COMPARISON OF ANTIMICROBIAL TREATMENTS APPLIED VIA CONVENTIONAL SPRAY OR ELECTROSTATIC SPRAY TO REDUCE SHIGA-TOXIGENIC *ESCHERICHIA COLI* (STEC) ON CHILLED BEEF OUTSIDE ROUNDS

A Thesis

by

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ABSTRACT

The purpose of this study was to compare the efficacy of different antimicrobial interventions: lactic acid, lauric arginate ester, cetylpyridinium chloride or peracetic acid applied with either conventional spray or hand-held electrostatic spray to reduce populations of Shiga-toxin producing *Escherichia coli* (STEC).

Hot-boned beef outside rounds were inoculated within 1 hour after harvest with a cocktail of 8 serotypes of Shiga-toxigenic *E. coli* (STEC8). Outside rounds were chilled upon transport back to Texas A&M University, and then hung on flame-sterilized meat hooks at 4°C for 36 hours to simulate a contaminated full carcass side in the chiller. Then, outside rounds were treated with lactic acid, lauric arginate ester, cetylpyridinium chloride, peracetic acid, or water (control) using conventional spray or electrostatic spray. Pre-treatment and post-treatment samples were excised from all rounds to evaluate the efficacy of all interventions and the mode with which they were applied.

Lactic acid applied with conventional spray achieved the greatest reduction of STEC8 populations compared to all other treatments. In most cases, reductions obtained by conventional spray and electrostatic spray were not significantly different. The only treatment that differed significantly between conventional spray and electrostatic spray was lactic acid. Among the treatments applied with electrostatic spray, lauric arginate ester produced the greatest reduction of STEC8. If carcass interventions have similar performance between conventional and electrostatic spray, the use of the electrostatic

spraying system would be advantageous by saving water and antimicrobial. Spray type and intervention must be paired appropriately for optimum beef safety.

DEDICATION

To my parents, Barb Krause and Reg Hudson

for giving me the gift of life and loving and supporting me

every step of the way.

To my brother, Ian

for his love, support and guidance.

To my grandparents, Barbara and Bill Krause

for supporting me in all of my endeavors

and encouraging me to reach my goals and dreams.

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INTRODUCTION

Among the different virulence groups of *Escherichia coli*, Enterohemorrhagic *E. coli* (EHEC) has caused the greatest concern due to the severity of resulting disease, specifically in young children and the elderly. This group is also known as Shiga-toxigenic *E. coli* (STEC), as it produces Shiga toxins.

The most commonly studied and recognized serotype of STEC is O157:H7, however recently 6 more serotypes have been declared adulterants in non-intact beef products. While hemorrhagic colitis affects mostly adults, HUS is common in children infected with EHEC. Although leafy greens are acquiring relevance as vehicles of STEC resulting in foodborne illness outbreaks, beef continues to be the leading food associated with STEC infection. The danger associated with non-intact beef products is the inevitable comingling of potentially contaminated meat. The beef industry has been developing and implementing interventions to reduce pathogen presence on carcasses, with STEC being the target pathogen. Extensive work has been done to study the efficacy of carcass interventions, including the application of water washes and organic acids. Many beef slaughter facilities traditionally use hot water as well as lactic acid, as it is an effective and inexpensive intervention. Other interventions not frequently used in the beef industry include cetylpyridinium chloride, lauric arginate and peracetic acid.

Traditional spray application methods include either automated spray cabinets or hand-held sprayers in smaller scale facilities. Electrostatic spray (ESS) is a novel spray application method, which uses less solution while achieving full coverage of the object

being sprayed. This is due to the attraction of negatively charged particles emitted from the spray gun to the positively charged object. Therefore, electrostatic spray is expected to reach pathogenic bacteria in hard to reach crevices of the beef surfaces so long as the charge permits. This technology may be beneficial for conservation of resources in the beef industry.

The objective of this study was to compare the efficacy of lactic acid, lauric arginate, cetylpyridinium chloride and peracetic acid applied both via conventional spray and electrostatic spray.

REVIEW OF LITERATURE

Foodborne Illness

In the United States between 1960 and 2000, the percentage of money spent on food outside the home has increased from 20% to over 40% *(64)*. According to Kant et al. *(64)* the percentage of adult Americans eating outside the home 3 or more times a week increased from 36% to 41% between 1987 and 1999. Demand for fresh, less-processed foods has also increased. With factors such as these, foodborne illness prevalence has steadily increased over the years. According to the Centers for Disease Control and Prevention (CDC), about 48 million people develop foodborne illness in the span of a year. Of these people, 128,000 are hospitalized with 3,000 resulting in fatalities *(32)*.

There are 31 major pathogens which are known to cause foodborne illness, and the rest are identified as unspecified agents which cause 80% of foodborne illnesses annually, with 56% resulting in death *(31)*. The top 5 foodborne pathogens acquired in the home resulting in hospitalization are *Salmonella*, Norovirus, *Campylobacter spp.*, *Toxoplasma gondii*, and *Escherichia coli* O157 *(31)*. According to Foodborne Diseases Active Surveillance Network (FoodNet) 2013 Annual Report, 19,162 laboratory-confirmed cases of foodborne illness were identified with 4,276 hospitalizations and 88 deaths resulting from these illnesses *(33)*. Two sources of contamination of a food of animal origin include primary and secondary contamination. Primary contamination results when the food is contaminated directly from the animal itself, i.e. contamination

with fecal matter. Secondary contamination occurs when some outside source, such as humans or other animals come into contact with the food *(58)*.

Generic and Pathogenic *Escherichia coli*

Escherichia coli

Escherichia coli was first described and isolated from human infant stools by Theodor Escherich in 1885 *(15)*. Although generally a harmless organism of the gut microflora, it can also be an opportunistic pathogen, causing neonatal meningitis, urinary tract infections, and gastroenteritis *(1, 63)*.

A member of the *Enterobacteriaceae* family *(1)*, the minimum growth temperature of *E. coli* is 7-8°C and maximum 44-46°C with optimum temperatures ranging from 35-40°C *(59)*. *E. coli* is a gram-negative, non-sporulating rod that is catalase-positive and oxidase negative. Members of the *Enterobacteriaceae* family can be differentiated based on a group of tests called IMViC. *E. coli* is indole positive, methyl red positive, Voges-Proskauer negative and citrate negative *(1)*. Minimum growth pH of pathogenic *E. coli* is 4.4 and minimum a_w is 0.95 *(59)*.

The human infant is said to acquire *E. coli* within days of birth from a combination of exposure to the mother by fecal to oral route as well as his or her environment *(15)*. Distinct serogroups of *E. coli* were able to be identified based on their somatic (O) antigen by the 1940s *(68)* and have been further divided into serotypes based on flagella (H) antigens and capsular (K) antigens. Knowledge of *E. coli* serogroups and serotypes have enabled the recognition of those which are pathogenic to humans *(15)*.

Pathogenic *E. coli*

According to Jay et al. *(61)*, there are five virulence groups of *E. coli* including enteroaggregative (EAggEC), enteroinvasive (EIEC), enteropathogenic (EPEC), enterotoxigenic (ETEC) and enterohemorrhagic (EHEC), also known as VTEC or STEC.

Enteroaggregative *E. coli* (EAggEC)

Characterized by "stacked brick" adhesion *(14, 61)*, this virulence group is common in developing countries and causes persistent (more than 14 days) diarrhea in children and other immunocompromised patients. Organisms in this group carry a 60-MDa plasmid, which is responsible for adhesion. Consisting of 40 different O-types, the EAggEC group produces two types of toxins. The first is the Shiga-like toxin and the second is the hemolysin-like toxin, which unlike hemolysin, does not lyse red blood cells *(14)*. Symptoms of EAggEC infection include stool containing mucous, persistant diarrhea, and low-grade fever in immunocompromised patients. Members of this virulence group have not shown a clear association with foodborne illness *(61)*.

Enteroinvasive *E. coli* (EIEC)

Members of this group carry a 140-MDa plasmid, which is responsible for invasion of the epithelial cells *(14, 61)*, and are very similar to *Shigella* spp. Symptoms include watery diarrhea, fever, headache, chills, and dysentery, which is linked to the presence of a toxin of 63 kDa *(14)*. Infected patients will get better after 7 to 12 days but may be shedders of EIEC for the rest of their life. Enteroinvasive *E. coli* was first recorded in a foodborne outbreak in 1947 with salmon *(61)*.

Enteropathogenic *E. coli* (EPEC)

The first virulence group of *E. coli* to be described, enteropathogenic *E. coli* mostly affects children and infants. Though EPEC do not produce Shiga toxins *(61)*, they are characterized by intimate attachment to epithelial cells, and the formation of "attaching and effacing" lesions or pedestal-like structures. Microvilli in the small intestine are worn down and unable to absorb nutrients, resulting in a potentially fatal condition *(14)*. The first recorded foodborne disease outbreak due to enteropathogenic *E. coli* (EPEC) infection occurred in 1961 in Romania, linked to coffee substitute *(1, 61)*.

Enterotoxigenic *E. coli* (ETEC)

Members of this virulence group are a common cause of Traveler's Diarrhea in underdeveloped countries *(61)*. ETEC is frequently ingested by travelers via contaminated water or contaminated food. While these organisms may not affect adult natives, ETEC is a major cause of infantile diarrhea in these developing countries. Enterotoxigenic *E. coli* attach via fimbrial colonization and then produce a heat-labile toxin or a heat-stabile toxin. An infected individual generally exhibits watery diarrhea and vomiting for 3 to 4 days resulting in extreme dehydration *(14)*. Foodborne illness as a result of ETEC infection has occurred due to consumption of mold-ripened soft cheese *(1)*.

