, and be easily

differentiated from other microorganisms that may be present *(62)*. Marshall et al. *(81)* compared 5 indicators with 5 isolates of *E. coli* O157:H7. The isolates were subjected to 7 different antimicrobial treatments and the results showed these indicators in a combined cocktail could serve to evaluate and validate antimicrobial interventions for beef carcasses. Niebuhr et al. *(90)* used these *E. coli* biotype I strains to compare their responses to that of a mixed culture of *Salmonella.* Results from this study showed 4 of the 5 surrogates exhibited a higher survival rate when exposed to the same antimicrobial interventions, indicating the isolates could also be used as surrogates for *Salmonella*. Cabrera-Diaz et al. *(16)* compared the growth, acid and thermal resistance, and attachment properties of the non-pathogenic *E. coli* strains to those of *E. coli* O157:H7 and *Salmonella* strains. The results of the trial found thermal and acid resistance of the nonpathogenic *E. coli* strains were not different or slightly higher than that of the *E. coli* O157:H7 and *Salmonella* strains, which would allow them to be a suitable surrogate organism for validating hot water and lactic acid interventions on beef carcasses. Keeling et al. *(72)* conducted trials to determine the effect of freezing, refrigerating, fermentation, and thermal inactivation on the *E. coli* biotype I isolates compared to *E. coli* O157:H7. Their research showed 3 of the isolates, BAA-1427, BAA-1429, and BAA-1430, showed no difference for refrigeration conditions and had slightly better survival in the frozen, fermentation, and thermal inactivation studies than *E. coli* O157:H7, which could allow for a margin of safety.

2.8 Antimicrobial Interventions for Controlling STEC in Beef

Due to foodborne pathogen outbreaks associated with meat products and the need to continuously improve safety strategies against foodborne pathogens, research has been focused on controlling pathogens at an initial fresh beef carcass level. A vast amount of research has been done on methods to decontaminate freshly slaughtered beef carcasses which are contaminated by feces from intestines, hide, or hooves *(53, 59, 86, 104, 105, 108)*. Various techniques used to control STEC contamination during beef processing may include: use of bacteriophage treatment, live animal cleaning, hide decontamination after stunning, trimming and steam vacuuming of defined carcass areas, whole-carcass steam, hot water, and/or chemical intervention sprays, and proper carcass chilling *(18-21, 28, 30, 32, 49, 51, 53, 80)*. In the beef harvest establishment, these types of interventions are used in a 'multiple-hurdle' approach applied in combination throughout the process as an attempt to control microorganisms and increase the safety of the end products *(59, 86, 87, 120)*. These practices are ultimately cumulative with the final intervention, most likely a critical control point (CCP), immediately before chilling.

McEvoy et al. *(82)* reported a reduction in prevalence on carcasses after chilling for 24 h and hypothesized that chilling may stress the bacterial cells due to the synergistic effect of low A_w and temperature. Similarly, Gill et al. *(50)* reported a reduction in coliforms and *E. coli* on carcasses following cooling processes of between 0.5 $\log_{10}$ units and 2 $\log_{10}$ units. As it pertains to food safety, the primary objective of chilling is to limit or slow growth of microorganisms; not necessarily to reduce their

numbers. When the carcass is processed and trimmed in to smaller cuts, the concentration of STEC should not increase if chill conditions are well controlled, but cross-contamination may occur to other cuts and surfaces with distribution of the pathogen throughout the ground meat.

Research has been conducted for many chemical interventions applied to meat to reduce pathogens. Most of this research has focused on applications intended for use on the slaughter floor or on chilled subprimals and trimmings. In the early 1990s, hot water, chlorine and short chain organic acids were the most common sanitizing agents. Their effectiveness was dependent on the concentration used, temperature of the sanitizer, contact time, and the sensitivity of the microorganisms to the specific compound *(31)*. Some current chemical antimicrobial interventions include: polyphosphates, chlorine, cetylpyridinium chloride (CPC), sodium metasilicate (SMS), lactic acid, peroxyacetic acid (PAA), hypobrombous acid, hydrochloric acid, acidified sodium chlorite (ASC), acetic acid, citric acid, as well as some blends of organic acids. Organic acids exhibit greatest antimicrobial efficacy in their undissociated form, as this form facilitates greater penetration of the lipid bilayer of the cell membrane. Once inside the cell, the acid molecules begin to dissociate because of the near neutral pH of the cytoplasm. In an attempt to re-balance the cytoplasmic pH, the cell must use adenosine triphosphate (ATP) to pump protons out of the cell across the cytoplasmic membrane, depleting the cell of its energy source *(29)*. Some organic acid treatments have been shown to be more effective at reducing bacterial contamination on adipose tissues compared to lean tissue *(28, 53)*. After application of organic acid, the adipose tissue surface pH does not return

to normal as soon as it may for lean surfaces, thereby creating an environment that is unfavorable for most pathogens *(30, 53)*. However, Cutter and Siragusa *(28)* reported that the surface pH of acid treated lean tissue did not differ from untreated pieces 24 h after acid application. Dickson *(30)* theorized that the pH differences between the lean and adipose tissues were likely due to acid dilution caused by differences in lean and adipose tissue water content, 75% and 20%, respectively, or even different buffering mechanisms.

2.8.1 Lactic Acid Solutions

Lactic acid can be applied to beef carcasses, subprimals, and trimmings at a range of 2.0% to 5.0% concentration, and shall not exceed a temperature of 55°C, according to FSIS Directive 7120.1 *(116)*. Hardin et al. *(53)* found water washing followed by 2.0% organic acid solution (55°C) significantly reduced *E. coli* O157:H7 and *Salmonella* Typhimurium on beef. Furthermore, reduction of *E. coli* O157:H7 was greatest for those receiving the lactic acid spray compared to those that received the acetic acid spray *(53)*. Harris et al. *(55)* found no difference among organic acid types or concentration (lactic or acetic; 2.0% or 4.0%) and acidified sodium chlorite sprays in their ability to reduce *E. coli* O157:H7 and *Salmonella* Typhimurium on beef trim. However, ground beef samples from the acidified sodium chlorite treated trimmings generally had a greater amount of *E. coli* O157:H7 present. Yoder et al. *(123)* stated organic acids including lactic acid are generally more effective as concentration increases from 1.0 to 5.0%. Lactic acid and acetic acid sprays did not differ statistically in their ability to reduce *Salmonella* Typhimurium in the Hardin et al. *(53)* study.

However, a study conducted by Anderson et al. *(3)*, showed 3.0% lactic acid was more effective than acetic acid at reducing *Salmonella* Typhimurium on inoculated beef muscle cores. In fact, the 3.0% lactic acid treatment was the most effective acid treatment for reducing *Salmonella* Typhimurium at all application temperatures (20°C, 45°C, and 70°C) *(3)*. Furthermore, reduction of *Salmonella* Typhimurium increased as lactic acid application temperature increased. A 2.0 log reduction of *Salmonella* Typhimurium was achieved using the 3.0% lactic acid (70°C) dip, while reductions between 1.0 and 1.5 log were still achieved with the 20°C and 45°C lactic acid applications *(3)*. Castillo et al. *(21)* found application of a 2.0% lactic acid solution (55°C) following a water wash to inoculated pre-chilled beef rounds reduced *E. coli* O157:H7 and *Salmonella* Typhimurium by 5.2 log cycles each. Additionally, the ground beef produced from products that received a pre-chill and post-chill acid spray, presented lower pathogen levels *(21)*. Application of a 4.0% lactic acid solution (55°C) to chilled, inoculated beef rounds reduced *E. coli* O157:H7 and *Salmonella* Typhimurium an additional 2.0 and 1.6 log cycles, respectively. King et al. *(74)*, reported that 2% solution of lactic acid reduced *E. coli* O157:H7 and *Salmonella* Typhimurium counts on beef carcasses while entering the chilling cooler, and prevented growth during the chilling period. In another study by Castillo et al. *(17)*, 2.0% lactic acid spray in combination with hot water (95ºC at the source) had higher log reductions of *E. coli* O157:H7 and *Salmonella* Typhimurium than independent treatments. Cutter and Siragusa *(28)* reported a 2.6 log reduction in *E. coli* O157:H7 on beef carcass tissues following a 5.0% lactic acid (24°C) spray treatment. Heller et al. *(56)* observed a 1.1 log reduction of *E.*

coli O157:H7 on inoculated outside rounds destined for moisture enhancement or blade tenderization after application of a 5.0% lactic acid (55°C). Kalchayanand et al. *(65)* demonstrated that hot water (85°C), followed by lactic acid (4.0%) were the most effective at reducing STEC compared to 6 other treatment groups. Because lactic acid has been shown to effectively reduce *E. coli* and *Salmonella* across many different applications and parameters, it has become a very common intervention implemented by numerous beef processors. Regarding the vacuum storage after lactic acid spray on beef subprimals, the treatment improved the microbiological quality of meat for 14, 28, 56, 84, and 126 days of vacuum storage *(96)*. In a recent study, lactic acid was evaluated as an initial and secondary subprimal intervention for *E. coli* O157:H7, non-O157 STECs, and a nonpathogenic *E. coli* surrogate *(94)*. In that study, initial use of lactic acid was validated as a subprimal intervention during beef fabrication followed by a secondary application to a vacuum-packaged product. As a result, total inoculum counts were reduced from 6 log CFU/cm^2 to 3.6, 4.4, and 4.4 log CFU/cm^2 for the *E. coli* surrogates, *E. coli* O157:H7, and non-O157 STEC inoculation groups, respectively *(94)*. Following the second application, total inoculum counts were 2.6, 3.2, and 3.6 log CFU/cm^2 for the *E. coli* surrogates*, E. coli* O157:H7, and non-O157 STEC inoculation groups, respectively. This study concluded that lactic acid treatments significantly lowered counts of pathogenic and nonpathogenic strains of *E. coli* on beef subprimals *(94)*. In a different study, lactic acid spray was used to reduce the translocated *E. coli* O157:H7 and *Salmonella* Typhimurium DT 104 in inoculated USDA Choice strip loins (*53*). In this study, lactic acid spray before mechanical tenderization or enhancement reduced *E.*

coli O157:H7 and *Salmonella* Typhimurium DT 104 loads up to 3.0 and 2.3 $\log_{10}$, respectively *(37)*.

