Evaluation of individual and combined antimicrobial spray treatments on chilled beef subprimal cuts to reduce Shiga toxin-producing *Escherichia coli* populations

by

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B.S., Abilene Christian University, 2014

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Food Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2017

Approved by:

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Abstract

Due to the potential of Shiga toxin-producing *Escherichia coli* (STEC) contamination, beef processors use various antimicrobial interventions throughout the slaughter and fabrication processes to reduce risks of contaminating the food supply. Certain antimicrobials are approved and marketed for spraying onto chilled subprimal cuts; however, administering these treatments through commercial-scale equipment against foodborne pathogens is not fully validated. This study evaluated the efficacy of three common antimicrobial sprays, individually (Study 1) and combined (Study 2), against a rifampicin-resistant STEC cocktail (O26, O45, O103, O111, O121, O145, and O157:H7) using a commercial style subprimal spray cabinet. For Study 1, beef subprimals (n=16) were mist-inoculated with the cocktail (ca. 5 log CFU/cm^2), followed by spray-treatment with individual antimicrobials [200 ppm peracetic acid (PAA), 2% Centron™ (sulfuric acid, sodium sulfate anhydrous and water mixture; CEN), 4.5% lactic acid (LA), or water (W)]. Study 1 was designed as randomized generalized block. After each treatment phase, STEC population reductions were quantified. As individual antimicrobial treatments, LA and PAA provided greater ($P \leq 0.05$) STEC reductions (0.5 and 0.6 ± 0.08 log CFU/cm^2, respectively) compared to water (0.2 ± 0.08 log CFU/cm^2), but the CEN reduction (0.4 ± 0.10 log CFU/cm^2) was statistically similar to W.

To test the efficacy of combined treatments on subprimal cuts in Study 2, a split-plot design was used using three replications. The inoculated subprimals (n=4) were first treated with PAA, LA, CEN, or W; vacuum packaged; and stored for 72 hours at 4°C. Each subprimal was then divided (n=16) and treated with each of the four antimicrobials as a second treatment. Cumulative reductions from the two treatments and storage ranged from 0.5 to 1.5 log CFU/cm^2 (± 0.3 log CFU/cm^2); the greatest reduction was observed when subprimals were treated with LA

followed by vacuum packaged storage and another LA application. Nevertheless, there was no statistical significance among treatments for a particular combination of treatments in Study 2.

These studies indicate that the individual antimicrobial treatments evaluated are marginally effective for reducing STEC populations on chilled beef subprimal cuts during fabrication. Although there does not seem to be a specific combination of treatment that is more effective than another, the overall bacterial reduction may be improved by combining treatments when the beef is stored under vacuum packaged conditions and retreated upon bag opening, as typical of mechanical tenderization operations.

Keywords: antimicrobials, beef subprimal, lactic acid, peracetic acid, interventions

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Acknowledgements

The completion of this research and thesis greatly depended on many people, to whom I would like to express gratitude.

All funding of this project was generously provided by the USDA NIFA AFRI Shiga Toxin-Producing *Escherichia coli* Coordinated Agricultural Project Grant. The collaborators for Objective 3 (Antimicrobial Interventions to Control STEC on Beef) worked closely with myself and Dr. Phebus and me to plan and execute this project. I would like to particularly thank Dr. John Luchanksy and his laboratory at USDA ARS ERRC (Wyndmoor, PA) for providing the STEC strains used in the project. I would also like to thank the collaborators from Texas A&M University, Dr. Gary Acuff and Lisa Lucia, for traveling to Kansas State University to help with sample processing at the Biosecurity Research Institute (BRI) for Study 1.

To complete these studies, a great amount of effort from support staff was required. The BRI Biosafety Officers provided necessary guidance to complete the project in the Biosafety Level-3 facility, and their help was appreciated. Additionally, the Food Safety and Defense Laboratory staff aided in all portions of the experiment. I specifically thank the undergraduate and graduate students (Daniel Vega, Matthew Krug, Amanda Wilder, Nicholas Sevart, Sarah Jones, Austin McDaniel, Jessica Huffman, and Robert Sholl) for helping prepare supplies and execute the project protocol. Drs. Minto Michael and Keyla Lopez also provided invaluable guidance while planning and executing the research, for which I am extremely grateful. Lastly, I emphatically thank Dr. Phebus for the guidance and mentorship he provided before, throughout, and following the completion of this research.

These studies were designed to mimic common industry processes, so the equipment and supplies used were meant to be consistent with those used by beef processors. Zoetis Inc.

provided a commercial-style subprimal spray cabinet, as well as the Centron™ product. Lactic acid was provided by BIRKO and Corbion, and Zee Company provided peracetic acid (Microtox Plus). I greatly appreciate the materials and expertise offered by these companies.

Finally, I wholeheartedly thank my husband, Justin Fleer, for supporting me throughout my time in graduate school. His patience and encouragement was crucial to my ability to complete this research, and words cannot express my gratitude for him. I also thank my family, Gary and Awilda Acuff and Laura Tolentino, who have always supported my dreams and ambitions in life. I owe a great deal of who I am and what I have accomplished to their love and encouragement for the last 25 years.

Chapter 1 - Introduction

Each year, Shiga toxin-producing *Escherichia coli* (STEC) cause an estimated 265,000 illnesses, some of which result in over 3,600 hospitalizations and 30 deaths (Scallan 2011). One of the main STEC serotypes responsible for these diseases is O157:H7. Foodborne outbreaks associated with O157:H7 were first recognized in 1982 in Oregon and Michigan, and since then, outbreaks of various sizes have occurred each year (Rangel 2005). While approximately one-third of STEC illnesses are attributed to O157:H7, non-O157 STEC are responsible for nearly two-thirds of illnesses each year (Scallan 2011). From 1996 to 2013, the incidence of confirmed O157 cases decreased, but the incidence of non-O157-related illnesses increased (Figure 1; CDC 2016).

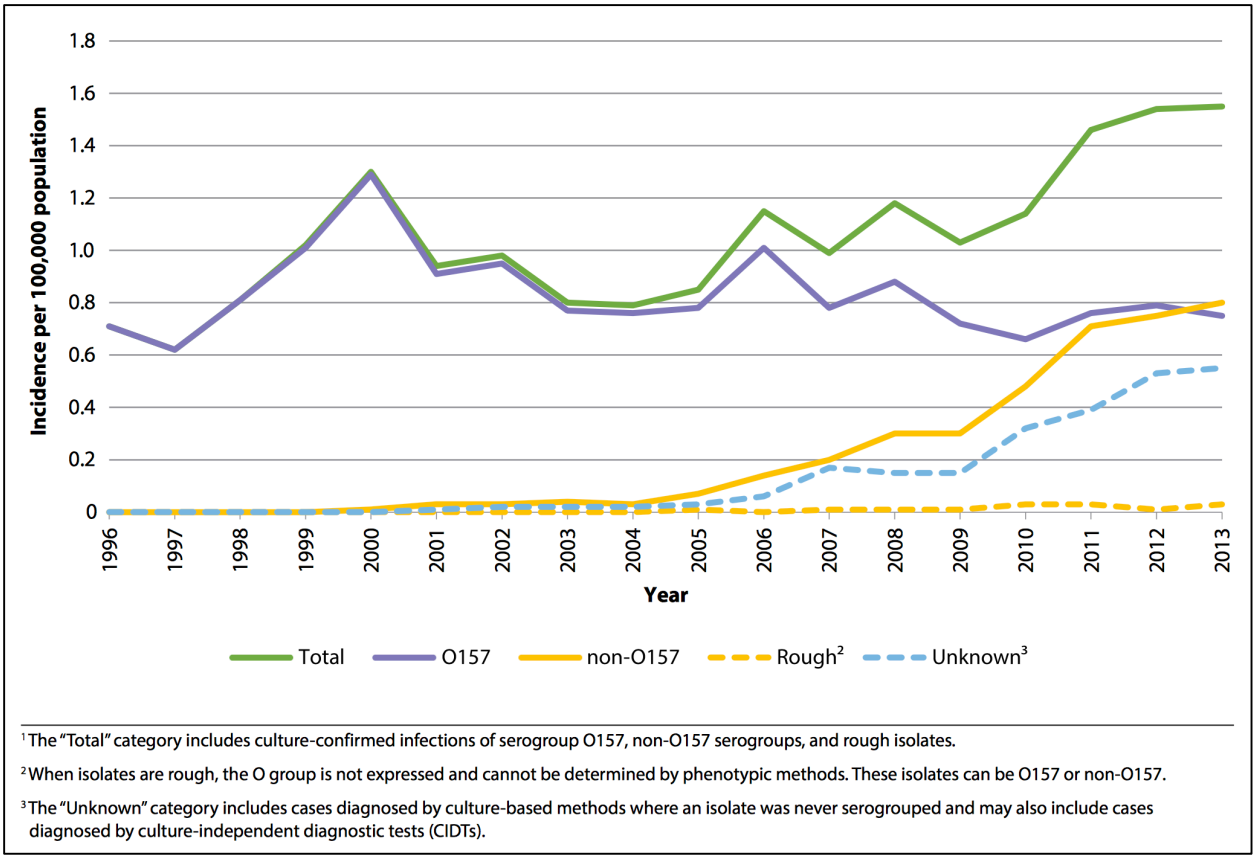

Figure 1.1. Incidence rate of human Shiga toxin-producing *Escherichia coli* (STEC) infection reported to CDC, by serogroup and year, United States, 1996-2013 (CDC 2016).

STEC-related infections often lead to diarrhea, which may at times be either watery or bloody (Hughes 2006). If the infection is severe, patients may develop hemolytic uremic syndrome (HUS) and renal failure (Mead 1998). These serious side effects result from the extreme virulence of some STEC strains caused by the combination of Shiga toxin-producing genes, Stx1 and/or Stx2, and the attaching and effacing gene, eaeA (Law 2000). The eaeA gene allow STEC to intimately attach to the intestinal lining and cause severe disease (Donnenberg 1997, Lai 1997).

STEC enter the food system through many vectors, one of which is cattle that serve as a natural reservoir for the bacteria (Moxley 2015, Persad 2015). Once cattle enter the slaughter plant, cross contamination from the potentially contaminated hides onto dressed carcasses is common and not easily prevented. However, due to research over the last several decades, scientists have found various antimicrobial interventions that can be used by processors to reduce the impact of carcass-level contamination, such as hot water carcass washes and antimicrobial sprays. More recently, antimicrobial applications subsequently applied to raw chilled cuts of beef have been a focus of industry and food safety researchers to further reduce foodborne risks associated with beef, particularly ground beef and other non-intact products (i.e., mechanically tenderized, reformed, and/or marinated). These interventions can be used to reduce spoilage, as well as pathogenic bacteria (Acuff 1987, Dickson 1992, Phebus 1997, Castillo 1998).

Beef processors are particularly interested in utilizing interventions to efficiently create a safer product. Due to recent advances in genome sequencing of pathogens from outbreaks and recalls, and the decision in 1994 to regulate *E. coli* O157:H7 as an adulterant in raw ground beef (expanded to include raw non-intact beef products in 1999 and to include six additional STEC serogroups in 2011), processors risk facing regulatory actions, negative business consequences,

recalls and consumer lawsuits if their products are contaminated with any of the seven regulated STEC serogroups (STEC-7) (FSIS 1999, 2011). The public health, social, and economic impacts that result from an outbreak have led to more government and industry funding being awarded to advance STEC-related research. The scientific community seeks to further elucidate issues regarding STEC contamination and methods that can improve beef safety.

Research Objectives and Questions

The research described in this thesis was designed to evaluate the antimicrobial efficacy of individual spray treatments that are commonly used in the beef industry, specifically lactic acid, peracetic acid, and a sulfuric acid blend for application on raw, chilled subprimal cuts to reduce artificially inoculated populations of STEC-7. Additionally, two-treatment combinations of these antimicrobials incorporating a short vacuum-packaged storage period were also assessed in an effort to simulate a scenario commonly practiced by beef processors and retailers that includes treatment, storage, shipping, and a second treatment upon opening the subprimal bags.

To achieve these research objectives, the research sought to answer the following questions:

1. *Are particular antimicrobials more successful in reducing STEC on the surface of chilled subprimal beef cuts than others or water alone?*
2. *When paired with a 72-hour vacuum-packaged storage period, are certain combinations of chemical antimicrobial spray treatments more successful than others in reducing STEC-7 on the surface of chilled beef subprimal cuts?*
3. *Does vacuum-packaged storage influence STEC-7 populations on subprimal cuts?*

Chapter 2 - Literature Review

The purposes of this chapter are to present findings from a literature search, as well as explain research issues regarding beef subprimal antimicrobial treatments. The document summarizes the general characteristics of Shiga toxin-producing *Escherichia coli* (STEC), provides information on previous laboratory studies on the topic, and postulates direction for future research.

Contextual Information & Background

In 1993, after a fast-food chain in the United States was associated with a deadly outbreak of *Escherichia coli* O157:H7 from serving undercooked hamburgers, STEC became of great concern to consumers, public health officials, the beef industry, and food microbiologists. The dangers of contaminated and undercooked meat products were made clear when over 500 individuals were sickened and four children died from hemolytic uremic syndrome (HUS) associated with STEC infections (Bell 1994). While there was some knowledge of the cellular properties of STEC prior to the outbreak, it was not until after O157:H7 was declared an adulterant that the food industry and regulatory agencies became more concerned with food safety risks in the beef industry. This heightened interest gave scientists more tools and funding to apply STEC research to the food industry.

STEC of greatest concern in beef have been labeled as the "Big 7" and contain the serotypes O26, O45, O103, O111, O121, O145, and O157:H7, as they are more commonly associated in foodborne illness outbreaks and considered adulterants in non-intact beef products (FSIS 1999, FSIS 2012). These are classified based on particular gene markings that set them apart and impact their virulence. The danger of some of these pathogens lies in their ability to

develop attaching and effacing (A/E) intestinal lesions, but not all STEC have the genes that produce these adherence abilities (Kaper 2015). The Shiga toxin gene (Stx) is found in these pathogens, which allows them to produce a verotoxin that is clearly linked to HUS, a prominent cause of acute renal failure, specifically in children (Kaper 2015). There are more reported incidences of non-O157 illnesses, but illnesses caused by O157:H7 are generally considered to be more severe (Gould 2013).