Shiga-Toxin Producing *E. coli* (STEC)

According to the 2013 FoodNet Annual Report *(33)*, Shiga-toxin producing *E. coli* (STEC) are known to cause approximately 265,000 foodborne infections in the United States annually with August being the highest month of incidence. In 2013, O157 STEC caused 211 hospitalizations and 2 deaths with an incidence rate of 1.15 in 100,000 *(33)*. In contrast, non-O157 STEC caused 76 hospitalizations, 2 deaths, and had an incidence rate of 1.18 in 100,000 *(33)*. While the incidence rate of *E. coli* O157 infections has generally gone down since 1996, non-O157 infections have made a steady increase resulting in similar incidence rates in the past few years. Costs associated with STEC illnesses include medical care, decreased quality of life, and even death *(97)*. According to Scharff et al. *(96)*, O157 infections on average cost $10,446 per case whereas non- O157 infections cost $1,764.

Enterohemorrhagic *E. coli* (EHEC) is known to produce Shiga-like toxins (Stxs), which are toxic against Vero cells (African green monkey kidney cells). This is why EHEC is also known as Shiga toxin-producing *E. coli* (STEC) or verotoxin-producing *E. coli* (VTEC) *(14)*. Shiga toxin-producing *E. coli* are able to produce two different Shiga-like toxins including Stx1 and Stx2. Stx2 is much more toxic than Stx1 and poses a higher risk of the host developing hemolytic uremic syndrome (HUS) resulting in acute renal failure which affects 5-10% of infected persons *(14)*. For children under the age of 5, HUS affects 3 in 100,000 children per year and frequently results in death or life-long kidney dialysis *(75)*. In 1994, the USDA Food Safety and Inspection Service (FSIS) declared *E. coli* O157:H7 as an adulterant in any non-intact, ground beef product *(51)*.

Ground beef is a particular food safety concern due to comingling of potentially contaminated surface pieces with internal, essentially sterile, pieces. Not until 2012 did USDA-FSIS declare six additional STEC *(51)* adulterants in ground beef, also known as non-O157 STEC or "The Big Six". These include serogroups O26, O103, O45, O111, O121, and O145 which cause illness similar to that caused by O157 *(6)*. Foodborne illness caused by STEC has most often been associated with undercooked ground beef products *(1)*. Ground beef hamburgers sold to the consumer should be 160°C in the center of the patty per USDA regulations. Outbreaks associated with foods of animal origin such as ground beef or raw milk are generally caused by inadequate cooking or heating temperatures *(1)*.

STEC O157:H7

First identified by the Centers for Disease Control and Prevention (CDC) as a human pathogen of a rare serotype in 1982 *(93, 100)*, *E. coli* O157:H7 was implicated in a landmark outbreak in the United States in 1993 resulting from a national fast food chain serving undercooked ground beef hamburgers *(14)*. *E. coli* O157:H7 was also the cause of an outbreak resulting from contaminated spinach in 2006, resulting in 199 illnesses, 102 hospitalizations, and 3 deaths *(14, 30)*. In 2013, an outbreak caused by contaminated ready-to-eat (RTE) salads resulted in 33 illnesses and 7 hospitalizations *(34)*. While *E. coli* O157:H7 is largely known to be found in meats and other foods of animal origin, it is important to note that other food products such as leafy greens can become contaminated with contaminated irrigation water, soil, fecal material from wildlife, or cross-contaminated with raw meat. These food products exhibit a risk not

seen in most meat products: they do not have a lethal cooking step, and are generally consumed fresh. This pathogen is able to survive the high acidity of the human stomach due to its acid tolerance and colonizes the small intestine which occurs in 3 stages including localized adherence, signaling event, and intimate contact *(14)*.

Non-O157 STEC

Non-O157 STEC include serogroups O26, O45, O103, O111, O121, and O145, also known as "The Big Six". These serogroups are now considered adulterants in ground, non-intact beef products. Beef is the primary vehicle of foodborne illness associated with non-O157 STEC *(11)*. After 1993, *E. coli* O157:H7 received the most attention as it was commonly known to cause foodborne illness in the United States. However, it can be seen from cattle hide prevalence data *(10)* that both O157 and non-O157 STEC are a threat to food and beef safety. According to Hale et al. *(55)*, domestically acquired non- O157 STEC infections account for 59.7% of all STEC infections whereas 40.3% are caused by O157 STEC. Based on these percentages, it seems as though non- O157 serogroups are causing more STEC-related illnesses in the United States at present. Non-O157 serogroups can cause hemolytic uremic syndrome, but more often O157-infected patients with HUS require life-long treatment such as kidney dialysis *(97)*.

Secondary Sequelae of STEC Illness

Hemolytic Uremic Syndrome (HUS)

Enterohaemorrhagic *E. coli* (EHEC) is particularly dangerous very young children and the elderly. The severity of EHEC infections is wide ranging from non-bloody diarrhea to secondary sequalae such as hemolytic uremic syndrome (HUS). HUS is known to cause acute renal failure, hemolytic anemia and thrombocytopaenia. Ten percent of children with EHEC infections will develop HUS. Children with HUS either require lifelong kidney dialysis (50%) or will die as a result of this secondary sequelae (3-5%) *(1)*.

Thrombotic Thrombocytopenic Purpura (TTP)

Thrombotic thrombocytopaenic purpura (TTP) is a much less common secondary sequelae that affects adults infected with STEC. Instead of targeting the kidneys as does HUS, TTP causes neurological symptoms due to brain blood clots and fever, resulting in a fatal condition if left untreated due to damage to vital organs *(1)*.

Meat Safety

Since the 1993 outbreak of *E. coli* O157:H7 linked to hamburgers served at a national fast food restaurant which caused 501 reported illnesses, 45 incidences of HUS, and 4 deaths, the beef industry has made huge strides to improve beef safety *(13, 63)*. Shiga-toxigenic *E. coli* (STEC) naturally reside in healthy cattle intestines, maintaining a symbiotic relationship with these animals; these organisms are not pathogenic to the animals like they are to humans. Cattle are known to be a primary reservoir for STEC

(65), present in the intestinal tracts of 10-28% of North American cattle *(66)*. Bovine intestines do not have the Shiga-toxin binding receptor, allowing carriage of STEC without susceptibility to disease *(100)*. STEC is also found frequently in the environment as well as the intestines of many warm-blooded animals *(100)*.

Hides are contaminated with animal fecal material and these microorganisms can then be transferred to raw carcass surfaces as well as other areas in the processing environment *(63)*. Elder et al. *(44)* reported the prevalence of *E. coli* O157:H7 to be in 28% of feces and 11% of hides in the late summer months of July and August. The main source of carcass contamination is the hide *(10)*; According to Arthur et al. *(4)*, *E. coli* O157:H7 can survive on the hide for up to 9 days, suggesting pre-harvest interventions should occur within 9 days prior to slaughter. Removal of the hide can occur in one of two ways: manual removal by personnel or an automatic hide puller, both of which introduce possible modes of cross-contamination of the carcass surface *(100)*.

Interventions For Reducing Pathogens in Beef and Beef Products

Pre-Harvest Interventions in Beef

Studies have shown some pre-harvest preventive methods to be useful in reducing the colonization and shedding of O157:H7 in harvest-ready cattle, thus reducing the potential for fecal contamination on the hide surface.

Grain type and the method with which the grain is processed is thought to affect the colonization of *E. coli* O157:H7 in cattle. According to Jacob et al. *(60)*, there is a positive relationship between colonization and shedding of O157:H7 and a barley-based

diet. Fox et al. *(49)* reported decreased prevalence of *E. coli* O157:H7 in fecal waste when cattle were fed dry-rolled grains as opposed to steam-flaked grains.

According to Cray et al. *(36)* calves shed much higher numbers of O157:H7 than do adult cattle. Reducing animal density or separating young cattle from adult cattle may be advantageous in reducing the colonization and ultimate shedding of the pathogen in adults *(72)*.

Direct-fed microbials, also known as competitive enhancement strategies *(53, 63)*, employs introduced bacteria to have an antagonistic effect on pathogens in the gut of the animal. *Lactobacillus* and *Bifidobacterium* are the most commonly used probiotics *(63)*. An efficient, *Lactobacillus* –based direct-fed antimicrobial is known as NP51 has been shown to reduce the fecal shedding of O157:H7 by 49% *(21)*. NP51 is commercially available and approximately 10-15% of fed cattle are given this product in the United States *(100)*. Other direct-fed microbials, such as *Bacillus subtilis* strain 166, are not as effective at reducing *E. coli* O157:H7 shedding in cattle and should not be used as a pre-harvest intervention *(3)*. Callaway et al. *(23)* reported a 3 log reduction in fecal *E. coli* O157:H7 populations when cattle were exposed to a sodium chlorate supplement.