2.8.2 Citric, Acetic, and Peroxyacetic Acid Solutions

Currently, there are several antimicrobial solutions on the market that utilize a blend of organic acids for use on particular products. Examples of some of these blends are as follows: Beefxide® (lactic acid and citric acid blend, Birko Corporation, Henderson, CO), Citrilow™ (citric acid and hydrochloric acid blend, Safe Foods Corporation, Little Rock, AR), Inspexx™-150 (acetic acid, peroxyacetic acid, and hydrogen peroxide blend; Ecolab, St. Paul, MN) and Inspexx™-200 (acetic acid, peroxyacetic acid, hydrogen peroxide, octanoic acid, and peroxyoctanoic acid blend; Ecolab, St. Paul, MN)

Pohlman et al. *(95)* found that beef cuts treated with Citrilow™ had the lowest counts of *E. coli* and coliforms for day 1 and 2 of retail display. Laury et al. *(76)* conducted a trial in which beef trim was treated with either water (control) or Beefxide®, and was then swabbed to determine the final pathogen load. Findings suggested Beefxide® significantly reduced *E. coli* O157:H7 by 1.4 log CFU/ 100 cm², and *Salmonella* by 1.1 log CFU/100 cm², as compared to the control samples. A study conducted by Hendricks et al. *(57)* evaluated the effectiveness of 2.9% lactic acid and 2.4% Beefxide® applied to beef cuts using a commercial spray cabinet. After application of an antimicrobial, each product was passed through a blade tenderizer. Lactic acid treatments had a 1.3 log reduction while Beefxide® treatments had a 1.4 log reduction. These researchers concluded that lactic acid and Beefxide® were similar in the

efficiency of reducing *E. coli* in the production of non-intact beef products. Cutter and Siragusa *(28)* found while acid concentrations 1.0%, 3.0%, and 5.0% had varying effects on pathogen reductions, there was no difference in efficacy between acetic, lactic, and citric acid solutions.

The active component of buffered vinegar is acetic acid, which is appearing frequently as a natural preservative in processed meat as well as in poultry products. Typical household vinegar has a pH of 2.0-3.0. In one trial, 2.0% acetic acid was applied to beef flank sections before chilling and the treatment was able to reduce *E. coli* O157:H7 by 0.65 log CFU/cm^2 and *Salmonella* by 0.87 to 0.91 log CFU/cm^2 *(7)*. In another study, beef carcasses were treated with 2% acetic acid spray or acetic acid plus pulsed-power electricity treatment, which significantly reduced the incidence of *E. coli* O157:H7 and caused a 1-log CFU/cm^2 reduction of *Salmonella* Typhimurium *(109)*.

Certain peroxyacetic acid solutions permitted for use as antimicrobials on red meat carcasses and parts are listed in FSIS's Directive 7120.1 *(116)*. Yoder et al. *(123)* conducted a study which tested the efficacies of 8 chemical sprays to reduce pathogens on meat surfaces. The 8 chemicals used in the study were citric acid, lactic acid, acetic acid, peroxyacetic acid, acidified sodium chlorite, chlorine dioxide, sodium hypochlorite, and ozone-enriched water. Yoder et al. *(123)* reported that a 200 ppm peroxyacetic acid solution reduced *E. coli* O157:H7 and *Salmonella* Typhimurium 0.44 and 1.03 log CFU/cm^2, respectively. Although reductions were accomplished, these reductions did not differ from those achieved using tap water alone. All other treatments were more effective than the 200 ppm peroxyacetic acid solution at reducing *E. coli*

O157:H7 and *Salmonella* Typhimurium, with acetic acid, lactic acid, and citric acid being the 3 most effective. Penney et al. *(93)* studied the efficacy of peroxyacetic acid and water washing to control *E. coli* O157:H7 on beef and bob veal carcasses. Treatments evaluated in the study were: 180 ppm peroxyacetic acid, water wash, and water wash followed by 180 ppm peroxyacetic acid wash. Water washing followed by peroxyacetic acid spray resulted in reductions of 2.73 and 3.21 log CFU/cm^2 of *E. coli* O157:H7 on veal and beef, respectively. The peroxyacetic acid treatment alone was most effective at reducing *E. coli* O157:H7 on veal and beef with reductions of 3.56 and 3.59, respectively. King et al. *(74)* evaluated the effects of peroxyacetic acid and its ability to reduce *E. coli* O157:H7 and *Salmonella* Typhimurium on beef carcasses. Multiple variables including peroxyacetic acid treatment before or after chilling, following a water wash, and at different concentrations and application temperatures were evaluated. Application of 200 ppm peroxyacetic acid to chilled beef resulted in no reduction of *E. coli* O157:H7 or *Salmonella* Typhimurium. Application of 200 ppm peroxyacetic acid to hot beef carcasses resulted in 0.7 log CFU/cm^2 reduction of *E. coli* O157:H7 and *Salmonella* Typhimurium *(74)*.

2.8.3 Other Chemical Antimicrobials

Cetylpyridinium chloride (CPC) is a water-soluble compound that has been used for more than 50 years in oral hygiene products. CPC is a quaternary ammonia, which kills or injures cells by disruption of the cytoplasmic membrane, resulting in leakage of cell constituents. This compound has a low surface tension with hydrophilic and lipophilic properties which allows it to work well to hydrate and penetrate tissue *(61,*

85). CPC has been effective for reducing *Salmonella* contamination of poultry carcasses *(73, 121, 122)* as well as for preventing cross-contamination during poultry slaughter. However, CPC is not currently approved for use on any beef product other than hide-on washes *(116)*. There have nonetheless been several trials that have researched the efficacy of CPC in several different application scenarios. In a study by Cutter et al. *(27)* examining spray-washing lean beef surfaces with 1.00% CPC, this compound reduced 5 to 6 $\log_{10}$ CFU/cm^2 of inoculated *E. coli* O157:H7 and *Salmonella* Typhimurium to practically undetectable levels (0 $\log_{10}$ CFU/cm^2). This same study on adipose beef surfaces also reduced 5 $\log_{10}$ CFU/cm^2 of inoculated *E. coli* O157:H7 and *Salmonella* Typhimurium immediately (>2.5 $\log_{10}$ CFU/cm^2). Ransom et al. *(98)* demonstrated that 0.5% CPC applied to beef surfaces and trimmings resulted in the best reductions of *E. coli* O157:H7 (4.8 $\log_{10}$ CFU/cm^2 and 2.1 $\log_{10}$ CFU/cm^2 for beef carcass tissues and lean tissue pieces, respectively) when compared to other commonly used antimicrobials. In another trial in the same laboratory, Stopforth et al. *(106)* observed the greatest reductions of *E. coli* O157:H7 by using CPC solutions in a spray-chilling application. Byelashov et al. *(15)* found 0.50% CPC included as a brine ingredient in moisture-enhanced beef resulted in the lowest overall counts of pathogens when compared to the other treatments. Sodium metasilicate (SMS) solutions have a strong alkalinity (pH of 12.3 at 1.00% solution) and have demonstrated efficacy for reduction of pathogens *(118)*. Several trials have been conducted that support utility of SMS and CPC as antimicrobial ingredients to reduce microorganisms in beef *(2, 44-46, 83)*. Adler et al. *(2)* reported either significant reductions or no detectable *E. coli* O157:H7 in samples

using CPC or SMS as a brine ingredient. Mehall et al. *(83)* found beef cuts in the CPC and SMS treatments had the lowest counts of APC and coliforms at day 2 of retail display. Continued investigation of the application methods for these compounds is necessary to fully understand their capabilities.

CHAPTER III

MATERIALS AND METHODS

3.1 Meat Selection

Various cuts of beef were used for the trials described in this chapter. For one experiment, beef briskets, deckle off, boneless (IMPS# 120) were collected from a local harvest facility to acquire pre-chilled product. All other beef was purchased as vacuum-packaged, boxed beef and were stored (2°C) until time of use. The following cuts were used throughout the trials using the Institutional Meat Purchase Specifications (IMPS) of the USDA *(110)* to describe them: beef round, bottom round flats (IMPS# 171B); beef loin, top sirloin butt, boneless (IMPS# 184); beef loin, strip loin, boneless (IMPS# 180); round, sirloin tip (knuckle), peeled (IMPS# 167A); beef loin, short loin (IMPS# 173); beef chucks, chuck roll (IMPS# 116A); beef rounds, (top) inside (IMPS# 169); beef loin, bottom sirloin butt, tri-tip, boneless (IMPS# 185C); and beef trimmings (80/20) (IMPS# 138).