STEC generally inhabit domestic and wild animals, particularly cattle or other animals that are frequently exposed to fecal matter (Moxley 2015, Baker 2016). These animals naturally harbor STEC in their intestinal tracts, due to constant exposure, and commonly remain asymptomatic (Persad 2015). The hide, skin, and carcass serve as vectors for STEC and can lead to easy transmission between animals and onto raw meat (Moxley 2015, Baker 2016). Poor management practices can encourage STEC presence in animal herds by means of contaminated drinking water, ground water contamination, or over-crowded pins (Persad 2015). STEC are most commonly transmitted to meat used for human consumption during slaughter and fabrication. For this reason, sanitation plays a key role in post-processing contamination (Moxley 2015). Most contamination occurs during hide removal and is usually unavoidable (Dickson 1992).

After contamination occurs during slaughter and processing, sanitation becomes extremely important, but increasingly difficult. Often, sprays designed to apply an antimicrobial chemical or heat treatment to carcass surfaces are applied as interventions at various points throughout processing to reduce the potential for contamination on the carcass (Dickson 1991). The fabrication process also provides several opportunities for intervention. All interventions must only be used within regulatory limits of food grade chemicals (FSIS 2017). When choosing

an antimicrobial treatment, careful consideration of the microorganisms' properties is required. Gram-negative bacteria, for example, have an outer membrane made of lipopolysaccharides, making them more resistant to hydrophilic antimicrobials (Davidson 2005). Generally, a chemical is chosen based on its interaction with the cell membrane, which controls much of the cell's metabolic function. The antimicrobial interventions chosen are often organic acids, as they are bactericidal at appropriate concentrations (Dickson 1992). Many organic acids interfere with cell membrane reactions and prevent growth and metabolism, which can ultimately cause cell death (Hardin 1995). Currently, the most commonly used antimicrobials in beef processing are lactic acid and peracetic acid, also known as peroxyacetic acid. Less commonly used sprays may include acetic acid, sulfuric acid, lauric arginate, or a combination of several organic acids.

Importance of Research

Social Significance

Though largely underreported, the Centers for Disease Control and Prevention (CDC) estimates approximately 48 million cases of domestically acquired foodborne illnesses each year, some of which lead to hospitalizations and death (CDC 2016). Further, *Escherichia coli* O157:H7 is the fifth largest contributing pathogen to hospitalizations due to foodborne illness (Scallan 2011). From 1982-2002, ground beef was the most common vehicle for foodborne O157:H7 infections (Figure 2.1). Other food commodities were also implicated in O157:H7-related infections, but to a smaller degree (Rangel 2005). A more recent study implicated beef with 13% of all bacterial foodborne illnesses from 1998-2008 (Painter 2013). From an economic standpoint, CDC estimated that each fatality from an infection related to O157:H7 results in costs of approximately $7 million, as a fatal infection is likely associated with hospitalization,

medical bills, insurance costs, product recalls, and possibly lawsuits that incur court fees (CDC 2016).

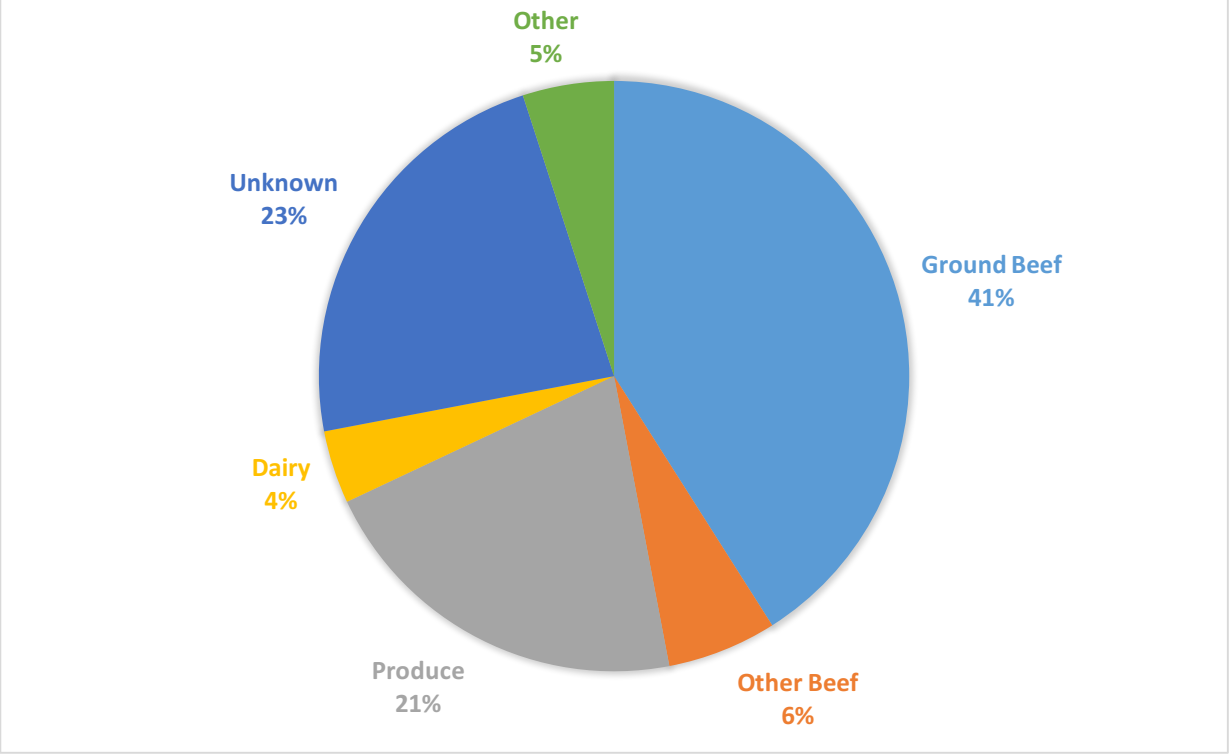

Figure 2.1. Food commodities implicated in *E. coli* O157:H7 infections between 1982-2002 (Adapted from Rangel 2005).

Infections usually occur when humans consume animal or vegetable products that have been contaminated with feces originating from cattle or other ruminants (Piérard 2012). People may also contract an infection through person-to-person contact. One such case of this form of transmission occurred when a young boy acquired an infection from another child at a daycare. Tragically, the baby died from kidney failure after being hospitalized for several weeks because the infection led to hemolytic uremic syndrome (HUS) (STOP 2014). When an STEC infection occurs, antibiotics are not recommended as a treatment (Tarr 2005). Some antibiotics are seen to

lyse the bacterial cells and release more Shiga toxin, heightening the probability of the development of HUS (McGannon 2010).

Industry Significance

Due to the massive multistate outbreak associated with Jack in the Box hamburgers in 1993, FSIS declared *E. coli* O157:H7 an adulterant in ground beef in 1994 (FSIS 1999). As a result, processors were expected to prevent contamination of beef with the pathogen during processing and then verify process control with microbiological testing for the pathogen. When a test is positive, producers must recall the entire production lot, sometimes thousands of pounds. The recalled product may be destroyed or sold at a lower cost to be cooked, but the company loses money and the consumer's trust either way. As a result, members of the beef industry filed a lawsuit against USDA in an attempt to stop the required beef testing programs (Stentz 1994). Their defense claimed that declaring O157:H7 an adulterant in ground beef and trim unfairly and impulsively blamed one part of the industry for a component of a raw product that cannot be truly controlled. The suit was eventually overruled, and the new law led beef processors to find intervention strategies, develop cleaner and more sanitary ways to process beef, and implement testing for the pathogen.

Scientific Significance

Prior to the outbreak in 1993 that led to the decision to name O157:H7 and eventually six other STEC as adulterants in raw beef products, a predominance of microbiologically-based research related to beef was conducted to lower prevalence of spoilage microorganisms and select pathogens (*Salmonella*) on beef surfaces (Acuff 1987, Dickson 1991, Dickson & Anderson 1991, Barkate 1993). Following the legal decision, however, there was a shift to focus even more on studies that specifically examined interventions to increase beef safety. The desire to

understand STEC virulence and pathogenicity on a molecular and applied level took the research spotlight. Since then, it is estimated that over $30 million has been devoted to beef safety research, the majority of it concentrating on *E. coli* (Andrews 2013). While over the last two decades there seemed to have been a greater focus on STEC in the beef supply, the studies and interventions often targeted and examined other organisms, as well, such as *Salmonella* (King 2005, Schmidt 2014, Zhao 2014).

STEC Background and Molecular Information

E. coli O157:H7 first officially appeared as an emerging foodborne pathogen when it was identified as the cause of an outbreak in 1982 associated with undercooked ground beef from fast food hamburgers in Oregon and Michigan that sickened 47 individuals. After 1982, O157:H7 was associated with a number of outbreaks over the next decade that, on average, ranged from 2 to 50 illnesses per outbreak (Rangel 2005). However, it did not attract notable attention again until a large multistate outbreak in 1993 in the Pacific Northwest of the United States associated with the Jack in the Box quick service restaurant chain.

E. coli O157:H7 and other STEC, such as O26, O45, O103, O111, O121, and O145 ("Big Six") can carry the Shiga toxin (Stx) gene and produce Shiga toxin 1 and/or 2 (Stx1, Stx2). These Shiga toxin genes are encoded on the chromosome of *Shigella dysenteriae*, which likely inserted itself into the *E. coli* chromosome by means of transduction through a phage (Piérard 2012). Also called a verotoxin, Shiga toxin production is clearly associated with hemolytic uremic syndrome (HUS), though the mechanisms by which this is caused is unclear (Karmali 1985, Piérard 2012). Stx1 or both Stx1 and Stx2 production are not associated with serotypes that cause disease as often as those that only produce Stx2. However, the exact reasoning for this

epidemiological finding has not been fully elucidated (Girardeau 2005). It is known, however, that the cytotoxin that is released attacks Vero cells, leading to kidney failure (Piérard 2012). HUS is a prominent cause of acute renal failure, specifically in children (Kaper 2015).

STEC can be grouped into five different seropathotypes designated A-E based upon their incidence and association with hemolytic colitis, HUS, and outbreaks (Karmali et al. 2003). Seropathotypes A and B consist of O157 and the other "Big Six," respectively, and are characterized as serotypes that have caused outbreaks of hemolytic colitis and HUS (seropathotypes B less commonly associated with outbreaks). Seropathotype C isolates are associated with sporadic HUS cases (EFSA 2013). These two groups are separated based on frequency, as both can be extremely virulent and cause severe disease (Scheutz 2015). Seropathotypes D (associated with diarrhea but no outbreaks or HUS cases) and E (not associated with human disease) are of lesser public health emphasis. In the case of most STEC, their virulence comes from the genetic factor that gives them the ability to produce attaching and effacing (A/E) intestinal lesions (Kaper 2015). These lesions allow colonization on the epithelial tissues of intestines, which can allow the pathogen to cross the mucosal barrier and directly attack internal organs (Piérard 2012). The complexity of STEC phylogeny and virulence, and the resulting human disease severity, was described by Girardeau et al. (2005) and demonstrates the differences associated with different Stx subtypes.

While many strains of *E. coli* are nonpathogenic and can be found widely in the human microbiome, STEC generally inhabit domestic and wild animals. Cattle are most often identified as a natural reservoir, but other ruminants may also harbor STEC, especially those that are frequently in the presence of fecal matter (Moxley 2015, Persad 2015, Baker 2016). These

animals naturally contain STEC in the lower intestinal tract due to constant exposure, and generally remain asymptomatic (Persad 2015).

Animals may be immune to the observable illness and symptoms that humans experience from exposure to STEC, which makes contamination from animals to food products for human consumption even more common and inconspicuous. To mitigate these risks, various food-grade decontamination strategies can be utilized at the processing level, such as organic acids. However, given the right conditions, *E. coli* O157:H7 and some non-O157 serotypes can develop acid resistance (Skandamis 2009, Kim 2015). Some studies show that the microorganism may have the ability to sense the presence of acid and respond more quickly to the stress than others, thus building a tolerance to acidic environments (Rowbury 2001). Other studies indicate that O157:H7 may inherently have more resistance to organic acids than other organisms (Dickson 1991, Hardin 1995). Nevertheless, even without notably increased resistance to popular decontamination strategies, non-O157 STEC are responsible for the majority of reported STEC-related foodborne infections (Smith 2015).

Beef Processing

Areas of Contamination

While in a feedlot, cattle face constant exposure to feces that are likely contaminated with STEC and other pathogens. These organisms can be internalized and colonized in the intestinal tract, yet much of the contamination is located on the hide, hair, hooves, and oral cavity (Keen 2002, Moxley 2015, Baker 2016). This contamination spreads throughout processing, due to the non-aseptic nature of slaughter practices (Dickson 1992). Of no small consequence, sanitation throughout slaughter and processing operations has great impacts on post-processing

contamination (Moxley 2015). There are many strategies to limit STEC contamination of carcasses, such has hide washes, hoof removal, steam vacuuming, trimming, and carcass decontamination using USDA FSIS-approved antimicrobial technologies (FSIS 2017). Figure 2.2 indicates a normal processing sequence, with points of intervention indicated by dotted lines (Dickson & Anderson 1991).