Another pre-harvest intervention involves the use of vaccine technology. Potter et al. *(88)* described a 58.7% lower risk of recovery of O157:H7 as compared to untreated controls. The use of antibiotics, such as neomycin sulfate, has also been reported to be beneficial. In a commercial feedlot study, *E. coli* O157 was recovered from treated cattle in 0.4% of feces and 2.5% of hides, as compared to the control cattle

in which *E. coli* O157 was present in 22.1% and 50% of feces and hides, respectively *(73)*. While bacteriophages have limited application in the food industry currently, these bacterial viruses have been suggested for use in the reduction of pathogenic bacterial shedding in cattle *(63)*.

It is important to note that these pre-harvest interventions are not all-encompassing, but rather a step towards improving beef safety. Food Safety and Inspection Service recommends 5 different practices to reduce the prevalence of O157:H7 in harvest-ready cattle: 1) Maintaining clean water, 2) Maintaining clean feed, 3) Keeping living environments well-drained, 4) Separating calves and heifers in housing or reducing animal density, and 5) Keeping extraneous wildlife out of feed pens as they may carry pathogens *(90)*.

Post-Harvest Interventions on Beef

Antimicrobial interventions include but are not limited to chemical dehairing, trimming, application of acids such as acetic or lactic acids, acidified sodium chlorite, water washing, steam-vacuuming, steam pasteurization, and irradiation. Specific regulations regarding carcass interventions are explained in the Food Safety and Inspection Service Directive 7120.1: Safe and Suitable Ingredients Used in the Production of Meat, Poultry, and Egg Products *(52)*.

Due to the lethal effect shown against microorganisms, organic acids are popular antimicrobials used in carcass interventions. Commonly used organic acids in the beef industry are acetic and lactic acid. Hardin et al. *(56)* reported reductions in *E. coli*

O157:H7 and *Salmonella* Typhimurium ranging from 2.4 to 3.7 log CFU/cm^2 and 3.2 to 5.1 log CFU/cm^2 when treated with water wash followed by 2% acetic acid (pH 2.5, 55°C, 11s), respectively. In the case of both pathogens, reductions achieved on outside rounds were the greatest of all other cuts. With regards to *E. coli* O157:H7, Hardin et al. *(56)* reported a greater reduction with 2% lactic acid as opposed to acetic acid. Lactic acid, however, caused greater reductions in beef surface pH than acetic acid.

The following sections describe some of the various chemical and physical agents that have been used in carcass interventions.

Lactic acid

Lactic acid is a commonly used organic acid in the beef industry *(100)*, and its efficacy at different points in the beef harvesting process have long been investigated. Its mechanism of action is the degradation of cell membrane components and subsequent loss of cell membrane integrity *(22)*. When a weak, undissociated acid permeates the bacterial membrane, it subsequently dissociates due to the markedly higher intercellular pH and acidifies the bacterial cytoplasm *(16, 95)*. It is recommended to be used in a 2% solution in order to avoid meat discoloration or other quality issues *(99)*. With regards to preevisceration carcasses, Bosilevac et al. *(19)* reported a 1.6 log CFU/100 cm^2 reduction in aerobic plate counts and 1.0 log CFU/100 cm^2 reduction in *Enterobacteriaceae* counts with a 2% lactic acid solution applied at approximately 42°C. After this study, Bosilevac et al. *(19)* actually recommended the use of hot water only on preevisceration carcasses instead of lactic acid. However, reductions were still achieved

with 2% lactic acid. Hardin et al. *(56)* reported lactic acid to be the most effective treatment applied to outside rounds and briskets contaminated with *E. coli* O157:H7 and *Salmonella* when compared to acetic acid, water wash alone, or trimming. Castillo et al. *(29)* investigated the additional reduction potential in *E. coli* O157:H7 and *Salmonella* populations with a postchill application of lactic acid and reported an additional reduction of 2 to 2.4 log CFU/cm^2 with *E. coli* O157:H7 and 1.6 to 1.9 log CFU/cm^2 with *Salmonella* Typhimurium. Additionally, Castillo et al. *(26)* reported on the effects of combined carcass interventions which resulted in a 4.6, 4.9, and 4.4 log CFU/cm^2 reduction in *E. coli* O157:H7 with water wash (69 kPa, 90 s) followed by 2% lactic acid (55°C, 276 kPa, 11 s), water wash followed by hot water (95°C, 166 kPa, 5 s) and then lactic acid treatment, and water wash followed by lactic acid treatment then hot water, respectively. Trimming followed by lactic acid and trimming followed by hot water treatment then lactic acid application both produced a 4.9 log CFU/cm^2 reduction in *E. coli* O157:H7 *(26)*. The use of lactic acid dips have also been investigated in the poultry industry *(39)*.

Lauric arginate ester

Lauric arginate ester (LAE), more commonly known as lauric arginate, is recognized as a safe food preservative in the food industry. Derived from lauric acid, L-arginine and ethanol, this compound was granted GRAS status under 21 CFR §170.30(b) *(8)*. Lauric arginate works as an antimicrobial by destabilizing the plasma membrane lipid bilayer of microorganisms *(8)*. Few studies have investigated

lauric arginate as a potential application in beef carcass interventions. Dias-Morse et al. *(41)* reported a 1.4 log CFU/g reduction in Shiga toxin-producing *E. coli* (STEC) as compared to inoculated untreated controls when a 5% lauric arginate treatment was applied to inoculated beef trimmings.

Many studies *(74, 77, 87)* have reported the bacteriocidal activity of lauric arginate against *Listeria monocytogenes* on ready-to-eat (RTE) meats and frankfurters. Luchansky et al. *(74)* studied the reduction of *L. monocytogenes* in cooked hams after application with a 5% LAE solution, reporting a 5 log CFU/ham reduction within 24 hours at 4°C. Additionally, when applied via the "Sprayed Lethality In Container" (SLIC) delivery method, which consists of delivering the antimicrobial into a vacuum bag immediately prior to placing the product in the bag and vacuum sealing the product, LAE successfully inhibited outgrowth of *L. monocytogenes* for up to 40 days under refrigerated storage *(74)*. Martin et al. *(77)* reported a 1 log CFU/cm^2 reduction of *L. monocytogenes* on frankfurters upon initial application of 2.5% lauric arginate solution. Porto-Fett et al. *(87)* reported the initial reduction of *L. monocytogenes* achieved by lauric arginate using the SLIC delivery method to be about 1.8 log CFU/package within 2 hours. However, lauric arginate alone did not inhibit the outgrowth of *L. monocytogenes* over a period of 120 days. When combined with potassium lactate or sodium diacetate, the initial reduction of *L. monocytogenes* was achieved and outgrowth of the pathogen was inhibited for up to 120 days *(87)*.

Cetylpyridinium chloride

Traditionally used as an oral antiseptic *(85)* cetylpyridinium chloride (CPC) has also been frequently utilized in the poultry industry to reduce populations of *Salmonella* and *Campylobacter*, two commonly found pathogens in poultry products *(103)*. CPC is a quaternary ammonium compound, a positively charged polyatomic ion with a long alkyl chain. It is currently approved for use in poultry processing and functions as an antimicrobial due to the cations, chloride and cetylpyridinium it forms in solution *(43, 100)*. These cations make the CPC solution amphiphilic which allows it to damage the bacterial cell membrane *(100)*. CPC has been frequently used in the poultry industry as a post-chill antimicrobial in drench cabinets on meat destined for grinding, and its efficacy in such applications has been compared with chlorine and peracetic acid *(103)*. With regards to pre-chill applications, Xiong et al. *(104)* reported the capability of 0.5% CPC to reduce *Salmonella* Typhimurium populations on chicken skins by 1.9 log CFU/ml.

The success of CPC in the poultry industry has led way to research in the beef industry. As CPC has not yet been approved for use in a beef processing plant, much investigation on the antimicrobial has been done as a hide intervention immediately prior to stunning *(17)*. Studies with CPC applied to beef hides have shown up to 4 log CFU/100 cm^2 reductions in aerobic plate counts and *Enterobacteriaceae* counts *(18)*. Baird et al. *(7)* demonstrated that a 1% CPC hide wash prior to opening resulted in a 4.5 log CFU/100 cm^2 reduction in coliform counts on beef hides—the greatest among 2% L-lactic acid and 3% hydrogen peroxide. Although the most effective concentration of

CPC to reduce microbial populations is 1% *(18)*, the FSIS Directive 7120.1 only allows 0.8% CPC application. Bosilevac et al. *(18)* recommend applying CPC immediately prior to or after stunning the animal in order to avoid recontamination of the hide before dehiding. Application of CPC prior to dehiding could be a practical alternative to chemical dehairing *(80)*. Cutter et al. *(38)* also investigated the ability of 1% CPC at 35°C to reduce *Salmonella* Typhimurium and *E. coli* O157:H7 on lean as well as adipose tissue beef surfaces. CPC immediately reduced *E. coli* O157:H7 by 5-6 log CFU/cm^2 and *Salmonella* Typhimurium to undetectable levels on lean surfaces and >2.5 log CFU/cm^2 of both pathogens on adipose tissue surfaces. Ransom et al. *(91)* reported a reduction of *E. coli* O157:H7 by 4.8 log CFU/cm^2 on beef carcass surfaces when applied at 0.5%. With regards to ground beef trimmings, Pohlman et al. *(86)* reported smaller reductions after CPC treatment. In this case, CPC reduced *E. coli* and *Salmonella* Typhimurium in ground beef by 0.6 and 0.7 log CFU/g, respectively.