3.2 Microbiological Selection and Preparation Methods

Five nonpathogenic *E. coli* Biotype I (Non-Rif^{R}) strains (BAA-1427, BAA-1428, BAA-1429, BAA-1430, and BAA-1431) were obtained from the American Type Culture Collection (Manassas, VA, USA). Three of these strains (BAA-1427, BAA-1428, and BAA-1430) then were selected in the Food Microbiology Laboratory at Texas A&M University for their inherent ability to naturally resist rifampicin (Rif^{R}) using the methods published by Kaspar and Tamplin *(70)*. For this research, either a combination

of the 5 non-rifampicin-resistant strains, or a combination of the 3 rifampicin-resistant (Rif^R) strains were used, depending on the trial. Previous research *(16, 81)* has validated that these microorganisms demonstrate similar thermal and lactic acid resistance properties to the human pathogen *E. coli* O157:H7. These marker microorganisms were combined into a "cocktail" to represent possible contamination with enteric pathogens of fecal origin such as *E. coli* O157:H7.

Cocktail preparation began 48 h before each day of the trial; cultures of *E. coli* (parent strains of BAA-1427, BAA-1428, BAA-1429, BAA-1430, and BAA-1431 for the Non-Rif^R cocktail; and rifampicin-resistant strains of BAA-1427, BAA-1428, and BAA-1430 for the Rif^R cocktail) were proliferated by aseptically transferring a loop of the microorganism from a tryptic soy agar (TSA, Becton, Dickinson and Co., Sparks, MD) slant to a sterile 10 ml tryptic soy broth (TSB, Becton, Dickinson and Co., Sparks, MD) tube and incubating aerobically (35°C) for 18 to 24 h. Following incubation, each strain was transferred individually by depositing a loop of the solution containing the microorganisms from its 10 ml TSB tube into a new 10 ml TSB before incubating aerobically at 35°C for 18 to 24 h. After incubation, the cultures were ready to either be used as a cocktail, or further prepared as a pellet. The cocktail preparation method was the method by which microorganisms were prepared for inoculation in the lab.

The pellet preparation method was the method by which the microorganisms were prepared for transport and use for the in-plant inoculation portions of the study. To prepare the pellet, the tubes from each culture were combined in a 50 ml Falcon™ (Thermo-Fisher Scientific, Waltham, WA) conical centrifuge tube and cells were

collected by centrifugation at 2,205 x *g* for 15 min. The supernatant then was decanted and the pellet was re-suspended in 30 ml of 0.1% (w/v) peptone diluent. These washing and re-suspension procedures were repeated twice more identically. The final pellets were stored (2°C) for 2-8 days (depending on experiment) until needed for inoculation use. Before use as inoculum, pellets were re-suspended in 30 ml (30 ml for Rif^R; 50 ml for Non-Rif^R) of 0.1% (w/v) peptone diluent.

3.3 Product Inoculation, Sampling, and Microbiological Analysis

For each experiment, background microbiological samples (3M™ Sponge-Stick with Buffered Peptone Water Broth, 3M Food Safety, St. Paul, MN) of the exterior surface of randomly selected beef cuts were taken to verify that no existing rifampicin-resistant (when applicable) microorganisms were present before inoculation.

Inoculation methods were based on product type and volume needed for complete coverage of the surface. In experiments using whole beef cuts, inoculum was applied via handheld sprayer. For cutlets and trimmings, the beef was separated into batches and was placed into sterile zippered-top bags and inoculation was conducted by adding inoculum to the zippered-top bag, and hand tumbling for 1 min to completely cover all pieces with the inoculum. Inoculum volumes were based on complete coverage of the surface. For all experiments, pre-treatment surface samples were collected after a 30-minute attachment time was observed to allow for attachment of surrogate *E. coli* to meat surfaces. Post-treatment surface samples were collected approximately 5 min after application of the antimicrobial intervention (when applicable). For each sample, 5 surface tissue excisions (10 cm^2 x 2 mm deep) were collected via sterile stainless-steel

borer, scalpel, and forceps. Surface excisions were composited (50 cm^2, total sampled area) in a sterile stomacher bag with 100 ml sterile diluent (0.1% peptone [w/v]) and were pummeled for 1 minute at 260 rpm using a stomacher (Stomacher-400, Tekmar Company, Cincinnati, OH).

For beef trimmings and cutlets, a sample (50 g composite) was taken randomly from the surfaces of the pieces by use of sterile stainless-steel scalpel and forceps. The composite sample was placed in a sterile stomacher bag with 100 ml sterile diluent (0.1% peptone [w/v]) and pummeled for 1 minute at 260 rpm using a stomacher.

For beef tri-tips used in moisture-enhanced experiments, surface samples were collected by excising 5 (3 from 1 side, 2 from the opposite side) 10 cm^2 x 2 mm deep pieces by use of sterile stainless-steel borer, scalpel, and forceps, creating a composite sample of 50 cm^2 sample area. After tumbling, surface samples (50 cm^2 total) and 1 internal 'core' sample was taken from each cut using a sterile stainless-steel borer. The top and bottom surfaces of the core sample were discarded and the exterior surface of the core was flame-sterilized by immersing the core sample in 95% ethanol and flame sterilizing the outside surface so only the surviving internalized microorganisms were quantified. Core samples then were placed in a sterile stomacher bag, sterile diluent (0.1% peptone [w/v]) was added, and samples were pummeled for 1 minute at 260 rpm using a stomacher.

All samples were plated with a sterile bent glass rod using the appropriate serial dilutions onto pre-poured and dried agar plates. For trials using Non-Rif^R cultures, samples were plated onto pre-poured and dried tryptic soy agar (TSA)-loaded Petri

dishes, overlaid with 12 ml MacConkey Agar (Becton, Dickinson and Co., Sparks, MD), and were aerobically incubated 48 h (35°C). For trials using Rif^R cultures, samples were plated onto pre-poured and dried rifampicin-tryptic soy agar (Rif-TSA, Difco, Becton, Dickinson and Co., Sparks, MD) Petri dishes. Before sample collection, Rif-TSA was prepared by adding a solution of 0.1 g of rifampicin (Sigma-Aldrich, St. Louis, MO) dissolved in 5.0 ml of methanol to 1 liter of autoclaved and tempered (55°C) TSA. Once the samples were plated, Rif-TSA plates were incubated (35°C) for 24 h. For all samples, colonies were inspected, counted, recorded, transformed, and reported as $\log_{10}$ CFU.

3.4 Antimicrobial Solution Preparation Methods

For laboratory experiments, lactic acid (88% L-lactic acid, Purac® FCC 88, Corbion, Lenexa, KS) solutions were prepared by diluting the 88.0% stock solution into deionized water to achieve the appropriate concentrations for each experiment. Acid titrations were conducted per the manufacturer's directions using the supplied test kit to confirm proper lactic acid concentrations. Titrations were performed by pipetting 1 ml of lactic acid test solution in to a clean test tube. Next, 1 drop of phenolphthalein indicator solution was added. Finally, individual drops of 0.25 N sodium hydroxide were added and then shaken to stir the solution until the test solution turned from clear to pink in color. The number of sodium hydroxide drops added was recorded and divided by 10 to calculate the percent lactic acid concentration.

Citrilow™ (pH = 1.05, Safe Foods Corporation, Little Rock, AR) solutions used in laboratory experiments were prepared according to manufacturer's instructions by

mixing with deionized water, and pH was verified using a calibrated pH meter (sympHony pH meter, VWR, Suwannee, GA)

The in-plant preparation of the concentrations of antimicrobial solutions (lactic acid, Beefxide® [2.0% at 41ºC, Birko Corporation, Henderson, CO], Citrilow™, and peroxyacetic acid solutions [Inspexx™-150 and Inspexx™-200, Ecolab, St. Paul, MN]) were performed according to manufacturers' recommendations by the plant personnel to ensure it was the same as their daily preparation.

3.5 Preliminary Trials

Before conducting the in-plant portion of the study, several trials were conducted to establish methods to prepare and transport the microorganisms from the laboratory for use in a federally inspected processing facility.

3.5.1 Culture Viability in "Pellet" Form

Fourteen (n = 14) sets of Rif^R pellets were created for this experiment and stored at 2°C until re-suspended for plating. Starting on the second day of storage, 2 pellets were plated each day for 7 consecutive days. Colonies were counted, recorded, and transformed and reported as $\log_{10}$ CFU/ml.

3.5.2 Non-Rif^R Cocktail vs. Pellet #1

Three beef bottom round flats were used. Each bottom round flat was divided into 2 portions and each portion was assigned to one of the 2 different inoculum preparation methods used (n = 6).

For this experiment, Non-Rif^R cocktail and pellet inocula were used. Inocula (9.2 & 9.1 $\log_{10}$ CFU/cm^2 for cocktail & pellet, respectively) were sprayed onto beef (2 ml)

using a hand-held sprayer. Following 30-min attachment period, lactic acid (15 ml, 3.5% ± 0.1% at 25°C) was applied to the inoculated beef using a hand-held sprayer. Pre-treatment and post-treatment surface excisions were collected for enumeration of surrogate *E. coli*.

3.5.3 Non-Rif^R Cocktail vs. Pellet #2

Six beef bottom round flats were used. Each bottom round flat was divided into 2 portions and each portion was assigned to either the cocktail or the pellet group (n = 12).

For this experiment, Non-Rif^R cocktail and pellet inocula were used. Of the 6 cuts assigned to each inoculum preparation group, 3 cuts were treated with Citrilow™, and 3 were treated with lactic acid. Inocula (9.2 & 9.1 $\log_{10}$ CFU/cm^2 for cocktail & pellet, respectively) were spray-applied onto beef (2 ml delivered vol.) using a hand-held sprayer. Following a 30-minute attachment time, lactic acid (15 ml, 3.5% ± 0.1% at 25°C) or Citrilow™ (15 ml, pH 1.05 ± 0.02, 25°C) were sprayed onto inoculated beef. Pre-treatment and post-treatment surface excisions were collected for enumeration of surrogate *E. coli*.