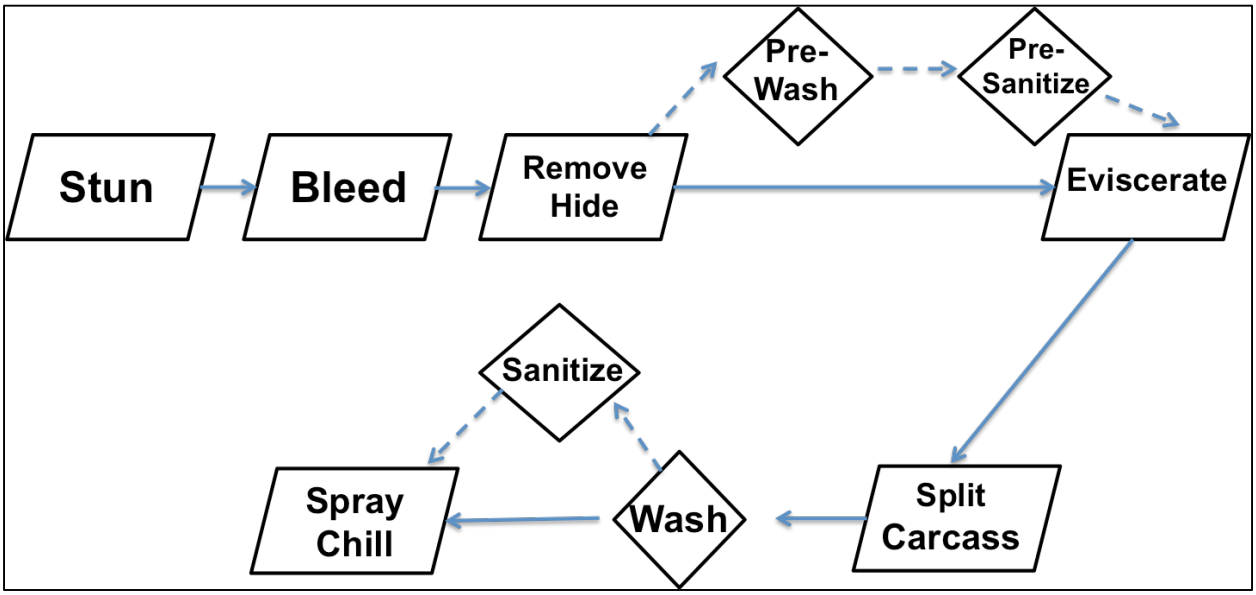

Figure 2.2. Diagram of beef slaughter operations. Solid lines indicate standard procedures. Dashed lines indicate additional procedures (Adapted from Dickson & Anderson 1991).

Wash Interventions

The beef industry has implemented numerous process interventions designed to decontaminate pre-chilled carcasses, such as steam vacuuming to sanitize defined areas of a dressed carcass, and steam pasteurization as a whole-carcass treatment (Castillo 1999, Phebus 1997). Today, the beef industry most commonly applies carcass washes such as hot water and organic acids, or a combination of both, during the slaughter operations. Choosing an acid as a decontamination treatment depends on the objects being sanitized and target organisms. While

an organic acid may provide a significant bacterial reduction and help control contamination, it is unlikely that an organic acid wash would totally remove a pathogen (Yoder 2012). Each acid operates under a specific mode of action and will have varied levels of efficacy (Davison 2005). It is preferable to decontaminate the carcass immediately after slaughter, while the temperature of the carcass is still warm and less time between contamination and decontamination limits bacterial attachment (Acuff 1987). This early intervention additionally reduces the likelihood of subsequent spreading of the contamination after the final wash, during carcass chilling, and throughout fabrication. When implemented appropriately, chemical spray treatments can lessen bacterial populations, both pathogenic and spoilage, thus improving the microbiological quality of the meat (Ransom 2003).

Hot Water

Hot water is a commonplace pre-rigor carcass treatment inside beef processing facilities. Its primary mode of action is to degrade bacteria cellular enzymes and cause the breakdown of DNA and RNA (Wheeler 2014). For most laboratory studies, hot water serves as a control or a standard treatment, as it is found to be very effective. Barkate, et al. (1993) found that spraying water for 10 seconds at 95°C can raise the carcass surface temperature to 82°C, killing exposed bacteria on the surface. A study comparing reductions on beef cheek meat from various solutions found that 10 seconds of immersion in hot water was more effective than lactic acid, peroxyacetic acid, hypobromous acid, a lactic and citric acid mixture, and a buffered sulfuric acid mixture (Schmidt 2014). Another study showed bacterial reductions as high as 4.2 log CFU/cm^2 when carcasses were sprayed with ambient water, followed by a hot water wash that was 95ºC at the source (Castillo 1998). Hot water may also be combined with a subsequent

organic acid treatment, such as lactic or citric acid to enhance bacterial reductions (Scott 2015). While the temperature of the water likely plays a significant role in the reduction, the water pressure undoubtedly provides physical removal of bacteria, as well (Barkate 1993).

Lactic Acid

Lactic acid remains one of the most common chemical interventions used by the beef industry over the last two decades. The organic acid proves useful in bacterial reductions demonstrated on both spoilage and pathogenic organisms, particularly STEC and *Salmonella* (Acuff 1987, King 2005). As with other organic acids, lactic acid has the ability to lower the surface pH of the meat (Hardin 1995). However, the ability of weak organic acids, such as lactic acid, to be bactericidal is more complex than simply acidifying the cellular environment. The weak acids are miscible, meaning they dissociate easily in water. This allows the acid to effectively pass through the cytoplasm. The accumulation of the protons in the cytoplasm force the bacterial cells to devote all energy to attempt to maintain homeostasis, abandoning other metabolic processes. The effects of the weak acid on membrane functionality eventually causes the cell to lyse (Hirshfield 2003). Additionally, in combination with specific concentrations of acetic acid, citric acid, and ascorbic acid, reductions in spoilage bacteria can be enhanced from lactic acid application (Acuff 1987).

FSIS Directive 7120.1 provides a list of approved substances and concentrations that may be used during meat, poultry, and egg product processing. This directive issues a lactic acid concentration limit of 5% (FSIS 2017). Many laboratory studies have examined the efficacy of lactic acid at varying concentrations and temperatures. When small portions of beef carcass tissue were treated with 2% lactic acid that was warmed to 55°C, large reductions of *E. coli*

O157:H7, more than 3 log CFU/cm^2, were observed (Ransom 2003). In another study that used a water wash prior to chilling the carcasses for 24 hours, warmed 4% lactic acid solutions showed reductions of >4.0 log CFU/cm^2 on carcasses inoculated with *Salmonella* and *E. coli* O157:H7, implying that carcass decontamination prior to fabrication, coupled with a warmed lactic acid treatment can provide heightened microbiological safety (Castillo 2001, King 2005).

Some studies indicate that lactic acid is the most effective intervention strategy that is approved for direct use on beef trim (Ransom 2003). However, even greater decontamination of inoculated carcasses can be observed when lactic acid is paired with other interventions, especially hot water washes and trimming (Castillo 2001). Many studies examine a range of application temperatures, with the general consensus being that a higher temperature of acid treatment generally gives greater reductions of bacterial populations (Dickson 1991, Castillo 1998). Even when not warmed, many studies have observed notable efficacy. In an experiment that tested the effects of 1, 2, and 5% lactic acid, Yoder et al. (2012) noted O157:H7 reductions of 2.9, 3.5, and 5.3 log CFU/cm^2, respectively. In a cost analysis that compared the results of these concentrations of lactic acid to water and similar concentrations of acetic acid, the authors determined that 2% lactic acid was the most suitable intervention choice.

Peracetic Acid

Peracetic acid (CH_3CO_3H), also known as peroxyacetic acid, is an organic acid that is much weaker than its counterparts, acetic acid and lactic acid. It is produced by reacting acetic acid with hydrogen peroxide. Rather than using low pH as its main mechanism of action, it oxidizes cell membranes, thus leading to cell destruction and death (Dias-Morse 2014). Peracetic acid serves several different roles within the food industry, serving as a decontaminant of

equipment and produce, as well as meat carcasses and beef trimmings (King 2005). Its use is approved up to 1800 ppm, but it is generally used at concentrations of 200-400 ppm due to hazardous conditions that the chemical poses to employees (FSIS 2017, Wheeler 2014).

While many within the beef industry have increased their uses of low concentrations of the peracetic acid, there is a wide range of antimicrobial results when applied in laboratory studies. Ransom et al. (2003) reported an average 1.0-1.4-log reduction with peracetic acid on inoculated beef tissue, but another study by King et al. (2005) only reported a 0.7-log reduction on hot carcasses. Yoder et al. (2012) designed an experiment to understand the effects of antimicrobial treatments on hot carcasses prior to chilling. They examined the effects of 200 and 1,000 ppm of peroxyacetic acid on O157:H7-inoculated beef plate pieces and they noted the bacterial reductions to be 0.4 and 3.8 log CFU/cm^2, respectively (Yoder 2012). However, when following a water wash, Penney et al. (2007) observed 2.7-3.4-log reductions on prerigor beef carcasses, which is more substantial than reductions shown in the aforementioned studies (Ransom 2003, Yoder 2012, Liao 2015). These differences could be due to the variations in experimental design and different choices for sample tissue types.

Lauric Arginate, Acetic Acid, and Sulfuric Acid

Lauric arginate, another food-grade antimicrobial, acts on the cell in a destructive manner by altering the metabolic processes within the cell due to disruption of the plasma membrane's lipid bilayer, but not causing cell death (Dias-Morse 2014). When paired with acetic acid, however, it is believed to penetrate the cell wall, as well as acidify the cell, leading to cell death. Dias-Morse et al. (2014) indicated that the order of application may affect the outcome. Specifically, it was shown that using an acetic acid solution followed by lauric arginate resulted

in a greater reduction of *Salmonella* on chilled skinless chicken meat than applying lauric arginate followed by acetic acid (Hawkins 2016). Acetic acid, in combination with pre- and post-washing during the spray chill cycle, has been observed to help lower bacterial populations, specifically on fatty tissue (Anand 2016). In one study that investigated interventions for hot carcasses in small beef plants, Yoder et al. (2012) inoculated beef plate pieces with O157:H7 and treated with 5, 2, and 1% acetic acid, which yielded bacterial reductions of 2.42, 5.24, and 2.16 log CFU/cm^2, respectively.

There are many proprietary formulas of chemical mixtures developed for the purposes of decontaminating animal carcasses and subprimals. One such mixture, a buffered sulfuric acid solution used by some beef processors called AFTEC 3000, was used in a validation study by Schmidt et al. (2014). When inoculated chilled beef cheek meat was immersed for 1-5 minutes, they found reductions of *E. coli* O157:H7, non-O157 STEC, and *Salmonella enterica* to be 0.8-1.5 log CFU/cm^2. These reductions were likely a response to the low surface pH of the meat that was observed, but they were less and not significantly different from a 2.5% lactic acid solution (Schmidt 2014). Another study by Geornaras et al. (2012) evaluated AFTEC 3000, which was used at a pH of 1.2. In this study, chilled beef trimmings inoculated with *E. coli* O157:H7 (ca. 3.1 log CFU/cm^2) were immersed in the solution and yielded a 0.3-log CFU/cm^2 reduction (Geornaras 2012). Aftec 3000, a sulfuric acid solution blend now marketed to beef processors as Centron™ (Zoetis, Parsippany, NJ) is approved for use at concentrations that lower the meat surface pH to 1.0-2.1 (FSIS 2017).

Chemically-based carcass treatments can be highly effective, each demonstrating different properties that can be bactericidal. However, microorganisms have the evolutionary capabilities to adapt to different environments and develop acid resistance. Improper sanitation

of equipment may allow bacteria to survive the cleaning, and provide an opportunity to resist future cleanings by means of resistance or biofilm formation (Skandamis 2009). These cells, known as habituated cells, can also form when acid and water runoff mix, diluting the sanitizing powers of the acid (Ricke 2003). Habituated cells may also grow when solutions are used for decontamination by immersion. Over time and due to exposure to organic matter, the solution may lose the ability to fully sanitize the food product, thus allowing pathogens the opportunity to build resistance (Samelis 2005). For this reason, plant sanitation and layout should be carefully considered and executed properly.

Conclusions

STEC as a Focus in the Scientific Community

STEC, like many other foodborne pathogens, are opportunistic pathogens, as they often take advantage of ingestion by an immunocompromised host. Various factors can make an individual immunocompromised or prone to an STEC infection (Paton 1998). Antibiotic use can cause intestinal microbiota to be less competitive, providing an easier path for STEC to colonize and cause infection within the GI tract (Eloe-Fadrosh 2013). Some STEC, such as O157:H7, carry attaching and effacing genes, which allow the bacterium to intimately attach to the epithelial wall and be internalized by host cells, as seen in Figure 2.3 (Mainil 2005). Depending on the severity and nature of the infection, hemolytic uremic syndrome or hemolytic colitis can result from the infection. While it is certain that ruminants play a role in transmitting O157:H7 to humans and infected humans may transmit the infection to other individuals, far less is known about non-O157:H7 strains (Mainil 2005). Further research is needed to discover more about the virulence of these strains and the relationship between the pathogens and the carriers and hosts.

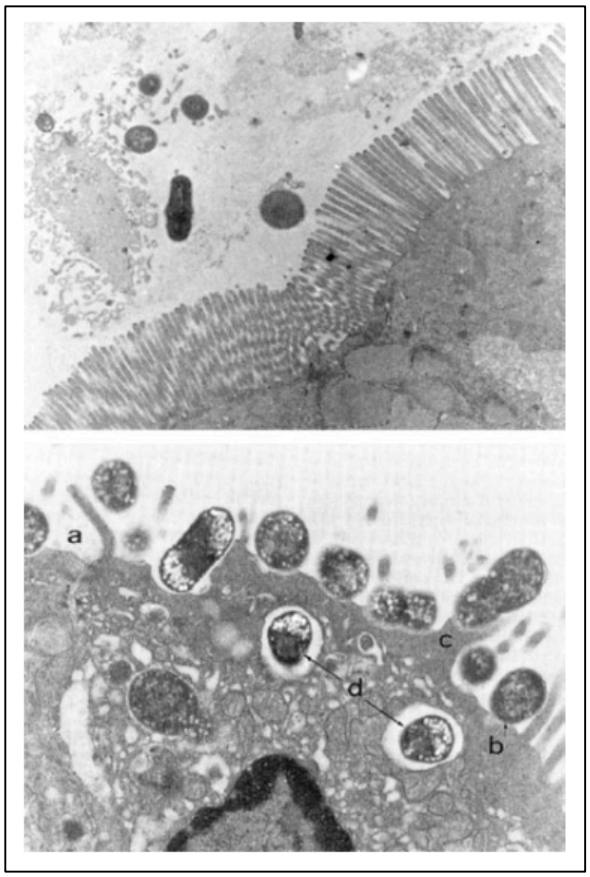

Figure 2.3. Intact intestinal microvilli and attaching/effacing lesions at the electron microscope. (a) Effacement of the microvilli and (b) intimate attachment of the bacteria to the enterocyte; (c) pedestal structure; (d) internalization of bacteria (Adapted from Mainil 2005).