Peracetic acid

Peracetic acid (PAA) is a strong oxidizing agent with the ability to disrupt the bacterial cell membrane *(39, 100)* which is used frequently as a sanitizer for surfaces in a processing facility that will come into contact with food, as well as a microbiological intervention on meats and produce *(67)*. According to Cords et al. *(39)*, PAA has three antimicrobial mechanisms: denaturation of cell proteins, inactivation of cell transport-crucial enzymes, and disruption of the permeability of the cell membrane. In solution, peracetic acid breaks down into water, oxygen and acetic acid, all non-toxic and safe for

use as a sanitizer in a food processing facility. Antimicrobial activity increases with increased concentration, and is not negatively affected at lower temperatures making PAA an advantageous sanitizer. Peracetic acid provides a better antimicrobial effect in an acidic environment *(39)*. The effectiveness of peracetic acid as a wastewater decontaminant with potentially less-harmful by-products than traditionally used chlorine has also been investigated *(62)*. Peracetic acid is the most commonly used antimicrobial with regards to postchill applications *(103)* and its efficacy of reducing microorganisms such as *Salmonella* and *Campylobacter* has been compared with other interventions such as chlorine, cetylpyridinium chloride, lysozyme, and even a mixture of peracetic acid and hydrogen peroxide *(12, 79, 103)*.

In contrast, the beef industry has investigated the efficacy of peracetic acid as an intervention against *Escherichia coli* O157:H7 and *Salmonella*. While peracetic acid has proven successful in significantly reducing *E. coli* O157:H7 prior to chilling *(82)*, post-chill application of peracetic acid (200 ppm, 43°C) is not recommended in beef production *(69)*. It is important to note that according to the USDA FSIS directive 7120.1, the concentration of peracetic acid used as an intervention must not exceed 200 ppm. King et al. *(69)* found that 1000 ppm peracetic acid, nearly 5 times the legal limit, still reduced *E. coli* O157:H7 and *Salmonella* Typhimurium less than 4% lactic acid. Ellebracht et al. *(45)* reported peracetic acid dips at 200, 500 and 1000 ppm applied to fresh beef trim to reduce *E. coli* O157:H7 by 0.7, 0.7 and 0.5 log CFU/cm^2, respectively.

Other Interventions

Chemical dehairing is a three step process patented by Bowling and Clayton *(20)* in which hair is removed with a sodium sulfide solution, followed by a hydrogen peroxide rinse and water rinse. This process is thought to minimize cross contamination from the hide to the carcass surface during dehiding. Nou et al. *(80)* tested the hypothesis that chemical dehairing, prior to dehiding would result in a reduced amount of cross-contamination of carcasses. Results obtained indicated that a chemical dehairing step effectively reduces the cross-contamination of beef carcasses, agreeing with previous research conducted by Castillo et al. *(25)*, where it was determined that inoculated *Salmonella* Typhimurium and *E. coli* O157:H7 were significantly reduced by a chemical dehairing treatment consisting of 10% sodium sulfide solution on hide pieces. Interventions such as chemical dehairing and application of organic acids go hand in hand, as no single intervention will completely remove pathogenic bacteria *(70)*.

The USDA's "zero-tolerance" rule *(50)* for fecal matter on carcass surfaces spurred research on various physical interventions. Prasai et al. *(89)* suggested trimming of visible contamination followed by a subsequent water wash, because of the possibility of cross-contaminating with an unsanitized knife between carcasses. Studies conducted by Gorman et al. *(54)* as well as Hardin et al. *(56)* have shown the beneficial effects of knife trimming in the reduction of microorganisms on beef carcass surfaces. However, other research has shown greater reductions can be achieved by subsequent treatments such as water washing or organic acid treatments *(26, 40, 56, 92)*.

Another antimicrobial intervention that has been investigated is acidified sodium chlorite (ASC). Castillo et al. *(28)* reported a 4.5 log CFU/cm^2 reduction of *E. coli* O157:H7 when treated with citric acid-activated acidified sodium chloride (140 ml, 10 s, 69 kPa, 22.4-24.7°C), as opposed to the 3.8 log CFU/cm^2 reduction achieved by phosphoric acid-activated acidified sodium chloride.

Different factors such as pressure and temperature of water wash have been investigated over the years. While some research has shown the beneficial effect of increased pressure applications *(2, 56, 71)*, some studies have not *(37, 105)*. In fact, DeZuniga et al. *(105)* reported a detrimental effect of wash water pressures exceeding 4.1 MPa, resulting in the potential bacterial penetration below the carcass surface. Increased temperatures have shown enhanced reductions in pathogenic bacteria. Castillo et al. *(26)* reported a 3.7 log CFU/cm^2 reduction in *E. coli* O157:H7 populations when treated with a hot (97°C, 166 kPa, 5 s) water wash. Barkate et al. *(9)* reported a 1.3 log CFU/cm^2 reduction in aerobic plate counts (APC) when treated (95°C sterile hot water, 40 s) before the final carcass wash and a 0.8 log CFU/cm^2 APC reduction when treated after the final carcass wash.

Steam- vacuuming can be used in lieu of knife trimming, and is approved by USDA-FSIS as such; its purpose is to remove visible fecal contamination *(101)*. Phebus et al. *(84)* reported steam pasteurization to be an effective post-harvest intervention, but recommended combining it with other interventions such as knife trimming followed by steam pasteurization to optimize bacterial reductions. Another potential point of contamination is the evisceration step, where fecal matter may come into contact with

carcass surfaces. Punctured or torn viscera can lead to contamination of the carcass with fecal matter. Steam vacuuming at this point in the process can remove fecal material *(100)*. Castillo et al. *(27)* reported a 2.8 log CFU/cm^2 reduction of *E. coli* after treatment with steam vacuum alone. However, additional treatments including hot water and lactic acid resulted in greater reductions ranging from 4.4-5.2 log CFU/cm^2, suggesting that a multi-hurdle intervention strategy is most effective. However, steam vacuum is designed to treat small areas on the beef carcass surface, not the entire surface *(27)*. Dickson *(42)* also reported that washing preevisceration carcasses may reduce further contamination in the slaughter process.

Although not accepted by certain groups of consumers, irradiation has been shown to significantly reduce pathogenic bacteria in raw meats. Three types of ionizing radiation that can be used for the treatment of food include gamma rays, X-rays, and e-beam. Gamma rays have the ability to penetrate several feet of material, whereas e-beams are much less powerful and will only penetrate a few centimeters. X-rays, introduced for use in foods in the mid 1990s, are as penetrative as gamma rays *(57)*. The Food and Drug Administration (FDA) currently approves of the irradiation of raw refrigerated meat products up to 4.5 kGy and frozen raw meat products up to 7 kGy *(48)*. Arthur et al. *(5)* investigated the efficacy of a 1 kGy dose on chilled beef carcass surfaces. One kGy was sufficient to reduce *E. coli* O157:H7 populations by at least 4 log CFU/cm^2. In 2011, USDA-FSIS denied the petition submitted by the American Meat Institute (AMI) for the use of e-beam on beef carcasses as a processing aid, which would have exempted such beef from labeling requirements. It was denied on grounds

that carcass geometry may lead to uneven absorbed dose and the petition did not detail control of treated carcasses so as to not exceed maximum approved dose *(46)*. At this time, ionizing radiation may be used if following requirements of 9 CFR 424.21 for total absorbed dose and 9 CFR 424.22 (c) (4) for labeling are followed *(46)*.

Importance of the Method of Application of Carcass Interventions

Conventional spray application, whether built into automated spray cabinets or hand-held pump sprayers, are generally used for application to full carcasses. However in the case of beef trim, it is possible to apply organic acids with dip or spray. According to Wolf et al. *(102)*, a lactic acid dip achieved significantly greater reductions of *E. coli* O157:H7, non-O157 STEC, and *Salmonella* when compared to lactic acid spray. The benefits of dip application include greater washing action, and even coverage. Spray application is easier to manage in a plant setting and further, avoids issues like excess water uptake or deterioration of sensory characteristics due to extended exposure time, which are both disadvantages of dip application *(102)*. Both conventional spray and dip application exhibit the disadvantage of excess water and antimicrobial usage. Electrostatic spray has the potential to combine the benefits of dip and spray application, i.e. even coverage and easily manageable process while using significantly less solution. Therefore, the method of application of the antimicrobials is a major factor in designing carcass interventions.

Electrostatic Spray System

The electrostatic spray system (ESS) is a relatively new technology being investigated in the beef industry. Pressurized air and solution meet at the spray nozzle where the solution is atomized into very small particles (30-60 microns in diameter). An electrode at the nozzle tip charges the atomized droplets. The negatively charged droplets are then attracted to the positively charged, grounded object which results in an even coverage of the object and less water and solution usage *(47)*. In the past, electrostatic spray systems (ESS) have been most commonly used in agricultural applications *(76, 81)*. This technology has been used in the application of pesticides and fungicides, delivering a much more even coverage than other spray technologies *(35)*. The poultry industry has investigated electrostatic spray as a means of applying sanitizers to food contact surfaces in a processing environment, with results confirming electrostatic spray application effective and more efficient than conventional spray systems *(94)*. Although originally aimed at agricultural applications, ESS is now a popular way to apply sanitzers in public restrooms, nurseries, locker rooms, and schools. A new company formed as a result of the success of ESS called SPRAYWell.E.D.D. which uses electrostatic spray systems to sanitize public areas such as restrooms *(98)*. The success of ESS in agricultural and sanitizer applications has spurred an interest in use of this technology in the beef industry. Very little research has been published regarding beef applications of electrostatic spray systems. Phebus et al. *(83)* has reported the potential for a multi-nozzle electrostatic spray cabinet to provide even coverage of beef products while using substantially less water and antimicrobial

solution. However, more research must be completed in order to determine the applicability of a hand-held electrostatic spraying system with a single nozzle for smaller beef processing facilities.