3.5.4 Rif^R Cocktail vs. Pellet

For this trial, 6 beef bottom round flats were used. Each bottom round flat was divided into 2 portions and each portion was assigned to either the cocktail or the pellet group (n = 12). Rif^R cocktail and pellet inocula were used for this trial.

Of the 6 cuts assigned to each inoculum preparation group, 3 cuts were treated with Citrilow™ and 3 were treated with lactic acid. Inocula (9.2 & 9.1 $\log_{10}$ CFU/cm^2 for cocktail & pellet, respectively) were spray-applied onto beef (2 ml delivered vol.)

using a hand-held sprayer. Following a 30-minute attachment time, lactic acid (15 ml, 3.5% ± 0.1% at 25°C) or Citrilow™ (15 ml; pH 1.05 ± 0.02, 25°C) were sprayed onto inoculated beef. Pre-treatment and post-treatment surface excisions were collected for enumeration of surrogate *E. coli*.

3.5.5 Pre-chill vs. Post-chill

Twenty-four (n = 24) pre-chilled beef briskets were obtained for this trial. Twelve briskets were assigned to the "post-chill" group and placed on ice in insulated coolers for transport and were stored (2°C) for 24 h. The remaining 12 briskets assigned to the "pre-chill" group were kept warm in an insulated container and transported to the Food Microbiology Laboratory at Texas A&M University. Upon arrival, the fat side (exterior of the carcass) of each cut was inoculated with 6 ml of the Rif^R cocktail. The $\log_{10}$ CFU/ml of initial inoculum cocktail was high enough (8.0 to 9.0 log CFU/ml) to ensure enough microorganisms could be recovered from the product, before and after the antimicrobial intervention. After inoculation, the "pre-chill" briskets were placed on a metal rack in refrigerated storage (4°C) for 24 h. After 24 h of chilling, the "post-chill" briskets were inoculated according to the methods described for the "pre-chill" briskets. For both "pre-chill" and "post-chill" briskets, temperature (28°C and 4°C, respectively) was recorded at the time of inoculation and at the time of antimicrobial treatment. Following collection of the pre-treatment samples, the 12 cuts (3 briskets per treatment) were subjected to approximately 100 ml spray of one of 4 treatments: 55°C lactic acid, 25°C lactic acid, 55°C Citrilow™, or 25°C Citrilow™ (lactic acid, 3.5% ± 0.1%; Citrilow™, pH 1.05 ± 0.05). Pre-treatment and post-treatment surface excisions were

collected for enumeration of surrogate *E. coli*. After chilling for 24 h, the "pre-chill" briskets were removed from refrigerated storage, sampled and treated according to methods described for the "post-chill" briskets. Pre-treatment and post-treatment surface excisions were collected for enumeration of surrogate *E. coli*.

3.6 In-Plant Validation of Antimicrobial Interventions to Reduce *Escherichia coli*

Texas A&M University worked with establishments in Nevada and Pennsylvania to complete the in-plant trials of this project. Both establishments are federally inspected, so the project was designed to comply with all USDA regulatory requirements related to process validation and use of surrogate microorganisms. According to USDA-FSIS *(115)*, "an establishment that chooses to conduct a validation study may use a surrogate indicator organism to measure change, but it should do so only after giving careful consideration to specific precautions. These precautions include ensuring that a properly trained individual introduces the non-pathogenic cultures within the establishment. In addition, the establishment should ensure that the introduction of the non-pathogenic cultures does not create an insanitary condition in the facility or cause the food to become adulterated. Finally, establishments should ensure that the non-pathogenic cultures are necessary and proven to be effective for the intended purpose."

"To better ensure insanitary conditions are not created, establishments are encouraged to apply surrogate indicator organism cultures in a manner to ensure that the establishment can conduct a full cleaning and sanitizing of the facility and equipment after the stage in the food safety application being evaluated. Generally, product

containing the surrogate indicator organism cultures would not automatically be considered adulterated."

To conduct this investigation, normal processing procedures were simulated on Saturday or Sunday when no other production was in process. This was to ensure sanitary conditions were maintained, products were controlled, and there was no potential for cross-contamination with non-experimental products. The processing environments were sampled before and after the validation to ensure no preexisting and/or residual contamination existed.

Beef cuts (whole subprimals) were selected by the establishment based on production volume. This trial focused on validating the efficacy of the application of the anti-microbial interventions such as lactic acid, Beefxide, Citrilow™, and peroxyacetic acid. The interventions were validated using processing parameters implemented by the commercial processing establishment.

3.6.1 In-Plant Trial #1

Eight cuts of each top sirloin, strip loin, knuckle, and short loin were used for this trial. The 8 cuts of each type were divided into 2 treatment groups: Inspexx™ 150 (150 ppm at 13°C) (4 cuts of each type) and Inspexx™ 200 (190 ppm at 13°C) (4 cuts of each type). Top sirloin and strip loin were treated with top sprayers only, whereas knuckle and short loin were treated with top and bottom sprayers. Top sirloin and strip loin cuts were inoculated on the fat (exterior of the carcass) surface with 6 ml of inoculum from the Rif^R pellet. The $\log_{10}$ CFU/ml of initial inoculum cocktail was great enough (8.0 to 9.0 $\log_{10}$ CFU/ml) to ensure a sufficient number of microorganisms can be recovered

from the product, before and after the antimicrobial intervention. Knuckle and short loin cuts were inoculated on the top and bottom surfaces of each cut with the cocktail; 6 ml per side. Pre-treatment and post-treatment surface excisions were collected for enumeration of surrogate *E. coli*. Each sample was placed in a sterile Whirl-Pak bag (Nasco, Atlanta, GA) and stored inside an insulated container with coolant packs, and transported to the Food Microbiology Laboratory at Texas A&M University.

3.6.2 In-Plant Trial #2

Twelve cuts of each striploin and knuckle were used for this trial. The 12 cuts of each type were divided into 3 treatment groups: Inspexx™ 150 (150 ppm at 19°C) (4 cuts of each type), Inspexx™ 200 (190 ppm at 23°C) (4 cuts of each type), and Beefxide® (2.0% ± 0.1% at 41°C) (4 cuts of each type). Each of the beef surfaces were sprayed with 6 ml of inoculum from the Rif^R pellet for this trial. All cuts were treated with top and bottom sprayers. Pre-treatment and post-treatment surface excisions were collected for enumeration of surrogate *E. coli*.

3.6.3 In-Plant Trial #3

For this trial, the following types of beef cuts were used: 4 chuck rolls, 4 inside rounds, 8 bottom round flats, 8 sets of cutlets from the bottom round, and 8 units of beef trimmings. Each of the beef cuts were sprayed with 6 ml of inoculum from the Non-Rif^R pellet for this trial. Pre-treatment and post-treatment surface excisions were collected for enumeration of surrogate *E. coli*.

For cutlets and trimmings, the beef was separated into 1 kg batches and was placed into sterile zippered-top bags and inoculation was conducted by adding 100 ml of

the Non-Rif^{R} pellet inoculum to the zippered-top bag, and hand tumbling for 1 min to completely cover all pieces with the inoculum. Pre-treatment and post-treatment surface excisions were collected for enumeration of surrogate *E. coli*.

All beef cuts, including cutlets, were treated with top and bottom sprayers. For beef trimmings, 4 units were treated using 2 different augers and their sprayers. Beef trimmings, as well as whole beef cuts, were treated with Citrilow™ (pH 1.05 ± 0.05 at 25°C). Beef cutlets were treated with lactic acid (3.5% ± 0.1% at 25°C).

3.6.4 In-Plant Trial #4

For this trial, the following types of beef cuts were used: 8 chuck rolls, 8 bottom round flats, and 8 bottom round roasts. Bottom round roasts was prepared by removing all fat and connective tissue from the surface of the original cut with a boning knife, to expose 100% of the lean muscle tissue. Each of the beef surfaces were sprayed with 6 ml of inoculum from the Rif^{R} pellet. All cuts were treated with top and bottom sprayers. Chuck rolls and outside rounds were treated with Citrilow™ (pH 1.05 ± 0.05 at 25°C). Bottom round roasts were treated with lactic acid (3.5% ± 0.1% at 25°C). Pre-treatment and post-treatment surface excisions were collected for enumeration of surrogate *E. coli*.

3.6.5 Water Comparison Trial

A trial was conducted using water from one of the processing facilities to prepare antimicrobial solutions for application onto beef cuts. The purpose was to determine if there was any difference in the efficacy of the antimicrobial solutions based on the source of the water used to prepare them.

Twenty-four beef bottom round flats were used. Six bottom rounds were used for each of the 4 replications. For each replication, each of the bottom rounds were cut in half, creating 12 individual units. Three of the cuts were assigned to each of the 4 treatment groups. A Rif^R cocktail was used for this experiment. The fat side (exterior of the carcass) of each cut was inoculated with 6 ml of the cocktail to ensure complete coverage of the surface. The $\log_{10}$ CFU/ml of initial inoculum cocktail was great enough (8.0 to 9.0 $\log_{10}$ CFU/ml) to ensure a sufficient number of microorganisms could be recovered from the product, before and after the antimicrobial intervention. Treatment solutions were prepared using either deionized water from the TAMU laboratory (TAMU) or water from a meat processing establishment in Pennsylvania (PA). Following collection of the pre-treatment samples, the 12 cuts (3 per treatment) were subjected to approximately 100 ml spray of one of 4 treatments: (TAMU-lactic acid, TAMU-Citrilow™, PA-lactic acid, and PA-Citrilow™) (Lactic Acid, 3.5% ± 0.1%) (Citrilow™, pH 1.05 ± 0.05). Pre-treatment and post-treatment surface excisions were collected for enumeration of surrogate *E. coli*.