Chosen Antimicrobial Spray Treatments

There are several spray interventions that have become common in beef processing: hot water, lactic acid, and peracetic (or peroxyacetic) acid. Hot water, arguably the most natural and affordable option, generally provides good bacterial reduction, especially at the carcass level (Barkate 1993, Castillo 1998, Schmidt 2014). Hot water as a treatment has the ability to degrade the cells' enzymes and cause the breakdown of DNA and RNA (Wheeler 2014). The temperature and pressure compete in a balancing act, however. A higher pressure can provide physical

removal of contamination, while a lower pressure allows for a higher temperature and longer contact time (Barkate 1993). Each study uses a unique set of parameters to validate washes, which leads to great variation between results. However, several studies target a temperature ca. 95°C, which when sprayed for 10-90 seconds, can raise the carcass temperature and effectively reduce bacteria (Barkate 1993, Castillo 1998). Another variation between studies is the combination of certain treatments. For example, lactic acid is often used to follow a hot water wash, which has been noted to be very effective (Castillo 1998). Table 2.1 describes a comparison of these treatments.

There are great differences between reductions observed from carcass-level treatments and subprimal-level treatments. Subprimals are typically chilled, which has seen to substantially impact the efficacies of treatments. Generally, processors do not spray water on subprimals, and instead spray an antimicrobial. Lactic acid at concentrations of 1-5% have been shown to be generally effective in lowering pathogenic and spoilage bacterial populations on the surface of chilled surfaces (Acuff 1987, Ransom 2003, King 2005, Schmidt 2014). Peracetic acid is also used at various concentrations, usually from 200-400 ppm, and has also been noted to help decontaminate beef tissue surfaces (Ransom 2003, King 2005, Penney 2007).

Table 2.1. Mean log reductions of STEC O157:H7 (log CFU/cm2) on beef cheek meat following immersion in lactic acid for the indicated durations (Adapted from Schmidt et al. 2014).

Adipose surface			Muscle surface		
1 min	2.5 min	5 min	1 min	2.5 min	5 min
1.9	1.9	2.1	1.2	1.6	1.8

Effective Experimental Design

Several inoculation strategies have been used for investigations, depending on the study and goals of the research. One of the more commonly used inoculation protocols is delivery of the inoculum to the meat surface through a liquid, in which a concentrated cocktail is made and diluted with a non-enriching substance (to prevent excessive protection for bacterial cells), and then applied to the meat surface by immersion, spraying, or spreading (Chancey 2013, Liao 2014, Gill 2016). Some prefer this technique, because of practicality and ease. However, other studies have indicated that it may not be fully representative of a true contamination scenario (Dickson 1992). To emulate contamination that likely occurs during slaughter and processing, several studies have shown the effectiveness of using fecal material contaminated with an organism during its stationary phase (Hardin 1995, Dickson 1991). This fecal material is then spread over the surface of the carcass in a uniform manner. Another study dispensed an inoculated, thinned fecal slurry to deliver the chosen pathogens to the meat surface (Yoder 2012).

As with any laboratory experiment, consistency between samples is key—particularly regarding bacterial attachment times after inoculation. Most studies determine desired attachment time based on previous work, but the selected times vary greatly between studies. Some experiments allow 30 minutes of uninterrupted contact time with the beef surface (Ellebracht 1999, Liao 2015). However, shorter times, such as 15 minutes, may also be used (Schmidt 2014). While most studies report attachment times generally range from 15-45 minutes, one study opted for a much longer time, allowing the inoculum to attach to the meat surface under refrigerated conditions overnight (Dias-Morse 2014).

In published reports on the efficacy of spray treatments, it is key to understand the mechanisms of action each spray uses to decontaminate the tissue. Several chemicals and antimicrobials, such as lactic acid and sulfuric acid, are most effective due to their low pH and pKa. Acetic acid, on the other hand, has a slightly higher pH and pKa, as does peracetic acid. Some studies have drawn connections between the surface pH of the meat after treatment and the color or quality of the meat. For the purposes of understanding the acids' effect on the samples, recording surface pH is a prudent experimental strategy reported by many researchers (Hardin 1995, Ellebracht 1999, King 2005, Schmidt 2014).

In this report, a summary of a literature search was presented, highlighting the key concepts of STEC contamination on beef subprimals and antimicrobial treatments to control this contamination. Contamination generally occurs during the slaughter and fabrication, leaving processors the difficult task of sanitizing a beef carcass during production. This attempt usually takes place in the form of a spray intervention, particularly using antimicrobials such as organic acids. These sprays should be challenged and validated to ensure efficacy for purpose under realistic commercial application scenarios.

Through laboratory studies, experimentation can compare various spray treatments. Treatments such as lactic acid, peracetic acid, and hot water are the most common and most supported interventions on beef carcasses and subprimals, so studies to compare these treatments and possible sequence effects or synergism would be extremely helpful to the beef industry.

While there is always value in repeating and replicating studies to confirm results and validity, there are clear 'next steps' to further research in this field. Some studies have shown greater efficacy from certain treatments, but these results all vary depending on the temperature of the meat and temperature, concentration and pressure of the treatment. Therefore, it would be

beneficial to do a validation study on the most popular treatments currently used in processing plants. This will either affirm or contradict the efficiency of interventions. Additionally, a study to examine possible synergy of treatment sequences would be helpful to the beef industry, since various interventions are used at different times throughout processing.

Chapter 3 - Materials and Methods

Overall Experimental Design

All experiments were conducted at the Kansas State University Biosecurity Research Institute, a biosafety level-3 biocontainment laboratory, which has full-scale slaughter and meat fabrication capabilities. All animal slaughter protocols were in compliance with USDA-FSIS standards and were approved by the Institutional Animal Care and Use Committee. Inoculated studies were conducted under an Institutional Biosafety Committee approved protocol, ensuring laboratory personnel safety in a beef processing operation, where encountering infectious aerosols was likely to occur. All laboratory personnel underwent intensive annual biosafety training and relied on personal protective equipment and operational procedures (double gloves, Tyvek suits, powered air purifying respirators, and validated disinfection protocols) to ensure safety.

The effectiveness of three individual antimicrobial spray treatments and water, when applied as single treatments immediately prior to vacuum packaging, against STEC-7 contamination on raw, chilled beef loins was evaluated in Study 1. The experiment was designed as a randomized complete block with three replications completed over a period of four weeks. For each replication, two head of grain-finished cattle were slaughtered in the same morning using USDA FSIS-approved protocols (FSIS 2011). These carcasses received standard ambient and hot water washes, followed by a light organic acid spray, using a commercial grade Chad wash cabinet (Chad Co. Inc., Olathe, KS). Carcasses were transferred to a chilling cooler where they received a sporadic spray chill during the initial six hours, and no spray during an additional 18 hours of chilled storage.

For each of the three replications, the four carcass sides were fabricated and all meat cuts were separated according to carcass side. The loin subprimal from each side was further divided into four pieces of approximately equal size, which were ultimately assigned to one of the four antimicrobial and control treatments (lactic acid, peracetic acid, Centron™, and water). All sixteen pieces for each replication were inoculated with a STEC-7 cocktail and randomly assigned to one of the four treatments; thus, each treatment was applied to four pieces and the average pre- and post-treatment STEC-7 counts were determined for statistical analyses. This design is depicted in Figure 3.1.

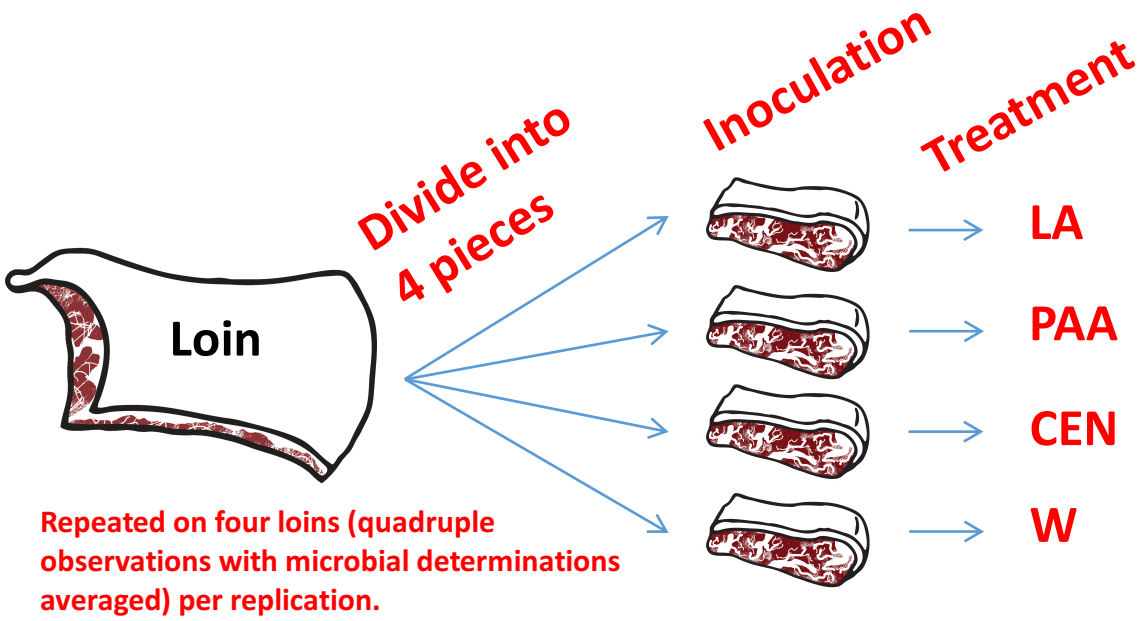

Figure 3.1. Study 1 experimental design. A total of four carcass sides [i.e., four loins with four different spray treatments; water (W), 4.5% lactic acid (LA), 200 ppm peracetic acid (PAA), and 1.1 pH Centron™ (CEN)] per experimental replication (total of three replications) were treated. The average microbial counts from these quadruplicate samples per treatment within replication were determined pre- and post-treatment application to establish STEC-7 reductions.

Study 2 occurred immediately after Study 1 and utilized the same fabricated carcass sides, but used the outside round subprimals. The subprimals were thawed following the

completion of Study 1 and used within one week, and no round had been frozen for more than 28 days. For Study 2, a split plot design was used to evaluate the efficacy of a two-treatment combination of chemical spray treatments incorporating a 72-hour vacuum-packaged storage period between chemical applications. On Day 0, four outside round subprimals were inoculated with the STEC-7 cocktail and treated with one of the four antimicrobial sprays. Following this first treatment, the four outside rounds were separately vacuum packaged and stored for 72 hours at ca. 5ºC. After this storage period, each outside round subprimal was removed from its packaging, divided into four pieces of approximately equal size that were randomly assigned to a second spray treatment, and then spray-treated using the assigned chemical. Thus, per replication, one outside round subprimal was treated with a primary antimicrobial spray or control (LA, PAA, CEN, or W) before vacuum packaging, following by a post-storage secondary spray treatment (LA, PAA, CEN, or W) of subunits cut from the opened outside round. The result of this experimental design yielded one STEC-7 population reduction value for each possible combination of primary and secondary treatment sprays per each of the three experimental replications. The Latin square design used for Study 2 is depicted in Figure 3.2.

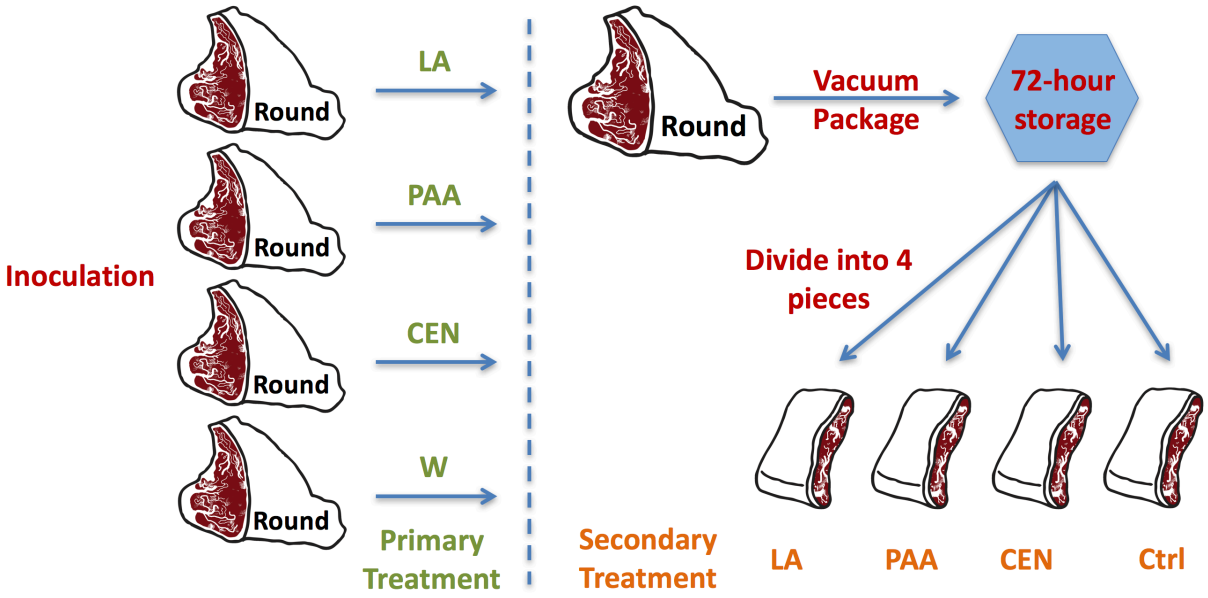

Figure 3.2. Study 2 experimental design per replication (three replications conducted). Four outside round subprimals from four carcass sides were assigned to one of four primary spray treatments; water (W), 4.5% lactic acid (LA), 200 ppm peracetic acid (PAA), and 1.1 pH Centron™ (CEN). Post-vacuum packaged storage, subprimals were opened and secondarily treated with one of the four sprays, thus providing total treatments that represented all possible primary and secondary spray applications. Microbial counts from each treatment combination were determined pre-and post-treatment applications to establish STEC-7 reductions overall, and after each individual processing step.