MATERIALS AND METHODS

General Procedures

Bacterial Cultures and Inoculum Preparation

Eight rifampicin-resistant isolates corresponding to a strain each of STEC serotypes O26:H11, O45:H2, O103:H2, O104:H4, O111:H-, O121:H19, O145:NM, and O157:H7, were obtained from the Texas A&M Center for Food Safety (TAMU CFS) culture collection in the form of CryoBeads™ at Texas A&M University, College Station, TX. These strains were originally obtained from Dr. John Luchansky, USDA. Each of these microorganisms was grown twice in tryptic soy broth (TSB; Becton Dickinson & Co., Franklin Lakes, NJ) at 35°C for 18-24 h for revival and then streaked for isolation on tryptic soy agar (TSA; Becton Dickinson & Co., Franklin Lakes, NJ) and TSA supplemented with 100 mg/L rifampicin (Sigma-Aldrich & Co., St. Louis, MO; TSAR). One colony from each strain streaked on TSA was confirmed as *E. coli* with API® strips (bioMérieux, Marcy-l'Étoile, France). Colonies on TSAR were checked for uniform size, shape and appearance. One colony from each strain on TSAR was transferred to TSA slants, where they were maintained at 5°C. Before use, working cultures were obtained by transferring a loopful of each strain from their respective TSA slant into separate 50 ml containers of TSB supplemented with 100 mg/L rifampicin, and incubated at 35°C for 18-24 hours. To prepare the inoculum, the entire contents of the 50 ml bottles with each strain were transferred into a sanitized, plastic spray pump bottle and swirled to mix into a STEC8 cocktail.

Inoculation Procedure

For all subsequent experiments, the inoculation of beef was achieved according to the following procedure, which was developed and tested by Dr. Randall Phebus of Kansas State University, as part of the standardized procedures for the multi-institutional Coordinated Agricultural Project funding this study:

A shoulder-length polyethylene glove was donned on the hand and arm that inoculated the beef surface. Prior to inoculation, the spray bottle containing the STEC8 cocktail was primed in a separate, empty biohazard bag inside a biosafety cabinet. The bottle was primed until an even mist of inoculum was emitted from the bottle. Once primed, the inoculation arm was carefully removed from the priming bag and transferred into the bag containing the beef tissue to inoculate. The bottle was calibrated to emit a spray with a total volume of 2 ml in 3 sprays of inoculum. This inoculum was sprayed three times onto the fat surface of the beef from a distance of approximately 30 cm. This inoculation procedure was intended to simulate contamination of the outside of a beef carcass, and was expected to result in a STEC8 population of approximately 6-7 log CFU/cm^2 on the beef surface. The inoculating arm was carefully removed from the bag and the shoulder-length glove was removed and placed in the biohazard trash. Nitrile gloves were removed and hands were washed prior to the next inoculation. Once clean gloves were donned, the bag containing the inoculated outside round was hand tumbled for 1 minute. Once tumbled, the bag was zip tied and placed outside the biosafety cabinet in a designated area for 30 minutes in order to allow bacterial attachment.

Preliminary Experiments

Utilization and Comparison of Neutralizers on Lactic acid-Treated Samples

The application of a neutralizer to a sample after treatment with an antimicrobial is thought to halt the effect of the antimicrobial during sample processing so as to eliminate the possibility of over estimation of antimicrobial efficacy. The purposes of this preliminary experiment were to determine whether a neutralizer is necessary for correct enumeration of STEC8 after treatment with L-lactic acid, and to compare the performance of various neutralizers to recover STEC8.

Preparation of Lactic acid Solution

A specified amount of water and stock L-lactic acid (Birko Corp., Henderson, CO, 88%) was measured out separately in order to obtain a final concentration of 2% L-lactic acid. Water was first heated to approximately 60°C prior to pouring the 88% lactic acid into the water. After adequate mixing, the solution was poured into a hand-pump sprayer (Roundup; model no. 190260, 10 psi) and sealed. The temperature of the solution being emitted from the nozzle was checked to be 55°C prior to spraying on the beef surface. Brisket pieces were hung inside a model spray cabinet within a biosafety cabinet and sprayed with the 2% lactic acid solution approximately 30 cm from the beef surface.

Product Procurement and Inoculation

Untrimmed briskets were obtained from a local grocery store and held at 4°C prior to use. To simulate the contamination of a carcass post-dehiding, the brisket pieces (approximately 6 X 8 inches in size) were warmed to an internal temperature of 37°C in

a water bath within 20 minutes prior to inoculation. Once 37°C internal temperature was reached, pieces were spray inoculated according to the protocol described previously. Pieces were left at room temperature for 30 minutes to allow for bacterial attachment.

Application of lactic acid treatment

After bacterial attachment, the inoculated briskets were hung in a model spray cabinet within a biosafety cabinet and treated by spraying with 2%, 55°C LA using the hand-pump sprayer for 30 (200 ml) or 45 s (250 ml) from a distance of 30 cm. Surface pH of the brisket was measured before and after treatment with a surface pH meter (ExStik®, model PH100).

Comparison of Neutralizers and Sample Collection

Prior to and after LA treatment application, three core samples of 10 cm^2 each were aseptically excised using a flame sterilized borer and scalpel. Samples were placed in sterile stomacher bags with 99 ml 0.1% sterile peptone water (PW) and stomached for 1 minute, serially diluted, and plated on TSAR. Samples were also collected from the brisket surface prior to inoculating to ensure that no rifampicin-resistant bacterial populations were present.

Immediately following treatment, 30-cm^2 surface samples were excised and placed in sealable bottles containing 100 ml of one of the following sterile neutralizing agents: peptone water (control), buffered peptone water (BPW), phosphate buffered saline (PBS) or Dey-Engley broth (D/E broth) for 2 minutes. Immediately following neutralization, samples were transferred using flame-sterilized forceps into a sterile

stomacher bag containing 99 ml peptone water to be stomached for 1 minute, serially diluted, and plated on TSAR. Because of the rifampicin resistance of the STEC8 inoculum, all colonies growing on the TSAR plates were assumed to be from the STEC8 cocktail and were therefore enumerated. Plate counts were converted into log values and compared between neutralizers and control.

In a second phase of this preliminary experiment, the methods described above were repeated except half of the samples were treated with a neutralizing step in sterile PW prior to stomaching, serial dilution, and plating on TSAR.

Fifty percent of samples treated with lactic acid were immediately placed in 100 ml sterile PW for 2 minutes before transferring samples to a sterile stomacher bag, diluting with 99 ml sterile 0.01% PW, stomaching, serially diluting, and plating on TSAR. The other half were treated with lactic acid and immediately placed in a sterile stomacher bag with 99 ml sterile 0.01% PW, stomached, serially diluted and plated on TSAR. Enumeration of STEC8 after treatment with or without a neutralizing step were compared.

Comparison of Lactic acid Brands in the Reduction of STEC8 on Beef Carcass Surfaces

As there were several brands available on the market, it was important to compare the efficacies of different brands before choosing which brand would be used for the remainder of experiments. The purpose of this preliminary experiment was to determine

whether a specific brand of L-lactic acid was more effective at reducing populations of STEC8 on beef carcass surfaces.

Inoculation

Untrimmed briskets were obtained from the local grocery store and held at 4°C and warmed to an internal temperature of 37°C prior to inoculation. Brisket pieces were inoculated according to an established spray inoculation protocol described in the following section, and then left at room temperature for 30 minutes to allow bacterial attachment.

Treatment and Sampling

Preparation of lactic acid solution was completed as described previously. All brisket pieces were sampled by aseptically excising three core samples of 10 cm^2 each with a flame sterilized borer and scalpel after the attachment period and prior to treatment with one of three brands of L-lactic acid. Samples were placed in sterile stomacher bags with 99 ml peptone water, pummeled for 1 minute, and serially diluted and plated on TSAR. Pieces were treated with 2% LA solutions prepared with 3 different brands of L-lactic acid (Sigma-Aldrich (85%, St. Louis, MO), Birko (88%, Henderson, CO), or Purac (88%, Lenexa, KS)). Post treatment samples were then aseptically excised as described above, stomached in 99 ml sterile peptone water, serially diluted and plated on TSAR. Log counts were compared to determine differences between brands.

Comparison of Slaughter Scenarios

Contamination of the carcass can occur at several points in the beef harvesting process. Because of this, it is important to understand the efficacy of interventions applied to carcasses at different points in the slaughter process. The purpose of this preliminary experiment was to determine if different points of contamination in the slaughter process affected the reduction of STEC8 by 2% lactic acid.

Inoculation

Untrimmed briskets were obtained from the local grocery store and held at 4°C prior to use. Briskets were cut into pieces approximately 6 inches by 8 inches. Brisket pieces were spray inoculated as described in the following section according to three possible slaughter scenarios: A) Pieces were warmed to an internal temperature of 37°C, inoculated according to the spray inoculation protocol described above, and then left at room temperature for 30 minutes to allow bacterial attachment. B) Pieces were warmed to an internal temperature of 37°C, inoculated according to an established spray inoculation protocol, and were placed at 4°C overnight to be sampled and treated the following day. C) Chilled pieces with an internal temperature of 5°C were inoculated according to an established spray inoculation protocol and then placed at 4°C for 30 minutes to allow bacterial attachment.