3.7 Evaluation of the Use of Lactic Acid to Reduce *Escherichia coli* on Frozen Beef Surfaces

3.7.1 Frozen Trimmings

Beef inside rounds were fabricated into small pieces to create beef trimmings. For each of the 3 replications, beef trimmings were weighed and segregated into three 1.2 kg batches for inoculation.

The $\log_{10}$ CFU/ml of initial inoculum cocktail was high enough (8.0 to 9.0 $\log_{10}$ CFU/ml) to ensure a sufficient number of microorganisms could be recovered from the product both before and after the antimicrobial intervention. Once segregated, each of the 1.2 kg batches of beef trimmings were placed into sterile zippered-top bags and were inoculated with a Rif^R cocktail. Inoculation was conducted by adding 120 ml (to completely cover the surface) of the cocktail to the zippered-top bag, and hand tumbling for 1 min to completely cover all pieces of trimmings with the inoculum. Once inoculated, the 1.2 kg batches were divided into two 600 g units of trimmings and assigned to one of the 2 treatments. Post-inoculation (before freezing), pre-treatment (after freezing), and post-treatment surface excisions were collected for enumeration of surrogate *E. coli*. Samples were taken from each of the 600 g units by use of sterile stainless-steel scalpel and forceps, creating a composite sample of 50 g. Each unit of trimmings was stored in a freezer (-20°C) for approximately 24 h. After freezing, trimmings were tempered (15 min at 25°C), broken apart, and sampled (50 g surface). Immediately after sampling, trimmings were subjected to warm (55°C) lactic acid (prepared from 88.0% L-lactic acid) spray (10 ml delivered vol) using a hand-held sprayer. Lactic acid solutions were heated by circulating hot water around the solution container until the solution temperature reached the desired temperature (close to, but not exceeding 55°C) for application in this trial. Lactic acid was applied to the trimmings at either 2.5% or 5.0% concentration, depending on their treatment group assignments. Post-treatment samples then were collected.

3.7.2 Fresh vs. Frozen

Beef bottom round flats were used. For each of the 4 replications, 6 bottom round flats split in half creating 12 individual pieces (48 total). These pieces then were trimmed to a smaller size (approx. 20 cm x 15 cm x 5 cm), while maintaining the original "medial" surface (near the attachment to the femur) of the bottom round. This was done so they could later be placed into containers for freezing.

A Rif^R cocktail was used for this experiment. Six ml of inoculum was applied to completely cover of the surface. The $\log_{10}$ CFU/ml of initial inoculum cocktail was high enough (8.0 to 9.0 $\log_{10}$ CFU/ml) to ensure a sufficient number of microorganisms could be recovered from the product both before and after the antimicrobial intervention. The 12 units were assigned to 4 separate lactic acid treatment groups (3 units per treatment): fresh - 2.5% lactic acid, fresh - 5.0% lactic acid, frozen - 2.5% lactic acid, and frozen - 5.0% lactic acid. The units assigned to the 2 "fresh" treatment groups were subjected to a spray of the designated lactic acid solutions (80 ml delivered vol, applied at 25°C), using a pump-up, garden-type sprayer. The units assigned to the 2 "frozen" treatment groups were placed into autoclaved aluminum foil pans, covered with foil, and stored in the freezer (-20°C) for approximately 24 h. After freezing, the cuts were tempered (15 min at 25°C), and were subjected to a spray of the designated lactic acid solutions (approx. 80 ml, applied at 25°C), using a pump-up; garden-type sprayer. Pre-treatment and post-treatment surface excisions were collected for enumeration of surrogate *E. coli*.

3.8 Evaluation of Antimicrobial Ingredients to Reduce *Escherichia coli* in Moisture Enhanced Beef Products

3.8.1 Moisture Enhanced Preliminary Trial

A preliminary trial using 4 beef tri-tips was conducted to determine if individual cuts could be vacuum tumbled while contained inside of a vacuum package, and if the packaging would have an impact on the internalization of the microorganisms within the product. Before inoculation, tri-tip roasts were trimmed to remove all exterior fat and connective tissue from the surfaces.

A Rif^R cocktail was used for this experiment. Two ml of the inoculum was applied to completely cover the surface. The $\log_{10}$ CFU/ml of initial inoculum cocktail was high enough (8.0 to 9.0 $\log_{10}$ CFU/ml) to ensure a sufficient number of microorganisms could be recovered from the product after vacuum tumbling. Following attachment, surface samples were collected. This sample was collected to determine the pre-tumbling load of the applied microorganisms. Two of the cuts were vacuum packaged (KOCH Vacuum Packaging Machine, Kansas City, MO) and 2 of the cuts were left un-packaged. The cuts were placed in a BIRO vacuum tumbler Model 12 VTS-42 (Biro MFG Co., Marblehead, OH). The product was tumbled for 30 min to simulate normal tumble marinating procedures. Post-tumbling core samples were collected.

3.8.2 Marinated Trial #1

Twenty-four beef tri-tips were used. Before inoculation, tri-tip roasts were trimmed to remove all exterior fat and connective tissue from the surfaces. Marinade formulation was REO - TAMU Fajita Marinade (REO Spice & Seasoning, Inc.,

Huntsville, TX). The base marinade consisted of 240.0 g of REO marinade seasoning, 26.4 g of sodium tripolyphosphate (STP, BK Giulini Corp., Simi Valley, CA) and 332.4 ml of deionized water. For the treatment group, 3.0 g of cetylpyridinium chloride (CPC, 0.02%, added as Cecure® 40.0% stock solution, Safe Foods Corp., North Little Rock, AR) were added to the marinade. Marinades were stirred using a hot plate/stirrer (no heat; VWR, Suwannee, GA).

For each of the replications, 3 tri-tip roasts were assigned to the treatment (CPC) group and 3 were assigned to the control (CON) group. For the 3 tri-tip roasts in each group, one of them was sampled on day 0, 2, and 4 after marination. A Rif^R cocktail was used for this experiment. Two ml of the inoculum was applied to completely cover the surface. Weights for each tri-tip were recorded and marinade was added for a 10% moisture enhancement (e.g., 34 g marinade added to 340 g tri-tip). Marinated cuts were individually vacuum packaged and vacuum tumbled for 30 min. One of the 3 pieces in each treatment group was sampled immediately (day 0). The 2 remaining pieces of product were held at 4˚C to be sampled at 48 h and 96 h. Pre-treatment and post-treatment surface excisions and internal core samples were collected for enumeration of surrogate *E. coli*.

3.8.3 Marinated Trial #2

Thirty-six beef tri-tips were used. Before inoculation, tri-tip roasts were trimmed to remove all exterior fat and connective tissue from the surfaces. A Rif^R cocktail was used for this experiment. Two ml of the inoculum was applied to completely cover the surface.

Marinade formulation was REO - TAMU Fajita Marinade. The base marinade consisted of 120.0 g of REO marinade seasoning, 13.2 g of sodium tripolyphosphate (STP) and 166.8 ml of deionized water. For the AvGard® (0.2%, sodium metasilicate added as AvGard® XP, Danisco USA, Inc., New Century, KS) treatment group, 6 g of AvGard® was added to the marinade. For the 0.05% and 0.10% CPC treatment groups, 3.75 ml and 7.5 ml of CPC were added to the marinade, respectively. Marinades were stirred using a hot plate/stirrer. Weights for each tri-tip were recorded and marinade was added for a 10% moisture enhancement (e.g., 34 g marinade added to 340 g tri-tip). Marinated cuts were individually vacuum packaged and vacuum tumbled for 30 min.

For each of the 3 replications, 3 tri-tip roasts were assigned to each of the treatment groups (Control, AvGard®, 0.05% CPC, and 0.10% CPC). All cuts were sampled after 4 days of storage at 4°C. Pre-treatment and post-treatment surface excisions and internal core samples were collected for enumeration of surrogate *E. coli*.

3.9 Statistical Analysis

Microbiological count data were transformed into logarithms before calculating means and performing statistical analysis. In the case of counts below the detection limit of the counting method (limit of detection [LOD] = 2.0 $\log_{10}$ CFU), a number (1.7 $\log_{10}$ CFU) between 0 and the lowest detection limit was used to facilitate data analysis. All data were analyzed using JMP® Software (JMP Pro, v12.2, SAS Institute Inc., Cary, NC). The Fit Model function was used for analysis of variance (ANOVA), determining interactions from the full model. Least squares means comparisons were performed

using Tukey-Kramer honestly significant difference (Tukey's HSD) tests with an $\alpha = 0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Preliminary Trials

4.1.1 Pellet Viability

Storing the pellets for up to 8 days had an impact ($P < 0.05$) on the concentration ($\log_{10}$ CFU/ml) of the microorganisms (Table 1). Days 2, 3, 4, 7, and 8 were not different. The reason for the slight decrease in concentration on days 5 and 6 is unknown, but we speculate that if this experiment were replicated with a greater number of samples, the difference in concentration across all 7 days would be minimized. These results suggest these cultures could be prepared as a pellet and stored (4˚C up to 8 days) for in-plant validation trials.