Bacterial Strains and Inoculum Preparation

For both studies, the inoculum was prepared following a protocol similar to Channaiah et al (2016). Rifampicin-resistant derivatives (100 µg/mL) of STEC cultures (human isolates) representing serogroups O26:H11 (H30); O45:H2 (CDC 96-3285); O103:H2 – 90-3128; O111:H- – JB1-95; O121:H19 – CDC 97-3068; O145:NM – 83-75), commonly referred to as the "Big 6" serogroups, were obtained from Dr. John Luchansky (USDA ARS Eastern Regional Research Center, Wyndmoor, PA) and were stored at -80°C on cryogenic beads in glycerol (Key Scientific, Stamford, TX), and rifampicin-resistant O157:H7 (USDA_FSIS 011-82) was stored in glycerol at -80°C. To activate cultures from frozen beads, a single bead was transferred to 10 mL

of tryptic soy broth (Bacto™, Sparks, MD; TSB) containing 50 µL of rifampicin solution (TSB_{rif}; containing 0.1 g/L rifampicin). To activate O157:H7, a sterile loop was used to transfer 10 µL of the thawed culture into 10 mL of sterile TSB_{rif}. Inoculated tubes of TSB were incubated at 37°C for 18-24 hours. A loopful (10 µL) of each activated culture was then transferred into 10 mL TSB_{rif} and incubated at 37°C for 18-24 hours to create parent strains.

To make a stock solution of rifampicin, 5 g of rifampicin powder (TCI America, Portland, OR) was added to 45 mL of methanol (Fisher Chemical, Fair Lawn, NJ) to create a 500-ppm solution (0.1 g/mL), mixed thoroughly, and filter-sterilized through a 0.22-µm syringe filter (Fisherbrand™). The stock solution was stored in a sealed container at 5-8°C and shielded from light.

Strain Confirmation

Each parent strain utilized in the inocula was biochemically assayed using API 20E strips (bioMérieux, Durham, NC). Strains were streaked for isolation on tryptic soy agar (Difco™, Sparks, MD; TSA), and a single colony was picked and added to sterile vials of 5% NaCl solution (bioMérieux, Durham, NC). Each well of the 20E strips was filled with the inoculated 5% NaCl solution according to directions provided by bioMérieux and then incubated at 37°C for 24 hours. Using apiweb™, each strain was confirmed as *Escherichia coli*.

Upon receipt of the culture set from Dr. John Luchansky at USDA ARS, all cultures were confirmed as to their serogroup designation, and possession of genetic markers for Stx 1 and/or 2 and eae by the laboratory of Dr. T.G. Nagaraja (Kansas State University Department of Diagnostic Medicine and Pathobiology) utilizing an 11-plex polymerase chain reaction assay (Jacob 2012, Shridhar 2016).

Preparation of Working Inoculum

For the first study, a loopful (10 µl) from each parent strain broth culture was transferred separately into a 9-mL tube of TSB_{rif}, incubated at 37°C for 18-24 hours, and then the seven tubes containing individual STEC-7 serogroups were combined with 937 mL of 0.1% peptone water (Bacto™ Peptone, Becton, Dickinson and Company, Sparks, MD) to form the STEC-7 cocktail inoculum containing ca. 7 log CFU/mL. The cocktail was transferred to calibrated 8-oz spray bottles (The Bottle Crew, Farmington Hills, MI) that were used to mist 7 mL of inoculum (ca. 3.5 mL onto the top and bottom surfaces) onto each of the loin's four treatment subunits.

In the second study, the 10-mL tubes of parent strains were combined (a total of 70 mL) and mixed in 930 mL of 0.1% peptone water to prepare the inoculum cocktail, again targeting an inoculum concentration of 7 log CFU/mL. This cocktail was transferred into calibrated spray bottles that were used to mist 30 mL of inoculum onto the surface of each whole outside round subprimal.

Inoculation of Meat Surfaces

Study 1: Each of the four pieces cut from each loin subprimal was placed on a sanitized cafeteria-style tray and placed inside a large biohazard bag. The misting spray bottle with the inoculum cocktail was primed to ensure even sprays by spraying into the biohazard trash until the pumps were even and consistent. The biohazard bag was tented so that it was not touching the meat, while also not allowing spray to exit the opening. Each piece was misted evenly by applying 3.5 mL to the top, using sanitized tongs to flip the meat piece, and applying another 3.5 mL to the bottom surface (delivering a total of 7 mL to the subprimal surface). The tray was carefully removed from the biohazard bag and allowed to sit, undisturbed, for approximately 45

minutes at ca. 10°C to allow bacterial attachment. An inoculation level of ca. 5 log CFU/cm^2 was targeted.

Study 2: Each large outside round subprimal was placed inside a large biohazard bag and was mist-inoculated as described for Study 1, but delivering a total of 30 mL (15 mL each to the top and bottom) to the subprimal's surface (Figure 3.3). The tray was removed and the bag was then closed and tumbled to ensure even distribution of inoculum. Subprimals were aseptically removed from the bag and placed on sanitized trays for approximately 30 minutes at ca. 10°C to allow bacterial attachment. An inoculation level of ca. 5 log CFU/cm^2 was targeted.

Figure 3.3. Inoculation of outside round subprimal for Study 2.

Subprimal Spray Cabinet

The commercial-style subprimal spray cabinet used in these studies was engineered and provided by Zoetis, Inc. (Parsippany, NJ). It was designed to use a conveyor belt delivery system consisting of flexible bands that could support a large subprimal, but spaced enough to allow nozzles positioned beneath to fully spray the subprimal underside (Figure 3.4). Additionally, nozzles above the belt provided upper surface spray coverage. Once inside the cabinet, 24 nozzles (8 above, 8 below, and 4 on each side) sprayed the subprimal at a rate of 0.1 gallons/minute and at 15 psi. The subprimal was sprayed in the cabinet for a total of 11 seconds.

Figure 3.4. Subprimal entering spray cabinet.

The spray cabinet was designed with the capability of spraying any chemical of choice through a pump (Dosatron International Inc., Model HSPK58LB, Clearwater, FL) that was pressurized by flowing water. A hose was connected to a municipal water source, which flowed through a series of valves to regulate the amount of chemical uptake and then mixed with the designated percentage of water. The pump was constructed to provide a spray with an antimicrobial concentration range between 0 and 10% by injecting an adjustable amount of chemical into a mixing chamber where the appropriate amount of water was automatically added.

Chemical Preparation

Three USDA-approved chemical intervention products and a water-only control were used to spray the subprimals and subunit pieces cut from full subprimals. L-lactic acid (4.5% v/v) was prepared from 88% L-lactic acid provided by BIRKO (Henderson, CO) for the first study and by Corbion (Purac®, Lenexa, KS) for the second study. The antimicrobial solution was applied at ambient temperature (ca. 10-15°C). To confirm an accurate lactic acid concentration, a sample aliquot from the spray cabinet was obtained and used to perform the following titration:

1. Add 5 mL of sample solution from the spray cabinet to a clean beaker.
2. Add 50 mL of deionized water to the beaker.
3. Add 5 drops of phenolphthalein indicator solution (ChemWorld, Kennesaw, GA) while swirling the beaker continuously.
4. Titrate using 0.25 N NaOH (BDH, VWR International, LLC, Radnor, PA) until the solution turns pink for more than 10 seconds.

5. Confirm concentration of lactic acid by using the following calculations:

[(0.25 × 0.09008 × mL NaOH) / 5] × 100 = % LA in application solution

Peracetic acid (200 ppm) was prepared from Microtox Plus™ (ZEE Company, Chattanooga, TN) and mixed with tap water. The antimicrobial solution was applied at ambient temperature (ca. 10°C). An aliquot from the spray cabinet was collected and used in a titration to determine the exact ppm concentration. The following procedure was used:

1. Add 5 mL of sample from the spray cabinet to a clean beaker.
2. Add 20 mL of 10% KI (BDH, VWR International, LLC, Radnor, PA) while swirling the beaker continuously.
3. Add 5 drops of 1% starch indicator (BDH, VWR International, LLC, Radnor, PA) to the sample while swirling.
4. Titrate using 0.1 N sodium thiosulfate (Ricca Chemical Company, Arlington, TX) until sample turns from a dark brown color to a light pink color for more than 10 seconds.
5. Confirm concentration of peracetic acid by using the following calculations:

[(0.38 × mL sodium thiosulfate) / 5] × 10,000 = ppm PAA in solution

Centron™ was provided by Zoetis, Inc (Florham Park, NJ) and was prepared by mixing with tap water and collecting a sample to confirm that the solution pH was between 1.0-1.2 using an ExStik® Waterproof pH meter (Model Number PH100, Extech Instruments, Waltham, MA). The antimicrobial solution was applied at ambient temperature (ca. 10°C)

Study 1 – Single Antimicrobial Application Evaluation on Chilled Loin Pieces

The 16 loin pieces per replication were inoculated with the STEC-7 cocktail according to the procedure described above. After attachment for approximately 45 minutes, each subprimal piece was sampled using a sterile corer (5.2 cm diameter; 21.13 cm^2). One core from both the top and the bottom of each subprimal piece was aseptically excised and combined (total inoculated surface area of 42.25cm^2) in a sterile Whirl-Pak™ sample bag (Nasco®, Fort Atkinson, WI) filter bag containing 75 mL of D/E Neutralizing Broth (Difco™, Becton and Dickinson Company, Sparks, MD) supplemented with 0.375 mL of rifampicin stock solution. Sample bags were placed in an insulated cooler for transport to the analytical laboratory on site. The subprimal pieces were then sent through the conveyer spray cabinet (Zoetis, Parsippany, NJ) and sprayed with one of four treatments: tap water (W), 4.5% lactic acid (88% BIRKO concentrate; LA), 200 ppm peracetic acid (Microtox Plus™; PAA), or pH 1.1 Centron™ sulfuric acid/sodium sulfate blend (Zoetis; CEN). After a five-minute post-spray drip time, core surface excision samples were obtained and analyzed in the same manner as described above (Figure 3.5).

Figure 3.5. Core surface excision sampling of loin pieces for Study 1.

Study 2 – Combination Antimicrobial Treatments

After allowing the STEC-7 cocktail inoculum to attach to subprimal surfaces for 30 minutes, pre-treatment samples were taken by using a sterile corer (3.9 cm diameter; 11.95 cm^2 inoculated surface area). A composite sample of four cored surface areas was created per subprimal by combining two cores from both the top and from the bottom of the subprimal. This sample, representing a total inoculated surface area of 47.80 cm^2, was placed in sample bag with 75 mL of DE Broth supplemented 0.375 mL of rifampicin stock solution. The surface pH of each subprimal was also recorded using a surface-reading meter (Extech Instruments, Waltham, MA).

The four subprimals were sent through the spray cabinet, each receiving a different treatment (W, LA, PAA, or CEN) as described previously. The conveyer belt was sanitized and

the system was completely cleared of all chemical between each treatment. Immediately after the treatment (within one minute of leaving the spray cabinet), the pH of the subprimal surface was recorded. After five minutes, composite samples formed from two cores from both the top and the bottom of the subprimal, again representing a total inoculated surface area of 47.80 cm^2, were taken as post-treatment samples. Each of the four treated subprimals were then vacuum packaged and stored in a dark walk-in cooler for 72 hours at 5°C.

After three days of storage at 5°C, each subprimal was aseptically removed from its bag and divided into four pieces for subsequent secondary spraying using the same four chemical treatments. This provided a total of 16 pieces across all four outside rounds. Upon opening the vacuum bags, pre-treatment tissue surface samples were obtained from each piece using a sterile 3.9-cm diameter core (11.95 cm^2). Two cores were taken from both the top and the bottom (total inoculated surface area of 47.80 cm^2) and placed in a bag with 75 mL of DE broth supplemented with 0.375 mL of rifampicin stock solution. The surface pH was also measured and recorded for each piece as previously described. The four pieces from each subprimal were assigned one of the four chemical spray treatments, using a split-plot experimental design. Following treatment of the pieces, the surface pH of the meat was immediately recorded (within one minute of leaving the spray cabinet) and the spray cabinet and conveyer belt were sanitized. After a five-minute drip time, post-treatment samples were obtained in the same manner as those prior to treatment (Figure 3.6). All collected samples contained in Whirl-Pak™ sample bags (Nasco®, Fort Atkinson, WI) were placed into an insulated cooler and transported to the on-site laboratory for immediate analysis.

Figure 3.6. Sampling outside round subprimal for Study 2.

Bacterial Enumeration

To confirm the STEC-7 population level in the inoculum cocktail that was applied to the subprimals, serial dilutions of the 24-hour cocktail mix were plated in duplicate on Petrifilm™ *E. coli* / Coliform (ECC) plates (3M™ Corporation, St. Paul, MN) using 0.1% peptone water diluent supplemented with rifampicin stock solution to achieve a 0.1 g/L concentration (45 µl of rifampicin stock solution per 9 mL peptone water; PW_{rif}). Each excised tissue sample in DE broth supplemented with rifampicin stock solution (0.1 g/L) was serially diluted using rifampicin-supplemented PW_{rif} and plated in duplicate on Petrifilm™ ECC. The plates were incubated at 37°C for 24 hours. Following incubation, colonies were counted according to manufacturer's instructions and were consistent with pure culture colony morphologies. Viable population levels were calculated and then transformed logarithmically to determine STEC-7

population reductions due to spray treatments (log CFU/cm^2 pre-treatment minus log CFU/cm^2 post-treatment).