Treatment and Sampling

Prior to treatment, three core samples of 10 cm^2 each were aseptically excised as described above from all briskets. Samples were placed in 99 ml 0.1% sterile peptone water to be stomached for 1 minute, serially diluted, and plated on TSAR. Pre-treatment

pH was recorded. All brisket pieces were then treated with 2% lactic acid upon completion of bacterial attachment. Immediately following treatment, three core samples of 10 cm^2 each were aseptically excised as described above and placed in 99 ml 0.1% sterile peptone water to be stomached for 1 minute, serially diluted, and plated on TSAR. Post treatment pH was recorded. The log reduction of STEC8 populations between different scenarios were compared.

Core Research

Comparison of Chemical Antimicrobials in the Reduction of STEC on Beef Carcass Surfaces

Procurement of Outside Rounds

Hot outside rounds (OR) were harvested at an FSIS-USDA inspected slaughter facility in Stephenville, TX immediately after slaughter. Then, these OR were placed in coolers containing instant hot packs (Dynarex, 5" X 9") and Mylar thermal blankets (Ever Ready First Aid) to ensure they stay warm, and transported to a nearby Texas A&M Extension Facility with a biosafety level 2 laboratory for inoculation within 1 hour after fabrication. Once at the biosafety level 2 laboratory, the surface pH and temperature as well as internal temperature were recorded and rounds were transferred into a large biohazard bag for inoculation. Prior to inoculation, three randomly selected outside rounds were sampled as described below to ensure that there were no naturally existing rifampicin-resistant organisms present.

Inoculation Procedure

Outside rounds ready for inoculation were moved into a biosafety cabinet and were inoculated following the standard protocol described above. Briefly, the STEC8 cocktail was sprayed on the fat side of the OR with a glove-protected hand and inside a large bag to prevent spills and aerosols. This inoculation procedure was expected to result in a STEC8 population of approximately 6-7 log CFU/cm^2 on the beef surface. After inoculation, the bag containing the inoculated outside round was hand tumbled for 1 minute. Once tumbled, the bag was zip tied and placed outside the biosafety cabinet in a

designated area for 30 minutes to allow bacterial attachment. Following the attachment period, the OR was placed in coolers with refrigerant to begin the chilling process during transport to the Texas A&M cooler. The total time between inoculation and arrival to the Texas A&M cooler did not exceed 3.5 h. Figure 1 depicts the steady temperature reduction during the chilling process between inoculation and upon arrival at Texas A&M University.

Chilling Process

To continue the chilling process begun during transport, the OR was hung on flame sterilized meat hooks inside a walk in cooler (4 m X 6 m) at 4 °C at the Texas A&M Food Microbiology Laboratory. A series of sanitized shelving units lined with aluminum foil to prevent dripping and cross-contamination between OR were set up in the walk in cooler and were used to hang the rounds to simulate the chilling process post-contamination. Outside rounds were left in the chiller for 36 hours prior to application of carcass interventions.

Application of Carcass Interventions

Pre-Intervention Sampling and Hanging of Outside Rounds

Following the chilling period, outside rounds were unhooked from the shelving unit (leaving the hook in the meat) and carefully placed in clean biohazard bags. Bags were placed in coolers containing ice packs for transport to the TAMU CFS, located off-campus. The transport time to the CFS did not exceed 30 min. Following transport, the rounds were immediately placed in the refrigerators (4°C) at the TAMU CFS to maintain cold temperatures prior to application of interventions. Surface temperature was

measured (ExStik®, model PH100) before transport to the CFS and immediately upon receipt at the CFS. The surface pH and temperature were measured and recorded using an ExStik®, model PH100 (ExTech Instruments, Nashua, NH) pH and temperature recorder. In addition, the internal temperature was measured with a type K needle probe thermocouple attached to a Total-Range digital thermometer (VWR, Radnor, PA). After pH and temperature recording, a pre-intervention surface sample of 30 cm^2 was aseptically excised as described above and placed in a sterile stomacher bag to be pummeled in a Stomacher at 230 RPM for 1 minute with 99 ml 0.1% sterile peptone water prior to serial dilutions and plating on TSAR. Once this sample was taken, the outside round was hung inside a model spray cabinet (Birko Corp., Centennial, CO) in a bioBUBBLE (bioBUBBLE, Fort Collins, CO) which consists of a 46 m^2 enclosure designed to contain biological hazards possibly present in the aerosols produced by application of treatments. The OR were hung in such an orientation to simulate a full carcass side hanging under commercial settings.

Preparation of Solutions

Each solution was prepared by calculating the amount of sterile tap water needed to be added to stock solutions in order to prepare a specified percentage solution to be applied. Heated solutions including 4.5% lactic acid and 0.02% peracetic acid were prepared by first heating the sterile tap water to approximately 5° above the target temperature, followed by adding the calculated amount of stock solution. Next, the temperature of solution being emitted from the conventional nozzle was measured to be 55°C for lactic acid and 42°C for peracetic acid. Due to the air-assisted nozzle present

on the electrostatic spray system, solutions emitted from this system were significantly cooled to approximately 25°C. All other solutions were not heated and therefore the stock solution could be mixed with the specified amount of water immediately prior to application of the treatment.

Treatment of Outside Rounds

The OR pieces were subjected to one of the following 5 treatments: sterile tap water, 4.5% (55°C) lactic acid (Purac, LA), 3% lauric arginate (A&B Ingredients: Cytoguard LA2X, 0.8% cetylpyridium chloride (Safe Foods: Cecure, CPC), and 0.02% (42°C) peroxyacetic acid (Birko: Birkocide, PAA). Each of these treatments were applied using a conventional hand pump sprayer (CS; Roundup, Marvsville, OH, 7.6 L, 10 psi (69 kPa)) or a hand-held electrostatic spraying system (ESS; Electrostatic Spraying Systems, Inc., Watkinsville, GA, 11.4 L, 30 psi (207 kPa)). The charge of solution emitted from the ESS was validated with a digital multimeter (Amprobe, model 15XP-B, ≤-10 amps). Consulting with the manufacturers was conducted to ensure that our model cabinet was sufficient to ground the outside round to be charged positively. For all treatments, the spray time was 1 minute, which resulted in the emission of 500 ml from the conventional sprayer and 126 ml from the ESS. Following treatment, the OR was allowed to drip for approximately 20 seconds before being removed from the spray cabinet. The OR were placed on a tray with the treated surface face-up. Post treatment surface samples of 30 cm^2 were aseptically excised as described previously and stomached at 230 RPM for 1 minute in 99 ml 0.1% peptone water prior to serial dilutions and plating on TSAR. Post treatment surface pH and temperature were

measured with a ExStik®, model PH100 hand-held pH and surface temperature reader, and the internal temperature of the OR was measured with a type K needle probe thermocouple attached to a Total-Range digital thermometer.

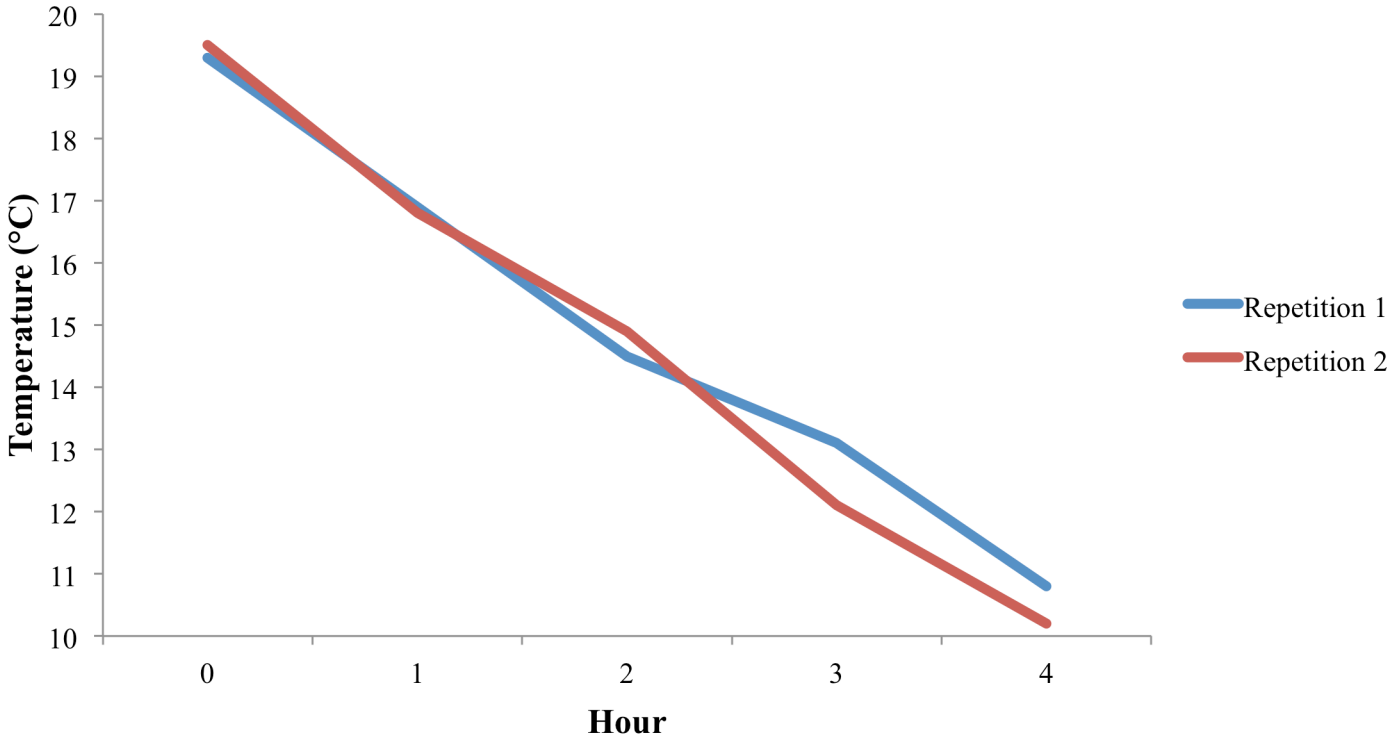

Figure 1. Beef surface temperature by chilling during transport after inoculation by hour