4.1.2 Cocktail vs. Pellet

There was no difference ($P > 0.05$) between starting (pre-treatment) microbial counts for cuts inoculated with the cocktail and those inoculated with the pellet in the first experiment (Table 2). Pre-treatment counts were different ($P < 0.05$) between the cuts inoculated with the cocktail and pellet for the second and third experiments (Table 3 and 4, respectively). For all 3 cocktail vs. pellet experiments, there was no difference ($P > 0.05$) between reductions of microbial counts for cuts inoculated with the cocktail and those inoculated with the pellet. Finally, for the second and third experiments, there was no difference ($P > 0.05$) between reductions of microbial counts for cuts treated with Citrilow™ and those treated with lactic acid.

These results helped to establish the utility of cultures prepared for use in pellet form for in-plant validation trials. Analyses of data indicate method of pathogen surrogate preparation for in-plant food safety intervention validation impacted numbers of cells successfully inoculated onto experimental product surfaces. Method of inoculum preparation did not impact observed surrogate reductions following treatment. Researchers and industry specialists engaged in food safety intervention validation could benefit from the resulting data.

4.1.3 Pre-chill vs. Post-chill

There was no difference ($P > 0.05$) between reductions of *E. coli* for cuts inoculated pre-chilling and those inoculated after chilling (Table 5). Citrilow™ applied at 55ºC to pre-chill inoculated cuts resulted in greater reductions ($P < 0.05$) any of the groups treated with lactic acid (Table 6). These results indicating the efficacy of Citrilow™ agree with those presented by Pohlman et al. *(95)*. Also, Castillo et al. *(21)* demonstrated that lactic acid sprays applied to both pre-chill and post-chill beef can reduce *E. coli*.

This trial was conducted to determine the effects of "pre-chill" inoculation (to simulate contamination during the harvest process) versus "post-chill" inoculation of beef cuts while performing CCP validation in processing facilities and to determine if the temperature of the subsequent antimicrobial spray had an interaction with the inoculation temperature. These results suggest that chilled beef cuts can be inoculated and used during the validation of food safety interventions.

4.2 In-Plant Trials

4.2.1 In-Plant Trial #1

There was no difference ($P > 0.05$) between Inspexx™ 150 and Inspexx™ 200 for reductions of surface *E. coli*. More favorable reductions were achieved on the top sirloins and strip loins (0.7 to 0.8 $\log_{10}$ CFU/50 cm^2) than on short loins and knuckles (0.2 to 0.3 $\log_{10}$ CFU/50 cm^2) (Table 6). Sirloins that were subjected to either of the treatments had better reductions ($P < 0.05$) than knuckles treated with Inspexx™ 150.

4.2.2 In-Plant Trial #2

There was no difference ($P > 0.05$) in reductions of *E. coli* surrogates between the different treatments for beef cuts (Table 7). Reductions of *E. coli* surrogates ranged from 0.1-0.6 $\log_{10}$ CFU/50 cm^2.

4.2.3 In-Plant Trial #3

Reductions of *E. coli* surrogate from application of Citrilow™ to beef cuts and beef trimmings were minimal (0.2 to 0.4 $\log_{10}$ CFU) (Table 8). Among beef cuts and trimmings treated with Citrilow, there were no differences ($P > 0.05$) among cut types for pre-treatment or post-treatment counts, or reductions. Beef cutlets treated with lactic acid had the lowest ($P < 0.05$) pre-treatment and post-treatment counts, but were the same ($P > 0.05$) as the Citrilow treated groups for reductions (Table 8).

4.2.4 In-Plant Trial #4

Pre-treatment counts of surrogate *E. coli* were higher ($P < 0.05$) on bottom rounds that on bottom round roasts, while post-treatment counts were not different ($P > 0.05$) among all cuts (Table 9). Reduction of *E. coli* surrogates achieved by treatment

with Citrilow™ or lactic acid were minimal (0.2 to 0.4 $\log_{10}$ CFU/50 cm^2), and did not differ across cuts and treatments ($P > 0.05$) (Table 9).

4.2.5 Water Source Comparison

Source of the water used to prepare the treatments did not affect ($P > 0.05$) reductions of *E. coli*. Lactic acid solutions prepared from both water sources resulted in 0.8 $\log_{10}$ CFU/50 cm^2 reduction. Citrilow™ solutions prepared from both water sources resulted in 0.7 $\log_{10}$ CFU/50 cm^2 reduction (Table 10). These data suggest, in this specific case, the water source was not a factor in the efficacy of these antimicrobial solutions.

4.2.6 In-Plant Trials Discussion

The findings from these plant trials agree with previous research pertaining to the minimal efficacy of peroxyacetic acid solutions on chilled beef products *(40, 74)*. Also, the results of the use of Beefxide® in these trials are contradictory to those observed by Laury et al. *(76)* and Hendricks et al. *(57)*, who found that Beefxide® resulted in a 1.4 $\log_{10}$ CFU/cm^2 reduction of *E. coli*. Further investigation is needed to determine if other factors may be influencing the efficacy of these interventions.

These results illustrate the challenges that exist pertaining to the efficacy of antimicrobial interventions on chilled beef subprimals. These data contradict the literature pertaining to the efficacy of lactic acid and Citrilow™ on chilled beef *(37, 54, 94, 95)*. Further investigation is needed to determine if other factors may be influencing the efficacy of the interventions.

4.3 Frozen Beef Surfaces

4.3.1 Frozen Trimmings

There was no difference ($P > 0.05$) between the 2 treatments for reduction of surrogate *E. coli*. Reductions of 0.5 and 0.7 $\log_{10}$ CFU/g were observed for 2.5% and 5.0% warm lactic acid, respectively (Table 11).

4.3.2 Fresh vs. Frozen

There was a difference ($P < 0.0001$) for post-treatment counts of surrogate *E. coli* between the fresh and frozen treatment groups (Table 12). Reductions achieved on frozen cuts treated with 5.0% lactic acid were greater ($P < 0.0001$) than reductions from either of the fresh treatment groups (Table 12). There were no differences ($P > 0.05$) for reductions between lactic acid concentrations. These data suggest that freezing, in combination with a subsequent 5.0% lactic acid spray, may lead to more favorable reductions of *E. coli* than lactic acid applied to fresh beef.

4.3.3 Frozen Beef Surfaces Discussion

The literature suggests that freezing and thawing can result in the injury and subsequent death of a portion of microorganisms. However, the available literature also describes the inconsistent effects of freezing and the chance of pathogen survival throughout the freezing and thawing process *(23, 35, 48, 79)*. If the membrane and other components of bacterial cells are damaged during the freezing process, they may be more susceptible to treatment with organic acids. Damage to the cell could allow for easier introduction of the undissociated acid molecules into the cytoplasm. Therefore, further investigation is needed pertaining to the effects of freezing, thawing, and

antimicrobial spray on the survival of pathogens in meat products. Due to the lack of literature available pertaining to antimicrobial solutions applied to frozen meat, further research is needed to determine potential intervention strategies for frozen beef products.

4.4 Moisture Enhanced Beef

Based on the findings of the preliminary trial, internalization of the microorganisms was not affected based on the use of the vacuum packaging. Therefore, all cuts for marination trials #1 and #2 were vacuum packaged before vacuum tumbling.

4.4.1 Marinated Trial #1

There were no differences ($P > 0.05$) for reductions of surface *E. coli* between CPC and control, across all days of storage (Table 13). Also, there was no difference ($P > 0.05$) among treatment groups and days of storage for internal surviving *E. coli* surrogates (Table 14).

4.4.2 Marinated Trial #2

Pre-treatment counts were the same ($P > 0.05$) across treatments, but post-treatment surface *E. coli* were lower ($P < 0.05$) for the 0.10% CPC group (Table 15). Although there was not a statistical difference ($P = 0.0696$), the 0.10% CPC treatment reduced surface *E. coli* by 0.7 $\log_{10}$ CFU/50 cm^2, while all other groups yielded a 0.5 $\log_{10}$ CFU/50 cm^2 reduction (Table 15). There was no ($P > 0.05$) difference between treatments for surviving surrogate *E. coli* recovered from internal core samples (Table 16).

4.4.3 Moisture Enhanced Beef Discussion

The findings of trials #1 and #2 are similar to that of Adler et al. *(2)* and Byelashov et al. *(15)*, who found that brines containing CPC were among the most effective at reducing pathogens in the meat. Previous research has used ground or comminuted beef products with antimicrobial ingredients added to the brine solutions. However, very few trials have investigated its use in an enhancement solution for whole-muscle beef cuts. The levels at which CPC was utilized during these trials were chosen based on the minimum level that was predicted to be effective. The findings of these trials suggest that further investigation is needed to determine if using CPC as an antimicrobial ingredient is effective at reducing *E. coli* to an acceptable level in whole-muscle, marinated beef products.

CHAPTER V

CONCLUSIONS

This research supports the use of pellets for transporting surrogates of STEC and *Salmonella* spp. to establishments for in-plant validation trials. Antimicrobial treatments for chilled beef were not as effective during in-plant trials as in previously published research. Further investigation may be warranted to determine the best combination of antimicrobial compounds, concentration, and solution temperature to reduce pathogens without negatively impacting the organoleptic quality of the end product.

Results of these experiments were inconclusive pertaining to reduction of *E. coli* on frozen beef surfaces. With what we know about sub-lethal injury of cells due to freezing, it is possible that microorganisms are more susceptible to pH changes due to chemical interventions. Interaction between freezing and the application of lactic acid suggest that additional research should be conducted to determine the most effective methods for applying antimicrobial interventions to frozen beef for the reduction of pathogens.

Data suggest that use of these antimicrobial compounds in a brine solution may be effective at reducing *E. coli* in moisture-enhanced, whole-muscle beef cuts. While there were tendencies for reduction of microorganisms due to treatments, it is unclear if these ingredients would be a reliable method of pathogen control in marinated beef. Further investigation is needed to determine the most effective use of these antimicrobial ingredients for whole-muscle marination.