Statistical Design and Analysis

Study 1

Study 1 was designed as generalized randomized complete block with 3 blocks (Replication), with each treatment replicated four times within each block with the exception for Centron™ (all replicates for Centron™ were lost in Block 2 due to equipment malfunction). A linear mixed model was fit to the data in SAS Proc MIXED, where the response variable for each experimental unit was the difference in log CFU (Pre-Treatment – Post-Treatment) with Treatment as a fixed effect and Block and Block-by-Treatment interaction as random effects. The Kenward-Roger adjustment to the denominator degrees of freedom (DDFM = KR) was used to account for the unbalance due to the loss of Centron™ observations in Block 2.

Study 2

For the Day 0 analysis, the response variable was the difference in log CFU (Pre-Treatment – Post-Treatment). The design was assumed to be a randomized complete block design with 3 blocks (Replication) of size 4 (number of treatments). Initial analyses indicated unequal variances among the 4 treatment groups. Graphical diagnostics indicated the treatment could be divided into two groups. LA and PAA were in one group, and Water and Centron™ were in another group. A linear mixed model was fit in SAS Proc MIXED with Treatment as a

fixed effect and Block as a random effect. The Kenward-Roger adjustment to the denominator degrees of freedom (DDFM = KR) was used to account for the unequal variances.

For the analysis of storage effect, a linear mixed model was fit in SAS Proc MIXED with Treatment 1 as a fixed effect, and Block and Block-by-Treatment 1 interaction as random effects. Denominator degrees of freedom were adjusted using the DDFM = KR option.

For the analysis of the Day 3 treatment, a split-plot design was chosen and the log reductions were fit to a linear mixed model using SAS Proc MIXED with Treatment 1, Treatment 2, and Treatment 1 by Treatment 2 interaction as fixed effects, and Block and Block-by-Treatment 1 modeled as random effects. Denominator degrees of freedom were adjusted using the DDFM = KR option.

To analyze the efficacy of the secondary wash without regard to the treatment on Day 0, the primary wash (Treatment 1) was treated as a blocking factor. A linear mixed model was fit in SAS Proc MIXED with Treatment 2 (secondary wash) as a fixed effect, and Block and Treatment 1 nested with Block as a random effect. Denominator degrees of freedom were adjusted using the DDFM = KR option.

For the analysis of the total log reductions, a split-plot design was used. A linear mixed model was fit using SAS Proc MIXED with Treatment 1, Treatment 2, and Treatment 1-by-Treatment 2 interaction as fixed effects. Block and Block-by-Treatment 1 were modeled as random effects, and denominator degrees of freedom were adjusted using the DDFM = KR option.

The Day 0 pH levels were analyzed as a randomized complete block design (RCBD) with repeated measures. A linear mixed model was fit in SAS Proc MIXED with Treatment 1, Time, and Treatment 1-by-Time interaction as fixed effects, Block as a random effect, and Time as the

repeated measure. Heterogeneous compound symmetry was selected to model the covariance between the repeated measures based on AIC (Akaike's Information Criterion). Denominator degrees of freedom were adjusted using the DDFM = KR option.

The Day 3 pH levels were analyzed as a split-plot design with repeated measures on the subplot units. A linear mixed model was fit in SAS Proc MIXED with Treatment 1, Treatment 2, Time and all 2-way and 3-way interactions as fixed effects, Block and Block-by-Treatment 1 as random effects, and Time as the repeated measure. Heterogeneous compound symmetry was selected to model the covariance between the repeated measures based on AIC. Denominator degrees of freedom were adjusted using the DDFM = KR option.

Significance of treatments was analyzed at $P \leq 0.5$.

Chapter 4 - Results

Study 1 – Evaluation of Individual Treatments

To evaluate the efficacies of individual antimicrobial treatments, a generalized randomized complete block design was chosen with four independent replicates for each treatment group in each block. However, due to an equipment malfunction, only two blocks contained observations for CEN, making the dataset unbalanced. For this reason, the experiment was analyzed as a generalized randomized block design using a linear mixed model with the treatments as a fixed effect and blocks as a random effect. The statistical analysis was performed using SAS Proc MIXED (SAS version 9.4, SAS Institute, Cary, NC) with denominator degrees of freedom adjusted using the Kenward-Roger method. Statistically, there was an overall treatment effect ($P = 0.003$).

After inoculation, the least square means of the logarithmic counts indicated that on average 5.1-5.3 log CFU/cm^2 (SE = 0.057-0.070) were retained on the subprimal pieces after attachment. Following treatment, it was shown that LA, CEN, and PAA had mean logarithmic reductions of 0.5, 0.4, and 0.6 log CFU/cm^2, respectively (Table 4.1). The control, W, induced a 0.2-log CFU/cm reduction, though the p-value was insignificant, indicating that W was not a conclusively better treatment than no treatment ($P = 0.056$). These treatments did not differ from one another statistically, but PAA and LA were statistically different from water ($P = 0.007$ and 0.0031, respectively). However, CEN and W did not differ significantly ($P = 0.149$). These comparisons are graphically represented in Figure 4.1. Statistically different values are denoted by uppercase letters that are not the same.

Table 4.1. Least squares means of STEC reductions (log CFU/cm^2) on subprimal pieces following antimicrobial treatment for Study 1.

	LA	CEN	PAA	W
Rep 1	0.6	0.3	0.6	-0.2
Rep 2	0.6	N/A	0.6	0.3
Rep 3	0.4	0.5	0.6	0.2
LS Means	0.5	0.4	0.6	0.2
SE	0.08	0.10	0.08	0.08

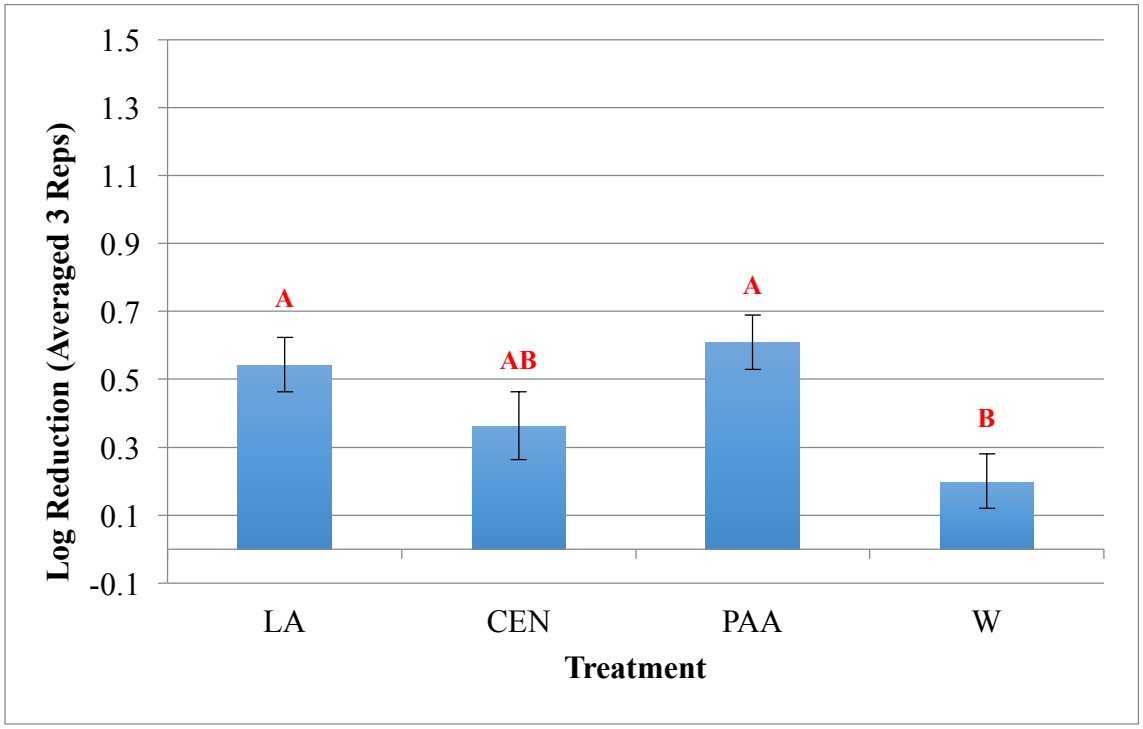

Figure 4.1. Least squares means of STEC reductions (log CFU/cm^2) on subprimal pieces following antimicrobial treatments for Study 1. Statistical differences are noted by dissimilar uppercase letters.

Study 2 – Evaluation of Combined Treatments

Study 2 was analyzed in several different steps corresponding to the stepwise execution of the experiment. The first portion of the experiment, Day 0, was treated as a randomized complete block design with the large subprimal as the experimental unit and the response was the reduction in logarithmic bacterial counts. The statistical analysis showed there was an overall

treatment effect ($P = 0.039$) despite the low power because of the small sample size (n = 16). Preliminary analysis indicated that the variances for the CEN and W treatment groups were similar but much larger than the variances for the LA and PAA groups. To account for this, the data was analyzed with two variances for residual error, one for CEN and W and a second for LA and PAA. The only statistically significant pairwise difference was for LA and PAA ($P < 0.038$). This can be partially explained by the fact that the variance for LA and PAA was much smaller than the variance of CEN and W. The larger variance for CEN and W hinders the ability to detect differences between those treatment groups.

Following the treatment on Day 0, the subprimals were vacuum packaged and stored for 72 hours. The bacterial reductions between the post-treatment samples on Day 0 and the pre-treatment samples on Day 3 indicate whether there was continued reduction during storage. This data was analyzed as a randomized complete block design, and there was no observed treatment effect ($P = 0.132$). However, examination of the least square means show numeric differences between the subprimals following storage. LA, PAA, CEN, and W showed average reductions of 0.6, 0.4, 0.4, and 0.8 log CFU/cm^2 with a cumulative standard error of 0.13 log CFU/cm^2, respectively. This variability could be due to randomness or the few number of samples.

Because the samples on Day 3 had received treatments on Day 0 and there was an overall effect due to Day 0 treatment, the Day 3 analysis included both the Day 0 and Day 3 treatments. Therefore, the Day 3 dataset was analyzed as a split-plot design where the whole plots were organized in a randomized complete block design with the Day 0 treatment as the whole-plot factor and the Day 3 treatment as the subplot factor. The Day 3 logarithmic counts were analyzed in SAS Proc MIXED in a linear mixed model with the fixed effects Treatment 1, Treatment 2 and Treatment 1 by Treatment 2, and the random effects of Block and Block by Treatment 1.

The Day 3 pre-treatment logarithmic counts (CFU/cm^2) were first analyzed as a check on the randomization and no significant effects were observed for Treatment 1 ($P = 0.212$), Treatment 2 ($P = 0.854$) or their interaction ($P = 0.756$), thus showing that the previous treatment on Day 0 and the storage did not bias the initial bacterial counts on Day 3. Analysis on the logarithmic reductions resulting from treatment on Day 3 showed average logarithmic reductions ranging from -0.4 to 0.5 log CFU/cm^2 (Table 4.2). However, the large standard errors (0.26 log CFU/cm^2) make it difficult to detect differences between the various treatments. No significant effects were observed for Treatment 1 ($P = 0.878$), Treatment 2 ($P = 0.178$) or their interaction ($P = 0.714$).

Table 4.2. Least squares means of STEC reductions (log CFU/cm^2) on subprimals following Day 0 treatment and on subprimal pieces following Day 3 treatment.

Day 0 Reductions (log CFU/cm^2 ± Std. Error)		**Day 3 Reductions** (log CFU/cm^2 ± 0.26 Cumulative Std. Error)			
	Treatment	**LA**	**Cen**	**PAA**	**Water**
0.3 ± 0.03	**LA**	0.4	0.1	0.3	0.2
0.2 ± 0.30	**Cen**	0.2	0.5	0.1	-0.1
0.6 ± 0.03	**PAA**	0.5	0.2	-0.1	0.1
0.0 ± 0.30	**W**	0.4	0.1	0.2	-0.4

Because there was no effect from the Day 0 treatment on the Day 3 pre-treatment samples, the Day 3 average logarithmic reductions were also analyzed as a randomized complete block design where treatment administered on Day 0 was treated as a blocking factor. The least square means reported logarithmic reductions ranging from -0.03 to 0.4 log CFU/cm^2, with a standard error of 0.12 CFU/cm^2 (Table 4.3). Treatments were not significantly different from one another except for W and LA ($P = 0.022$).

Table 4.3. Least squares means of STEC reductions (log CFU/cm^2) on subprimals following treatment on Day 3 without consideration of Day 0 treatment.

Treatment	Log Reduction (CFU/cm^2)
LA	0.4 ± 0.12
CEN	0.2 ± 0.12
PAA	0.1 ± 0.12
Water	0.0 ± 0.12

To understand the impact of the combination of two treatments and 72-hour vacuum package storage, the mean bacterial reductions from the initial starting levels prior to the first treatment were compared to the final mean bacterial levels after the treatment on Day 3. This experiment was also analyzed as a split plot design. Overall, the least square means that show the average logarithmic bacterial reductions indicate a microbiologically significant effect from the treatments (Table 4.4). LA followed by LA showed the largest overall reduction (1.5 log CFU/cm^2, $P < 0.0001$), closely followed by PAA/LA, LA/PAA, and CEN/CEN (1.3 log CFU/cm^2, $P < 0.0001$). CEN/PAA and W/W yielded the lowest reductions of 0.5 and 0.6 log CFU/cm^2, respectively ($P = 0.028$ and 0.007). Despite these seemingly adequate bacterial reductions, there was not a statistically significant effect for Treatment 1 ($P = 0.215$), Treatment 2 ($P = 0.167$) or their interaction ($P = 0.706$), which is likely due to the large standard errors of the least squares means (0.257 log CFU/cm^2).