RESULTS AND DISCUSSION

Preliminary Experiments

Utilization and Comparison of Neutralizers on Lactic acid-Treated Samples

Little research has been published on the need for neutralizers after antimicrobial application and as a result there is not a standard protocol for their use. It is thought that some antimicrobials may have a residual effect, leading to an overestimation of antimicrobial efficacy. Martinez-Gonzalez et al. *(78)* studied the impact of a neutralizing step on oranges after application of sodium chlorite spray or lactic acid dip and found that a neutralizing step did not affect enumeration of pathogenic bacteria as compared to samples not treated with a neutralizer.

In this study, 4 neutralizers were compared, including buffered peptone water (BPW), Dey-Engley broth (D/E), phosphate buffered saline (PBS) and peptone water (PW) as a control after application of 2% lactic acid spray. Log reductions in populations of STEC8 were observed by subtracting the pre-treatment counts from the post-treatment counts on the same piece of brisket.

As shown in Table 1, there was no significant difference ($P > 0.05$) in log CFU/cm^2 reductions of STEC achieved between different neutralizers used. Reductions for those samples treated with 2% lactic acid for 30 s followed by treatment with BPW, D/E, PBS, and PW were 1.2, 1.7, 1.6, and 1.3 log CFU/cm^2. Log reductions between samples treated with each neutralizer were not significantly different. In addition, there was no significant difference ($P > 0.05$) between samples treated for 30 s and those

treated with 45 s. Samples treated with 2% lactic acid for 45 s followed by treatment with BPW, D/E, PBS, and PW showed reductions of 1.0, 1.3, 1.6, and 1.5 log CFU/cm^2. Log reductions between samples treated with each neutralizer were not significantly different ($P > 0.05$). Based on these results, PW, the cheapest and easiest alternative, was chosen as the neutralizer to be evaluated in the following preliminary study. It was also made clear that under these circumstances, specifically treatment with 2% L-lactic acid, spraying for a longer amount of time (45 s versus 30 s) did not make a significant difference in log CFU/cm^2 reduction of STEC8. In fact, in the case of samples treated with BPW and D/E, samples treated with lactic acid for 45 s showed numerically smaller reductions than those samples treated for 30 s.

The next phase of this preliminary experiment examined the necessity of a neutralizing step and its effect on the populations of STEC after treatment with lactic acid. Those samples not treated with a neutralizing step were placed in diluent immediately to be serially diluted and plated. As seen in Figure 2, there was no significant difference ($P > 0.05$) between samples treated with a neutralizing step and those not. Samples treated with a neutralizing step showed a 1.5 log CFU/cm^2 reduction of STEC whereas samples with no neutralizing step showed only a slightly greater reduction of 1.6 log CFU/cm^2. These values were not significantly different ($P > 0.05$). Based on these results, it was concluded that a neutralizing step was not necessary for the proper enumeration of STEC on beef carcass surfaces after treatment with L-lactic acid.

Table 1. *Least square mean of the reductions*[a] *(log CFU/cm*^{2}*) of Shiga toxin-producing* E. coli *by treatment with 2%, 55°C lactic acid followed by neutralizer treatment*

	Spray Time	
Treatment[b]	30 s	45 s
Buffered peptone water (BPW)	1.2	1.0
D/E broth (D/E)	1.7	1.3
Phosphate buffered saline (PBS)	1.6	1.6
Peptone water (PW)	1.3	1.5

[a]Reduction = (log CFU/cm^2 pre-treatment) - (log CFU/cm^2 post-treatment)
[b]Immersion of samples in 100 ml BPW, D/E, PBS, or PW for 120 s
There was no significant difference ($P > 0.05$) between neutralizers or length of spray time; Root Mean Square Error = 0.3019.

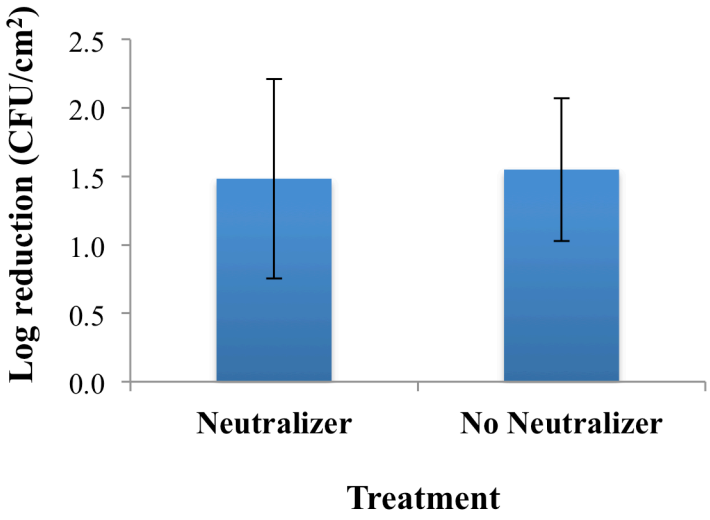

Figure 2. Log CFU/cm^2 reductions in Shiga toxin-producing *E. coli* by 2% lactic acid followed by a neutralizing step or no neutralizing step
There was no significant difference ($P > 0.05$) between log reductions achieved with or without a neutralizer.

Comparison of Lactic acid Brands in the Reduction of STEC8 on Beef Carcass Surfaces

Several brands of L-lactic acid were available on the market. It was necessary to determine whether one had a higher antimicrobial capacity than the others when used for reducing STEC populations on beef carcass surfaces. The three different 2% lactic acid solutions were prepared by mixing stock solutions (Sigma: 85%, Birko: 88%, and Purac: 88%) with the necessary amount of water to create a 2% solution as described in the previous section. As seen in Figure 3, brand 3 (Purac) achieved numerically greater reductions (1.5 log CFU/cm^2) compared to brands 1 and 2 (Sigma: 1.3 log CFU/cm^2 and Birko: 1.3 log CFU/cm^2, respectively), but there was no significant difference ($P > 0.05$) in reductions achieved between the 3 brands. For cost and availability reasons, Purac was determined to be the brand that would be used in all future experiments.

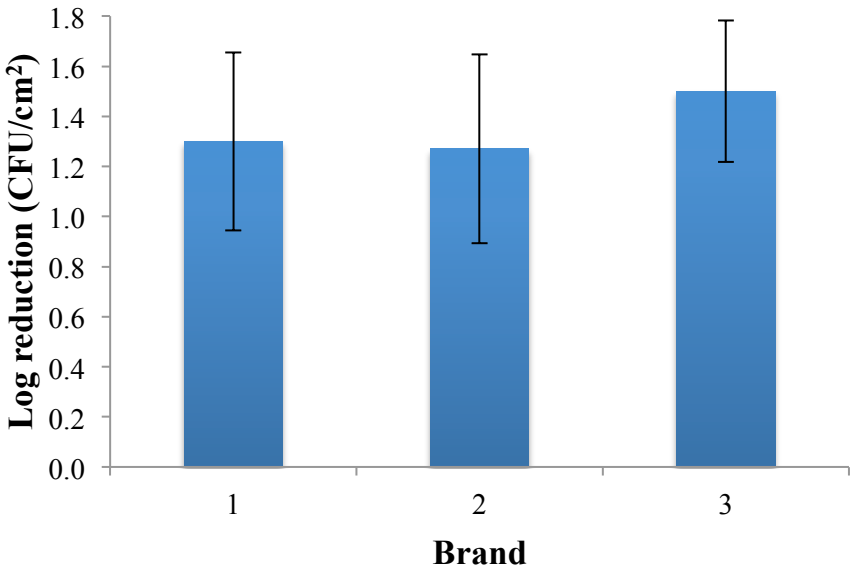

Figure 3. Log CFU/cm^2 reductions in Shiga toxin-producing *E. coli* by different brands of 2% lactic acid. There were no differences ($P > 0.05$) in reductions obtained by all brands of lactic acid.