The results of these trials demonstrate how surrogate microorganisms can be used to validate the application of antimicrobials on beef subprimals in-plant, frozen beef, and marinated beef cuts. Additional research in these areas are warranted to help provide more information to the beef industry. Beef processors can use this information to help design validation programs for their specific production processes.

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APPENDIX A

Table 1. Mean concentration (log_{10} CFU/ml) of *Escherichia coli* pellet inoculum after different days of storage[1].

Days of Storage	log_{10} CFU/ml
2	8.8^{A}
3	8.6^{AB}
4	8.7^{A}
5	8.2^{B}
6	8.3^{B}
7	8.8^{A}
8	8.7^{A}
SEM	0.077
P-value	0.0037

Means within the row with differing superscripts (A-B) differ ($P < 0.05$).
[1] Pellets were stored under refrigeration (4°C).

Table 2. Least squares means of *Escherichia coli* (log_{10} CFU/cm^2) on inoculated beef bottom rounds pre- and post-treatment[1], stratified by inoculum preparation method.

	Inoculum Preparation Method			
	Cocktail	Pellet	SEM	*P*-value
Pre-treatment	5.9^{A}	6.3^{A}	0.206	0.2205
Post-treatment	5.2^{A}	5.2^{A}	0.029	0.9857
Reduction[2]	0.7^{A}	1.1^{A}	0.199	0.2077

Means within rows with the same superscripts (A) are not different ($P > 0.05$).
[1] Treatment, lactic acid was prepared (88% L-lactic acid, Purac® FCC 88, Corbion, Lenexa, KS) to a concentration of 3.5% ± 0.1% (verified by titration) applied at 25ºC.
[2] Reduction = (log_{10} CFU/50 cm^2 before treatment) – (log_{10} CFU/50 cm^2 after treatment).

Table 3. Least squares means of pre- and post-treatment counts, and reductions of Rif resistant *Escherichia coli* (log_{10} CFU/50 cm^2) on inoculated beef bottom rounds, stratified by treatment and inoculum type.

	Inoculum Type / Treatments					
	Cocktail		Pellet			
	Lactic acid[1]	Citrilow[2]	Lactic acid[1]	Citrilow[2]	SEM	*P*-value
Pre-treat	6.6^{A}	6.5^{A}	6.1^{B}	6.0^{B}	0.105	0.0152
Post-treat	5.9^{A}	5.7^{A}	5.7^{A}	5.5^{A}	0.097	0.0776
Reduction[3]	0.7^{A}	0.8^{A}	0.4^{A}	0.5^{A}	0.149	0.3512

Means within rows with differing superscripts (A-B) differ ($P < 0.05$).

[1] Lactic acid was prepared (88% L-lactic acid, Purac® FCC 88, Corbion, Lenexa, KS) to a concentration of 3.5% ± 0.1% (verified by titration) applied at 25ºC.

[2] Citrilow™ was prepared (Safe Food Corp., North Little Rock, AR) to a pH of 1.05 ± 0.01 (verified by standardized pH instrument) applied at 25°C.

[3] Reduction = (log_{10} CFU/50 cm^2 before treatment) – (log_{10} CFU/50 cm^2 after treatment).

Table 4. Least squares means of pre- and post-treatment counts, and reductions of Rif resistant *Escherichia coli* (log_{10} CFU/50 cm^2) on inoculated beef bottom rounds, stratified by treatment and inoculum type.

	Inoculum Type / Treatments					
	Cocktail		Pellet			
	Lactic acid[1]	Citrilow[2]	Lactic acid[1]	Citrilow[2]	SEM	*P*-value
Pre-treat	6.4^{A}	6.3^{A}	6.0^{B}	6.2^{B}	0.068	0.0087
Post-treat	5.5^{A}	5.0^{A}	5.1^{A}	5.2^{A}	0.151	0.1110
Reduction[3]	0.9^{A}	1.3^{A}	0.8^{A}	1.0^{A}	0.127	0.0950

Means within rows with differing superscripts (A-B) differ ($P < 0.05$).

[1] Lactic acid was prepared (88% L-lactic acid, Purac® FCC 88, Corbion, Lenexa, KS) to a concentration of 3.5% ± 0.1% (verified by titration) applied at 25ºC.

[2] Citrilow™ was prepared (Safe Food Corp., North Little Rock, AR) to a pH of 1.05 ± 0.01 (verified by standardized pH instrument) applied at 25°C.

[3] Reduction = (log_{10} CFU/50 cm^2 before treatment) – (log_{10} CFU/50 cm^2 after treatment).

Table 5. Least squares means of the interaction of treatment x inoculation time on reductions[1] of Rif resistant *Escherichia coli* (log_{10} CFU/50 cm^2) on inoculated beef briskets.

	Inoculum Time[4]			
Treatment	Pre-chill	Post-chill	SEM	*P*-value
25ºC Lactic acid[2]	1.4^{B}	1.3^{B}		
55ºC Lactic acid[2]	1.6^{B}	1.3^{B}		
25ºC Citrilow[3]	2.7^{AB}	2.3^{AB}		
55ºC Citrilow[3]	3.4^{A}	2.1^{AB}	0.329	0.0030

Means with differing superscripts (A-B) differ ($P < 0.05$) for treatment x inoculation time interaction.

[1] Reduction = (log_{10} CFU/50 cm^2 before treatment) – (log_{10} CFU/50 cm^2 after treatment).

[2] Lactic acid was prepared (88% L-lactic acid, Purac® FCC 88, Corbion, Lenexa, KS) to a concentration of 3.5% ± 0.1% (verified by titration) applied to chilled beef briskets at 25ºC and 55ºC.

[3] Citrilow™ was prepared (Safe Food Corp., North Little Rock, AR) to a pH of 1.05 ± 0.01 (verified by standardized pH instrument) applied to chilled beef briskets at 25°C and 55ºC.

[4] Twelve briskets were inoculated pre-chilling (approx. 28°C) and 12 briskets were inoculated post-chilling (approx. 4°C).

Table 6. Least squares means of the interaction of treatment x cut type on reductions[1] of Rif resistant *Escherichia coli* (log_{10} CFU/50 cm^2) on inoculated beef cuts.

	Treatment			
Cut Type	Inspexx 150[2]	Inspexx 200[3]	SEM	*P*-value
Knuckle	0.2^{B}	0.3^{AB}		
Short loin	0.3^{AB}	0.3^{AB}		
Sirloin	0.8^{A}	0.8^{A}		
Striploin	0.7^{AB}	0.8^{AB}	0.102	0.0003

Means with differing superscripts (A-B) differ ($P < 0.05$) for treatment x cut type interaction.

[1] Reduction = (log_{10} CFU/50 cm^2 before treatment) – (log_{10} CFU/50 cm^2 after treatment).

[2] Inspexx™ 150 (Ecolab, St. Paul, MN) prepared to a concentration of 150 parts per million (verified by titration) applied at 13ºC.

[3] Inspexx™ 200 (Ecolab, St. Paul, MN) prepared to a concentration of 190 parts per million (verified by titration) applied at 13ºC.

Table 7. Least squares means of the interaction of treatment x cut type on reductions[1] of Rif resistant *Escherichia coli* (log_{10} CFU/50 cm^2) on inoculated beef cuts.

	Treatment				
Cut Type	Beefxide[2]	Inspexx 150[3]	Inspexx 200[4]	SEM	*P*-value
Knuckle	0.1^A	0.3^A	0.3^A		
Striploin	0.4^A	0.6^A	0.2^A	0.133	0.1421

Means with the same superscripts (A) are not different ($P > 0.05$).
[1] Reduction = (log_{10} CFU/50 cm^2 before treatment) – (log_{10} CFU/50 cm^2 after treatment).
[2] Beefxide® (Birko Corporation, Henderson, CO) applied at 2.0% ± 0.1% (verified by titration) at 41ºC.
[3] Inspexx™ 150 (Ecolab, St. Paul, MN) prepared to a concentration of 150 parts per million (verified by titration) applied at 19ºC.
[4] Inspexx™ 200 (Ecolab, St. Paul, MN) prepared to a concentration of 190 parts per million (verified by titration) applied at 23ºC.

Table 8. Least squares mean pre- and post-treatment counts, and reductions of *Escherichia coli* (log_{10} CFU[1,2]) on inoculated beef cuts and beef trimmings.

Cut Type	*Treatment*	Pre-treatment	Post-treatment	Reduction[5]
Bottom Round[1]	Citrilow[3]	6.0^{AB}	5.7^{AB}	0.3^{A}
Chuck Roll[1]	Citrilow[3]	6.0^{AB}	5.9^{AB}	0.2^{A}
Inside Round[1]	Citrilow[3]	6.2^{AB}	5.8^{AB}	0.4^{A}
Beef Trim[2]	Citrilow[3]	6.7^{A}	6.5^{A}	0.2^{A}
Beef Cutlets[2]	Lactic Acid[4]	5.2^{B}	5.2^{B}	0.2^{A}
SEM		0.252	0.277	0.090
P-value		0.0038	0.0312	0.3606

Means within columns with differing superscripts (A-B) differ ($P < 0.05$).
[1] Surface excision samples; means reported as log_{10} CFU/50 cm^2.
[2] Surface samples were collected to reach a target mass; means reported as log_{10} CFU/g.
[3] Citrilow™ was prepared (Safe Food Corp., North Little Rock, AR) to a pH of 1.05 ± 0.01 (verified by standardized pH instrument) applied at 25°C.
[4] Lactic acid was prepared (88% L-lactic acid, Purac® FCC 88, Corbion, Lenexa, KS) to a concentration of 3.5% ± 0.1% (verified by titration) applied at 25ºC.
[5] Reduction = (log_{10} CFU pre-treatment) – (log_{10} CFU post-treatment).