Table 4.4. Least squares means of total STEC reductions (log CFU/cm^2) over Study 2. Reductions reflect samples taken prior to Day 0 treatment and following Day 3 treatment.

Day 0 Treatment	Day 3 Treatment	Reduction (log CFU/cm^2)
LA	LA	1.5 ± 0.3
LA	CEN	1.0 ± 0.3
LA	PAA	1.3 ± 0.3
LA	W	1.0 ± 0.3
CEN	LA	0.8 ± 0.3
CEN	CEN	1.3 ± 0.3
CEN	PAA	0.6 ± 0.3
CEN	W	0.5 ± 0.3
PAA	LA	1.3 ± 0.3
PAA	CEN	1.2 ± 0.3
PAA	PAA	1.1 ± 0.3
PAA	W	1.2 ± 0.3
W	LA	1.2 ± 0.3
W	CEN	1.0 ± 0.3
W	PAA	0.9 ± 0.3
W	W	0.7 ± 0.3

Throughout the treatment phases in the combination study, surface pH of the subprimals was obtained and recorded. The pH values were analyzed in a linear mixed model using SAS Proc MIXED with the fixed effects of Treatment 1, Treatment 2, sampling time (pre- and post-treatment) and their 2-way and 3-way interactions as, and the random effects of Block and Block by Treatment 1. Sampling time was modeled as a repeated measure and a heterogeneous compound symmetry covariance structure was selected over compound symmetry based on AIC values. Prior to treatment on Day 0, the subprimals were generally between pH 5.2-5.8 (Table 4.5). Following treatment of CEN and LA, the pH dramatically dropped to an average of 1.4 and 2.7, respectively ($P < 0.0001$). After the PAA treatment, the pH dropped from 5.8 to 5.2 ($P = 0.005$); and after spraying with water, the difference in pH was not significant ($P = 0.146$).

Table 4.5. Comparisons of surface pH for Day 0 pre- and post-treatment.

Treatment	Pre-treatment pH	Post-treatment pH
LA	5.7	2.7
CEN	5.2	1.4
PAA	5.8	5.2
Water	5.5	5.8

The same analysis was conducted for Day 3 pH values, and similar results were recorded. Prior to the treatment on Day 3, the surface pH ranged from 5.1 to 6.0 (Table 4.6). Following treatment of CEN and LA, the surface pH dropped to an average of 1.7 and 3.0, respectively ($P < 0.0001$). Prior to treatment with PAA, the average surface pH of the subprimals was 5.9 and following treatment, the pH dropped to 5.4 ($P = 0.0002$). The W treatment caused the subprimal surface pH to drop from 6.0 to 5.9, on average. This difference was not significant ($P = 0.880$).

Table 4.6. Comparisons of surface pH for Day 3 pre- and post-treatment.

Treatment	Pre-treatment pH	Post-treatment pH
LA	5.1	3.1
CEN	5.2	1.7
PAA	5.9	5.4
W	6.0	5.8

The pH observations between the two days were also compared to detect any interaction between the two treatments with respect to time. No such interaction was noted ($P = 0.102$).

Chapter 5 - Discussion

Throughout fabrication of beef carcasses, meat is handled and trimmed by many people and various tools, thus creating many opportunities for contamination of beef subprimal cuts. For this reason, subprimal interventions can be very valuable to processors. However, some studies, including the current study, indicate that high bacterial reductions are difficult to achieve when the meat is chilled. One study suggested that less than an average of 1-log reduction is achieved when the treated meat is 4°C, but average reductions increase with the meat temperature (Zhao et al. 2014). Furthermore, it is possible that bacteria attach more easily to cold meat surfaces as opposed to warmed meat surfaces, thus making antimicrobial treatments on chilled meat surfaces less effective (Kirsch et al. 2014). The low reductions observed in the current study could likely be due to the colder meat surface, which was used to emulate commonly followed beef industry practices.

The treatments used in the current study were not heated, but some studies indicate larger STEC reductions on meat surfaces when using heated treatments (Castillo 2001, King 2005, Ransom 2003). The current study sought to mimic common interventions used by processors. Due to efficiency and perceived negative effects of warmed antimicrobials on surface color and texture, many processors prefer the use of cold, low-concentration antimicrobials. Still, studies often note the better efficacies of antimicrobials such as lactic acid and peracetic acid, which was also marginally observed in the current study.

Answering Research Questions

Are particular antimicrobials more successful in reducing STEC on the surface of chilled subprimal cuts than others or water?

Following analysis of Study 1 results, it was clear that certain antimicrobial spray treatments are more successful at reducing STEC on the surface of chilled subprimal cuts than water due to the overall treatment effect. Logarithmic bacterial reductions showed that both LA and PAA were significantly different from the control (W). However, no antimicrobial chemical treatment was significantly different from another. While these statistical analyses highlight the distinct and indistinct differences in the treatments, the microbiological results are not as clear. CEN, LA, and PAA only reduced surface bacterial levels by 0.4, 0.5, and 0.6 log CFU/cm^2. These reductions are minimal in microbiological terms, but they do reflect better success than W, which only reduced surface STEC levels by 0.2 log CFU/cm^2.

When paired with a 72-hour vacuum packaged storage, are certain combinations of treatments more successful than others in reducing STEC on the surface of chilled subprimals?

Study 2 yielded several different analyses, but all pointed to the conclusion that a certain combination of treatments, when paired with vacuum packaged storage, did not generate better STEC reductions than another combination. While there was no statistical difference between the treatment combinations, there was a microbiological difference between the treatment combination (LA/LA) that delivered the largest logarithmic reduction (1.5 log CFU/cm^2) and the treatment combination (CEN/W) that delivered the smallest logarithmic reduction (0.5 log

CFU/cm^2). However, there were large standard errors, which likely occurred due to the small sample size and blurred any definitive statement regarding these combinations, thus lessening any statistical significance. Still, another study indicated greater bacterial reductions when multiple treatments are used in a multi-hurdle approach (Pittman et al. 2012).

Study 2 also yielded conclusions regarding the pH of antimicrobials used on the surface. It was established that both LA and CEN significantly lowered the meat surface pH, while PAA and W did not significantly lower the surface pH of the meat tissue. Out of the three antimicrobials used in this study, only CEN application and concentration is determined and measured by pH. In the FSIS Directive of Safe and Suitable Ingredients Used in the Production of Meat, Poultry, and Egg Products, it is stated that Centron™ should not lower the surface of the meat to less than 1.0 pH (FSIS 2017). While this directive is clear, it should be mentioned that during this study, it was observed that the pH of the surface of the meat could only be accurately measured within one minute of leaving the spray cabinet. Following this timeframe, the acid seems to buffer out and the surface pH dramatically increases. If processors should choose to use this chemical, it is recommended that the pH be measured on the surface of the meat within one minute of spraying to confirm chemical concentration.

Does vacuum-packaged storage influence STEC populations of subprimals?

While there was no treatment effect observed from the bacterial reductions during the vacuum packaged storage time, there were differences between the least square means. The greatest average reduction (0.8 log CFU/cm^2) resulted from the subprimals that were treated with W, while the average lowest reductions were from those treated with CEN and PAA (0.4 log CFU/cm^2). The standard error for all means was 0.1 log CFU/cm^2, which lessened the ability of

the statistical analysis to note differences in the reductions. Nevertheless, the lack of treatment effect indicated that this study did not observe a significant effect of vacuum package storage on STEC populations of the subprimals.

Further research

The current study generated several answers to research questions, but also led to many more questions that should be addressed in future research. Based on the statistical results, it behooves researchers to repeat certain areas of the current study with greater power. The large standard errors and unequal variances in several parts of the study indicate that a larger sample size would give more conclusive results. While a great benefit to the current study was the freshness of the subprimal cuts, the number of cuts available was a limiting factor. A study that was not limited to such a small size would have greater statistical power.

The chemical treatments used in the current study were prepared using chilled tap water in a refrigerated room. It is possible that the lower temperatures of the spray lowered their efficacies. Several studies show greater bacterial reductions when certain antimicrobials are warmed or heated (Bracket et al. 1993, Heller et al. 2007). Future work should compare the heating of specific treatments to assess if the heat has greater antimicrobial abilities. There are many other antimicrobial sprays that are currently used or being considered for use by the beef industry, such as Citrilow™ or lauric arginate. These antimicrobials should also be validated against one another to prevent use of a chemical that is not truly effective on a chilled beef subprimal.

Because vacuum packaging of subprimals is largely practiced in the beef industry, further work should be conducted to fully elucidate reductions that may be related to the storage time

and conditions. In one study conducted by Liao et al, bacterial reductions of 2 and 1 log CFU/50cm^2 were observed on high- and low-level inoculated subprimals that were vacuum packaged and stored for 14 days (2015). Another study also indicated that storage had an effect on bacterial reductions (Wolf et al. 2012). A study should be designed to specifically analyze correlation between storage conditions and reductions of STEC on beef subprimals.

Prior to conducting the current study, parameters of the spraying cabinet were established. However, it would be beneficial to conduct research that evaluates the consistency and efficacy of the spray cabinet. Other studies also used cabinets that were designed to spray subprimal cuts with similar parameters as those used in the current study, but some studies use garden sprayers or other apparatuses to deliver the antimicrobial (Heller et al. 2007, Pittman et al. 2012). Moreover, some studies applied the antimicrobial by dipping (Ellebracht et al. 1999, Wolf et al. 2012). A comparative study between the spray cabinet used in the current study and a garden sprayer or dipping application would help processors know which parameters to prioritize when designing interventions.

Another aspect of antimicrobial intervention studies that was not investigated in the current studies was injury-recovery of bacterial cells. Because all media used was selective, no assertions regarding injured cells could be made. A protocol that compares the reductions observed on selective media to nonselective media, such as Aerobic Plate Count Petrifilm™, would allow investigators to ascertain if the antimicrobials have sub-lethal effects on pathogens, as seen in Schmidt et al. (2014).

Review of Significance

While contamination of beef generally occurs at the carcass level, where interventions are proven very effective, it remains necessary that subprimals and non-intact beef be decontaminated, as well. The fabrication process can lead to the spread of pathogens, such as STEC, which can be internalized if the meat is further processed or mechanically tenderized (Heller et al. 2007, Liao et al. 2015).

It is well known that STEC do not cause the greatest number of foodborne illness in the United States each year. However, STEC pose a great public health risk because of the infectious dose and severe symptoms that can result from an infection (Baker et al. 2016). It is believed that as few as 10 to 100 *E. coli* O157:H7 cells can cause illness, thus leaving the beef industry no choice but to be concerned about possible contamination (Feng et al. 2011).

Although contamination of subprimals likely occurs at extremely low levels, the low infectious dose of STEC causes these low levels to be an issue for the beef industry. This study emphasized the need for a multi-hurdle approach to decontaminate beef surfaces, as a single treatment likely does not fully remove STEC from the tissue surfaces.

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Appendix A - SAS Code Used for Statistical Analyses of Data

A.1. Analysis for Study 1: Efficacies of Individual Treatments

```
PROC IMPORT OUT= WORK.one
            DATAFILE= "C:\Users\Chris\Documents\KSU
Consulting\Jennifer Acuff\Acuff_Study 1.xlsx"
            DBMS=xlsx REPLACE;
               Range="Sheet2$A1:E89";
               GETNAMES=Yes;
RUN;

data pre; set one;
  if time="Pre";
  rename logcfu=prelogcfu;
run;

proc sort data=pre;
  by rep trt sampleid;
run;

data post; set one;
  if time="Post";
run;

proc sort data=post;
  by rep trt sampleid;
run;

data reduc;
   merge post pre;
   by rep trt sampleid;
   logreduc=prelogcfu-logcfu;
run;

ods rtf file="C:\Users\Chris\Documents\KSU Consulting\Jennifer
Acuff\output_study1.rtf" style=journal;

Title "Check on the randomization with respect to trt groups";
proc mixed data=pre covtest plots=none;
   class rep trt;
   model prelogcfu=trt/ddfm=KR;
   random rep rep*trt;
   lsmeans trt/pdiff;
run;

Title "Analysis of Trt Effect on Log Reductions";
```

```
proc mixed data=reduc covtest plots=none;
  class rep trt;
  model logreduc=trt/ddfm=kr;
  random rep rep*trt;
  lsmeans trt/pdiff;
run;

ods rtf close;
```

A.2. Analysis for Study 2: Efficacies of Combined Treatments

```
PROC IMPORT OUT= WORK.Day3
            DATAFILE= "C:\Users\Chris\Documents\KSU
Consulting\Jennifer Acuff\Acuff_Study 2.xlsx"
            DBMS=xlsx REPLACE;
               Range="Sheet1$A1:G97";
               GETNAMES=Yes;
RUN;

PROC IMPORT OUT= WORK.Day0
            DATAFILE= "C:\Users\Chris\Documents\KSU
Consulting\Jennifer Acuff\Acuff_Study 2 (Day 0).xlsx"
            DBMS=xlsx REPLACE;
               Range="Sheet1$A1:F25";
               GETNAMES=Yes;
RUN;

ods rtf file="C:\Users\Chris\Documents\KSU Consulting\Jennifer
Acuff\output_study2_v2.rtf" style=journal;

data pre0; set Day0;
  if time="Pre";
  rename logcfu=prelogcfu;
  drop pH;
run;

proc sort data=pre0;
  by rep trt1;
run;

data post0; set Day0;
  if time="Post";
  drop pH;
run;

proc sort data=post0;
  by rep trt1;
run;
```

```
data reduc0;
   merge post0 pre0;
   by rep trt1;
   logreduc=prelogcfu-logcfu;
   vargroup=1;
   if trt1="Cen" or trt1="Control" then vargroup=2;
run;

proc sort data=reduc0;
  by rep vargroup trt1;
run;

Title 'Day 0 Log Reductions';
proc mixed data=reduc0 covtest plots=none;
  class Rep Trt1 vargroup;
  model logreduc=Trt1/ddfm=KR;
  random Rep;
  repeated/group=vargroup;
  lsmeans trt1/pdiff;
run;


Title 'Day 0 pH Analysis';
proc mixed data=Day0 covtest;
  class rep trt1 time sp_id;
  model ph=trt1|time/ddfm=KR;
  random rep;
  repeated time/subject=sp_id type=csh;
  lsmeans trt1 time;
  lsmeans trt1*time/pdiff;
run;


data pre; set Day3;
  if time="Pre";
  rename logcfu=prelogcfu;
  drop pH;
run;

proc sort data=pre;
  by rep trt1 trt2;
run;

data post; set Day3;
  if time="Post";
  drop pH;
run;
```

```
proc sort data=post;
  by rep trt1 trt2;
run;

data reduc;
   merge post pre;
   by rep trt1 trt2;
   logreduc=prelogcfu-logcfu;
run;


Title "Check on the randomization with respect to trt groups --
Day 3 Data Only";
proc mixed data=pre covtest plots=none;
   class rep trt1 trt2;
   model prelogcfu=trt1|trt2/ddfm=KR;
   random rep rep*trt1;
   lsmeans trt1|trt2;
run;


Title "Analysis of Trt Effect on Log Reductions Using Day 3 Data
Only";
proc mixed data=reduc covtest plots=none;
   class rep trt1 trt2;
   model logreduc=trt1|trt2/ddfm=KR;
   random rep rep*trt1;
   lsmeans trt1|trt2/pdiff;
run;

data overall;
  merge pre0 post;
  by rep trt1;
  logreduc=prelogcfu-logcfu;
run;

Title "Analysis of Overall Effect of Two-Stage Wash Trts: Day0
pre vs Day3 post";
proc mixed data=overall covtest plots=none;
  class rep trt1 trt2;
  model logreduc=trt1|trt2/ddfm=KR;
  random rep rep*trt1;
  lsmeans trt1|trt2;
run;

data storage;
  merge post0 pre;
```

```
  by rep trt1;
  logreduc=logcfu-prelogcfu;
run;

Title "Analysis of In-Bag Efficacy of First Wash: Day0 post vs
Day3 pre";
proc mixed data=storage covtest plots=none;
  class rep trt1;
  model logreduc=trt1/ddfm=KR;
  random rep rep*trt1;
  lsmeans trt1/pdiff;
run;

Title "Analysis of Second Wash after Storage: Day3 pre vs Day3
post";
proc mixed data=reduc covtest plots=none;
  class rep trt1 trt2;
  model logreduc=trt2/ddfm=KR;
  random rep trt1(rep);
  lsmeans trt2/pdiff;
run;

ods rtf close;

PROC IMPORT OUT= WORK.one
            DATAFILE= "C:\Users\Chris\Documents\KSU
Consulting\Jennifer Acuff\Acuff_Study 2.xlsx"
            DBMS=xlsx REPLACE;
               Range="Sheet1$A1:G97";
               GETNAMES=Yes;
RUN;

proc sort data=one out=two;
  by rep trt1 trt2 time;
run;


ods rtf file="C:\Users\Chris\Documents\KSU Consulting\Jennifer
Acuff\output_study2_pH.rtf" style=journal;

Title "Analysis of Trt Effects on PH";
proc mixed data=two covtest plots=none;
   class rep trt1 trt2 time sampleid;
   model pH=trt1|trt2|time/ddfm=KR;
   random rep rep*trt1;
   repeated time/subject=sampleid type=csh;
   lsmeans trt1|trt2;
   lsmeans trt1*time trt2*time/pdiff;
```

```
   lsmeans trt1*trt2*time;
run;

ods rtf close;
```

Appendix B - Data for the Experiments

B.1. Raw data for Study 1: Efficacies of Individual Treatments

Rep	Treatment	Sample ID	Time	Log CFU/cm^2
1	LA	1	Pre	5.61
1	LA	1	Post	4.87
1	LA	2	Pre	5.14
1	LA	2	Post	4.70
1	LA	3	Pre	5.44
1	LA	3	Post	4.55
1	LA	4	Pre	4.94
1	LA	4	Post	4.78
1	PAA	5	Pre	5.34
1	PAA	5	Post	4.47
1	PAA	6	Pre	5.32
1	PAA	6	Post	4.53
1	PAA	7	Pre	4.86
1	PAA	7	Post	4.75
1	PAA	8	Pre	5.34
1	PAA	8	Post	4.54
1	Cen	9	Pre	5.38
1	Cen	9	Post	4.86
1	Cen	10	Pre	5.38
1	Cen	10	Post	5.06
1	Cen	11	Pre	4.89
1	Cen	11	Post	4.58
1	Cen	12	Pre	5.08
1	Cen	12	Post	5.20
1	Control	13	Pre	4.87
1	Control	13	Post	4.82
1	Control	14	Pre	5.44
1	Control	14	Post	4.86
1	Control	15	Pre	4.88
1	Control	15	Post	5.08
1	Control	16	Pre	4.61
1	Control	16	Post	5.04
2	LA	17	Pre	5.40
2	LA	17	Post	4.77
2	LA	18	Pre	5.19
2	LA	18	Post	4.41
2	LA	19	Pre	5.17
2	LA	19	Post	4.24
2	LA	20	Pre	5.06

2	LA	20	Post	4.80
2	PAA	21	Pre	5.34
2	PAA	21	Post	4.15
2	PAA	22	Pre	5.14
2	PAA	22	Post	4.91
2	PAA	23	Pre	5.10
2	PAA	23	Post	4.83
2	PAA	24	Pre	5.31
2	PAA	24	Post	4.56
2	Control	25	Pre	4.83
2	Control	25	Post	4.68
2	Control	26	Pre	5.20
2	Control	26	Post	4.52
2	Control	27	Pre	5.03
2	Control	27	Post	4.57
2	Control	28	Pre	5.23
2	Control	28	Post	5.25
3	LA	29	Pre	5.28
3	LA	29	Post	5.08
3	LA	30	Pre	5.41
3	LA	30	Post	4.78
3	LA	31	Pre	5.34
3	LA	31	Post	5.07
3	LA	32	Pre	5.40
3	LA	32	Post	4.81
3	PAA	33	Pre	4.95
3	PAA	33	Post	4.55
3	PAA	34	Pre	5.45
3	PAA	34	Post	4.58
3	PAA	35	Pre	4.95
3	PAA	35	Post	4.53
3	PAA	36	Pre	5.10
3	PAA	36	Post	4.49
3	Cen	37	Pre	5.15
3	Cen	37	Post	4.59
3	Cen	38	Pre	5.23
3	Cen	38	Post	4.70
3	Cen	39	Pre	5.16
3	Cen	39	Post	4.79
3	Cen	40	Pre	5.20
3	Cen	40	Post	4.78
3	Control	41	Pre	5.11
3	Control	41	Post	4.94
3	Control	42	Pre	5.12
3	Control	42	Post	4.90

3	Control	43	Pre	5.27
3	Control	43	Post	4.89
3	Control	44	Pre	5.02
3	Control	44	Post	5.06

B.2. Raw data for Study 2: Efficacies of Combined Treatments; Day 0

Rep	Treatment	Sample ID	Time	Log CFU/cm^2	pH
1	LA	1	Pre	5.30	5.57
1	LA	1	Post	4.91	2.81
1	PAA	2	Pre	5.12	5.63
1	PAA	2	Post	4.52	5.23
1	Cen	3	Pre	4.52	5.62
1	Cen	3	Post	4.89	1.58
1	Control	4	Pre	5.19	5.60
1	Control	4	Post	5.70	5.60
2	LA	5	Pre	5.24	5.80
2	LA	5	Post	4.97	2.55
2	PAA	6	Pre	5.20	5.55
2	PAA	6	Post	4.66	5.04
2	Cen	7	Pre	5.21	5.00
2	Cen	7	Post	4.96	1.48
2	Control	8	Pre	5.28	5.76
2	Control	8	Post	5.08	5.99
3	LA	9	Pre	5.22	5.78
3	LA	9	Post	4.92	2.84
3	PAA	10	Pre	5.25	6.36
3	PAA	10	Post	4.60	5.34
3	Cen	11	Pre	5.08	5.10
3	Cen	11	Post	4.41	1.22
3	Control	12	Pre	5.53	5.23
3	Control	12	Post	5.10	5.81

B.3. Raw data for Study 2: Efficacies of Combined Treatments; Day 3

Rep	1st Treatment	2nd Treatment	Sample ID	Time	Log CFU/cm^2	pH
1	LA	LA	1	Pre	3.67	5.18
1	LA	LA	1	Post	3.64	2.93

1	LA	PAA	2	Pre	4.50	5.27
1	LA	PAA	2	Post	4.28	4.65
1	LA	Cen	3	Pre	4.39	5.03
1	LA	Cen	3	Post	3.89	1.88
1	LA	Control	4	Pre	4.53	4.96
1	LA	Control	4	Post	4.13	4.95
1	PAA	LA	5	Pre	4.09	5.69
1	PAA	LA	5	Post	3.82	2.66
1	PAA	PAA	6	Pre	3.90	5.76
1	PAA	PAA	6	Post	4.12	5.36
1	PAA	Cen	7	Pre	3.67	5.69
1	PAA	Cen	7	Post	3.45	2.08
1	PAA	Control	8	Pre	3.98	5.82
1	PAA	Control	8	Post	4.07	5.91
1	Cen	LA	9	Pre	4.73	5.25
1	Cen	LA	9	Post	4.55	2.56
1	Cen	PAA	10	Pre	4.78	5.36
1	Cen	PAA	10	Post	4.26	5.09
1	Cen	Cen	11	Pre	4.19	5.46
1	Cen	Cen	11	Post	3.19	2.09
1	Cen	Control	12	Pre	4.12	5.21
1	Cen	Control	12	Post	5.00	5.28
1	Control	LA	13	Pre	4.73	6.38
1	Control	LA	13	Post	3.79	2.71
1	Control	PAA	14	Pre	4.40	5.79
1	Control	PAA	14	Post	4.11	5.35
1	Control	Cen	15	Pre	4.11	6.45
1	Control	Cen	15	Post	4.08	2.8
1	Control	Control	16	Pre	4.41	6.36
1	Control	Control	16	Post	4.36	5.92
2	LA	LA	17	Pre	4.49	4.93
2	LA	LA	17	Post	3.10	3.01
2	LA	PAA	18	Pre	4.30	5.01
2	LA	PAA	18	Post	4.37	4.88
2	LA	Cen	19	Pre	4.38	5.11
2	LA	Cen	19	Post	4.56	1.72
2	LA	Control	20	Pre	4.24	5.24
2	LA	Control	20	Post	4.79	5.05
2	PAA	LA	21	Pre	4.53	5.99
2	PAA	LA	21	Post	3.81	3.44
2	PAA	PAA	22	Pre	4.43	5.88
2	PAA	PAA	22	Post	3.95	5.26
2	PAA	Cen	23	Pre	4.28	5.62

2	PAA	Cen	23	Post	4.18	1.77
2	PAA	Control	24	Pre	4.28	5.86
2	PAA	Control	24	Post	4.10	5.87
2	Cen	LA	25	Pre	4.58	5.07
2	Cen	LA	25	Post	4.13	3.12
2	Cen	PAA	26	Pre	3.92	5.16
2	Cen	PAA	26	Post	4.16	4.85
2	Cen	Cen	27	Pre	4.60	5.14
2	Cen	Cen	27	Post	4.18	1.39
2	Cen	Control	28	Pre	4.44	5.05
2	Cen	Control	28	Post	4.37	5.38
2	Control	LA	29	Pre	4.63	6
2	Control	LA	29	Post	4.23	3.02
2	Control	PAA	30	Pre	4.75	6.06
2	Control	PAA	30	Post	4.26	5.87
2	Control	Cen	31	Pre	4.67	5.87
2	Control	Cen	31	Post	4.56	1.81
2	Control	Control	32	Pre	4.01	5.76
2	Control	Control	32	Post	4.37	5.73
3	LA	LA	33	Pre	4.52	5.12
3	LA	LA	33	Post	4.61	3.21
3	LA	PAA	34	Pre	3.97	5.08
3	LA	PAA	34	Post	3.27	4.95
3	LA	Cen	35	Pre	4.08	5.12
3	LA	Cen	35	Post	4.27	2.29
3	LA	Control	36	Pre	4.73	5.01
3	LA	Control	36	Post	3.88	5.32
3	PAA	LA	37	Pre	4.36	6.09
3	PAA	LA	37	Post	4.00	3.25
3	PAA	PAA	38	Pre	3.67	5.96
3	PAA	PAA	38	Post	4.33	5.54
3	PAA	Cen	39	Pre	4.45	5.94
3	PAA	Cen	39	Post	4.25	2.22
3	PAA	Control	40	Pre	4.23	6.08
3	PAA	Control	40	Post	3.92	5.77
3	Cen	LA	41	Pre	3.82	5.04
3	Cen	LA	41	Post	3.73	2.88
3	Cen	PAA	42	Pre	4.64	5.05
3	Cen	PAA	42	Post	4.62	5.24
3	Cen	Cen	43	Pre	3.64	5.09
3	Cen	Cen	43	Post	3.55	1.69
3	Cen	Control	44	Pre	4.47	5.07
3	Cen	Control	44	Post	4.05	5.33

3	Control	LA	45	Pre	4.15	5.79
3	Control	LA	45	Post	4.26	3.2
3	Control	PAA	46	Pre	4.78	5.84
3	Control	PAA	46	Post	4.84	5.37
3	Control	Cen	47	Pre	4.47	5.86
3	Control	Cen	47	Post	4.26	2.09
3	Control	Control	48	Pre	4.27	5.77
3	Control	Control	48	Post	5.07	5.82