Table 9. Least squares mean reductions of *Escherichia coli* (log_{10} CFU/50 cm^2) on inoculated beef bottom rounds, chuck rolls, and bottom round roasts.

Cut Type	*Treatment*	Pre-treatment	Post-treatment	Reduction[3]
Bottom Round	Citrilow[1]	5.7^{A}	5.3^{A}	0.4^{A}
Chuck Roll	Citrilow[1]	5.6^{AB}	5.3^{A}	0.3^{A}
Btm. Round Rst.	Lactic Acid[2]	5.4^{B}	5.2^{A}	0.2^{A}
SEM		0.057	0.063	0.065
P-value		0.0021	0.1670	0.5355

Means within columns with differing superscripts (A-B) differ ($P < 0.05$).

[1] Citrilow™ was prepared (Safe Food Corp., North Little Rock, AR) to a pH of 1.05 ± 0.01 (verified by standardized pH instrument) applied at 25°C.

[2] Lactic acid was prepared (88% L-lactic acid, Purac® FCC 88, Corbion, Lenexa, KS) to a concentration of 3.5% ± 0.1% (verified by titration) applied at 25ºC.

[3] Reduction = (log_{10} CFU/50 cm^2 before treatment) – (log_{10} CFU/50 cm^2 after treatment).

Table 10. Least squares mean reductions of *Escherichia coli* (log_{10} CFU/50 cm^2) on inoculated beef cuts, stratified by water source and treatment[1,2].

Water Source	*Treatment*	Reduction[3]
TAMU	Citrilow[2]	0.7^A
PA	Citrilow[2]	0.7^A
TAMU	Lactic Acid[3]	0.8^A
PA	Lactic Acid[3]	0.8^A
SEM		0.098
P-value		0.6178

Means with the same superscripts (A) are not different ($P > 0.05$).

[1] Citrilow™ was prepared (Safe Food Corp., North Little Rock, AR) to a pH of 1.05 ± 0.01 (verified by standardized pH instrument) applied at 25°C.

[2] Lactic acid was prepared (88% L-lactic acid, Purac® FCC 88, Corbion, Lenexa, KS) to a concentration of 3.5% ± 0.1% (verified by titration) applied at 25ºC.

[3] Reduction = (log_{10} CFU/50 cm^2 before treatment) – (log_{10} CFU/50 cm^2 after treatment).

Table 11. Least squares means of *Escherichia coli* (log_{10} CFU/g) on inoculated frozen beef trimmings post-inoculation and post-treatment, stratified by lactic acid[1] concentration.

	Lactic Acid Concentration[1]			
	2.5 %	5.0 %	SEM	*P*-value
Post-inoculation	6.6^{A}	6.6^{A}	0.054	0.3996
Post-treatment	6.1^{A}	5.9^{A}	0.059	0.1521
Reduction[2]	0.5^{A}	0.7^{A}	0.065	0.0505

Means within the same row with same superscripts (A) are not different ($P > 0.05$).
[1] Lactic acid was prepared (88% L-lactic acid, Purac® FCC 88, Corbion, Lenexa, KS) to a concentration of 2.5% and 5.0% ± 0.1% (verified by titration) applied at 55ºC.
[2] Reduction = (log_{10} CFU/g after inoculation) – (log_{10} CFU/g after treatment).

Table 12. Least squares means of *Escherichia coli* (log_{10} CFU/50 cm^2) on inoculated beef cuts pre- and post-treatment, and reductions, stratified by lactic acid[1] concentration and freezing[3] treatment.

	Freezing Treatments / Lactic Acid Concentrations					
	Fresh[2]		Frozen[3]			
	2.5%[1]	5.0%[1]	2.5%[1]	5.0%[1]	SEM	*P*-value
Pre-treat	4.8^{A}	4.8^{A}	4.8^{A}	4.7^{A}	0.061	0.8867
Post-treat	4.0^{A}	3.8^{A}	3.4^{B}	3.2^{B}	0.090	< .0001
Reduction[4]	0.8^{C}	1.0^{BC}	1.4^{AB}	1.5^{A}	0.113	< .0001

Means within rows with differing superscripts (A-C) differ ($P < 0.05$).

[1] Lactic acid was prepared (88% L-lactic acid, Purac® FCC 88, Corbion, Lenexa, KS) to a concentration of 2.5%± 0.1% and 5.0% ± 0.1% (verified by titration) applied at 25ºC.

[2] Cuts assigned to the Fresh groups were treated with either 2.5% or 5.0% lactic acid.

[3] Cuts assigned to the Frozen groups were stored in the freezer (-20°C) for approximately 24 h before treatment with either 2.5% or 5.0% lactic acid.

[4] Reduction = (log_{10} CFU/50 cm^2 after inoculation) – (log_{10} CFU/50 cm^2 after treatment).

Table 13. Least squares means of the interaction of treatment x days of storage on reductions[1] of surface *Escherichia coli* (log_{10} CFU/50 cm^2) of inoculated marinated beef tri-tips on day 0, 2, and 4 of refrigerated (4°C) storage.

	Days of Storage				
Treatment	0	2	4	SEM	*P*-value
CON[2]	0.5^{A}	0.6^{A}	0.5^{A}		
CPC[3]	0.5^{A}	0.6^{A}	0.5^{A}	0.075	0.6968

Means with the same superscripts (A) are not different ($P > 0.05$).
All cuts were vacuum tumbled for 30-min after treatments were applied, then stored under refrigeration (4°C) for 0, 2, or 4 days before post-treatment samples were collected.
[1] Reductions = (log_{10} CFU/50 cm^2 before treatment) – (log_{10} CFU/50 cm^2 after treatment).
[2] CON, Control, marinated with no antimicrobial added.
[3] CPC, cetylpyridinium chloride (0.02%, added as Cecure® 40.0% stock solution, Safe Foods Corp., North Little Rock, AR).

Table 14. Least squares means of the interaction of treatment x days of storage on surviving internalized *Escherichia coli* (log_{10} CFU/g) of inoculated marinated beef tri-tips on day 0, 2, and 4 of refrigerated (4°C) storage.

	Days of Storage				
Treatment	0	2	4	SEM	*P*-value
CON[1]	3.0^{A}	3.1^{A}	2.4^{A}		
CPC[2]	2.3^{A}	2.5^{A}	2.4^{A}	0.278	0.1775

Means with the same superscripts (A) are not different ($P > 0.05$).
All cuts were vacuum tumbled for 30-min after treatments were applied, then stored under refrigeration (4°C) for 0, 2, or 4 days before internal samples were collected.
[1] CON, Control, marinated with no antimicrobial added.
[2] CPC, cetylpyridinium chloride (0.02%, added as Cecure® 40.0% stock solution, Safe Foods Corp., North Little Rock, AR).

Table 15. Least squares means of *Escherichia coli* (log_{10} CFU/50 cm^2) on inoculated and marinated beef tri-tips pre- and post-treatment, and reductions; stratified by treatment.

	Treatments					
	CON[1]	Avgard[2]	0.05% CPC[3]	0.10% CPC[4]	SEM	*P*-value
Pre-treat	4.9^{A}	4.8^{A}	4.8^{A}	4.7^{A}	0.057	0.2486
Post-treat	4.4^{A}	4.3^{A}	4.3^{A}	4.0^{B}	0.057	0.0007
Reduction[5]	0.5^{A}	0.5^{A}	0.5^{A}	0.7^{A}	0.064	0.0696

Means within rows with differing superscripts (A-B) differ ($P < 0.05$).
All cuts were vacuum tumbled for 30-min after treatments were applied, then stored under refrigeration (4°C) for 4 days before post-treatment samples were collected.
[1] CON, Control, marinated with no antimicrobial added.
[2] AvGard, sodium metasilicate (0.2%, added as AvGard® XP, Danisco USA, Inc., New Century, KS).
[3] 0.05% CPC, cetylpyridinium chloride (0.05%, added as Cecure® 40.0% stock solution, Safe Foods Corp., North Little Rock, AR).
[4] 0.10% CPC, cetylpyridinium chloride (0.10%, added as Cecure® 40.0% stock solution, Safe Foods Corp., North Little Rock, AR).
[5] Reduction = (log_{10} CFU/50 cm^2 before treatment) – (log_{10} CFU/50 cm^2 after treatment).

Table 16. Least squares means of internal surviving *Escherichia coli* (log_{10} CFU/g) in inoculated and marinated beef tri-tips; stratified by treatment.

	Treatments					
	CON[1]	Avgard[2]	0.05% CPC[3]	0.10% CPC[4]	SEM	*P*-value
Internal Survivors	2.4[A]	2.4[A]	2.7[A]	2.1[A]	0.176	0.2226

Means with the same superscripts (A) are not different ($P > 0.05$).
All cuts were vacuum tumbled for 30-min after treatments were applied, then stored under refrigeration (4°C) for 4 days before internal samples were collected.
[1] CON, Control, marinated with no antimicrobial added.
[2] AvGard, sodium metasilicate (0.2%, added as AvGard® XP, Danisco USA, Inc., New Century, KS).
[3] 0.05% CPC, cetylpyridinium chloride (0.05%, added as Cecure® 40.0% stock solution, Safe Foods Corp., North Little Rock, AR).
[4] 0.10% CPC, cetylpyridinium chloride (0.10%, added as Cecure® 40.0% stock solution, Safe Foods Corp., North Little Rock, AR).