Comparison of Slaughter Scenarios

The purpose of this preliminary experiment was to determine how different points of contamination, whether occurring prior to or after chilling, in the slaughter process affects the efficacy of a carcass intervention to reduce STEC on beef carcass surfaces. The intervention used was a lactic acid spray. The three scenarios tested simulated potential points of carcass contamination during slaughter. The first involved inoculating the brisket piece when it had an internal temperature of approximately 37°C, allowing STEC to attach to the surface of the meat for 30 minutes at room temperature before applying the intervention. In the second scenario, brisket pieces were inoculated when they had an internal temperature of approximately 37°C, allowing STEC to then attach overnight at 4°C. For the third scenario, brisket pieces with an internal temperature of approximately 4°C were inoculated and STEC was allowed to attach to the brisket surface for 30 minutes at 4°C. As shown in Figure 4, reductions in STEC achieved by the three scenarios were 2.4, 2.7 and 1.3 log CFU/cm^2, respectively. Scenarios 1 and 2 resulted in significantly greater ($P < 0.05$) log reductions than scenario 3. Scenario two represents the most common scenario for carcass contamination, whereas scenario 1 represents a less common but likely risk of cross contamination in the slaughter process. The lower reduction associated with scenario 3 may have been due to the lower temperature of inoculation and attachment.

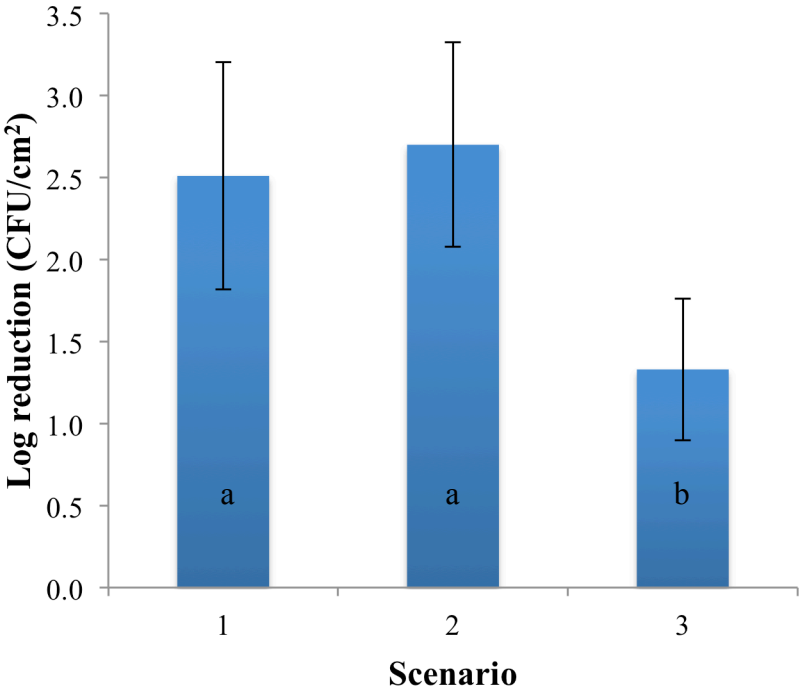

Figure 4. Log CFU/cm^2 reductions in Shiga toxin-producing *E. coli* with water wash followed by 2%, 55°C lactic acid spray. Scenario 1: Inoculate hot (37°C), attach at room temperature (25°C). Scenario 2: Inoculate hot (37°C), attach overnight in chiller (4°C). Scenario 3: Inoculate chilled (4°C), attach in chiller (4°C). Scenarios marked with different letters differ statistically ($P < 0.05$).

Core Research

Comparison of Chemical Antimicrobials in the Reduction of STEC on Beef Carcass Surfaces

The purpose of this research was to compare the efficacies of different antimicrobials in the reduction of STEC on chilled beef carcass surfaces, as well as to compare the efficacies of ESS and a conventional spray as application methods for these antimicrobials.

According to data in Table 2, W, LA, LAE, CPC and PAA applied with CS achieved reductions in STEC populations of 0.2, 3.2, 2.3, 1.6 and 1.0 log CFU/cm^2, respectively, whereas the same treatments applied with ESS reduced STEC populations by -0.1, 1.7, 2.2, 1.2, and 0.7 log CFU/cm^2, respectively. As shown in Figure 5, lactic acid applied with conventional spray yielded significantly greater reductions ($P < 0.05$) compared to lactic acid applied with electrostatic spray. This may have been due to the temperature differential between LA solution emitted by CS (55°C) and LA applied with ESS (55°C in tank, approximately 25°C coming out of the air assisted nozzle).

Table 3 shows the pre- and post-treatment surface pH readings resulting from application of various treatments W, LA, LAE, CPC and PAA. When using a conventional spray to apply LA, LAE and PAA, the pH was reduced from 6.2 to 2.8, 6.5 to 3.8, and 6.2 to 4.7, respectively. When using an ESS to apply these antimicrobials, the pH was reduced from 6.2 to 3.0, 6.2 to 4.1, and 6.1 to 4.9, respectively. As expected, the pH was not reduced by the application of STW or CPC regardless of the method of application. Lactic acid produced significantly greater pH reductions than all other

treatments. There were no significant differences ($P > 0.05$) in pH changes among treatments between CS and ESS. This suggests that if there was a difference in efficacy among treatments between CS and ESS, the difference was the result of some other factor, not pH.

Table 4 shows the increase in temperature after application of all treatments. As expected, the two heated treatments, LA and PAA, applied with CS achieved the significantly ($P < 0.05$) highest increase in beef surface temperature (14.4 and 12.7, respectively). Heated treatments applied with ESS were not heated upon nozzle exit, as explained previously. This increase in surface temperature has been proved to be only momentary. In a previous study, Castillo et al. *(24)* reported that spraying of cold carcasses with warm LA resulted in an increase of carcass surface temperature of 1.6°C, and this temperature returned to chilling temperatures within 10 s. This study was also completed under refrigerated conditions with full carcasses. This should eliminate potential concerns from the industry about carcass temperature increase due to LA spray. In the present study, the outside rounds were taken from refrigerated storage to a model cabinet inside a bioBUBBLE at room temperature. This could also have caused a relatively greater temperature increase. This is consistent with the findings of King et al. *(69)*, where a 12°C surface temperature increase was observed after application of 43°C PAA to carcass pieces when completed in a laboratory setting at room temperature.

As shown in Table 2, lauric arginate ester produced the greatest reductions (2.2 log CFU/cm^2) in STEC8 counts among treatments applied via ESS, however reductions produced by applying LA with ESS (1.7 log CFU/cm^2) were not significantly different.

Still, LAE applied with CS achieved reductions that were not significantly different than reductions produced by LAE applied with ESS. Peracetic acid was the least effective antimicrobial in the reduction of STEC with reductions of 1.0 and 0.7 log CFU/cm^2 for CS and ESS, respectively; Reductions produced by peracetic acid were not significantly different ($P > 0.05$) than those produced by water alone, in any case. Lactic acid applied with CS achieved the numerically greatest reduction (3.2 log CFU/cm^2) of STEC among all treatments, but reductions achieved by conventional application with LAE (2.3 log CFU/cm^2) were not significantly different ($P > 0.05$) than LA applied with CS.

These results, specifically those obtained after CS application of LA, can be loosely compared to reductions obtained in previous studies. Castillo et al. *(29)* described a post-chill reduction of 2.0-2.4 log CFU/cm^2 *E. coli* O157:H7 with 4% lactic acid. However, results obtained in the present study showed a greater reduction (3.2 log CFU/cm^2) in STEC compared to reductions reported by Castillo et al. *(29)*. It is important to note the concentration of lactic acid solution prepared in the present study was slightly higher at 4.5%, which may have resulted in a greater reduction. Other important differences between the previous study *(26)* and this study include the use of a cocktail of 8 strains of STEC as inoculum, as opposed to a cocktail of one strain each of *E. coli* O157:H7 and *Salmonella* in the previous study, and the method of inoculation. The previous study described inoculating the carcass surface using a pathogen-inoculated fecal suspension with washed cells that was spread on the beef surface whereas the present study involved a spray inoculation method with unwashed cells.

Finally, the present study included prechill interventions like the previous study, which resulted in reductions up to 5.2 log CFU/cm^2 when treated with water and lactic acid. However, the prechill interventions applied to the beef in the present study were applied prior to inoculation. Considering the differences in experimental design and pathogens used, the slightly greater postchill reduction by 4.5% lactic acid in the present study is still comparable to the results obtained in the previous study.

Lauric arginate ester has been studied less frequently with regards to beef and STEC. One study conducted by Dias-Morse et al. *(41)* reported a 1.4 log CFU/g in STEC. This study is not comparable to the present study, since Dias-Morse et al. *(41)* applied the intervention at a much higher concentration (5% as opposed to 3%), and the volume of solution applied by these authors (about 160 ml) to the beef trimmings was lower than the volume of lauric arginate ester applied in the present study (500 ml) applied to the beef outside round. The volume and concentration applied in the present study was calculated based on the weights of the outside rounds and percentage pickup in the interest of following federal guidelines. However, many studies have been completed regarding the use of lauric arginate ester in the reduction of *Listeria monocytogenes* in RTE meats *(74, 87)*. More work must be completed to understand the antimicrobial capacity of LAE against STEC on chilled beef carcass surfaces.

Although cetylpyridinium chloride is not currently approved for use in beef processing, its success in postchill applications to poultry carcasses for the reduction of *Salmonella* has spurred interest in its application in the beef industry *(103, 104)*. Due to the fact that the majority of research with CPC on beef has been its application in poultry

or as a beef hide intervention *(18, 80, 103, 104)*, it is difficult to compare the present studies to any previous studies published in the literature. The very low reductions (1.0 log CFU/cm^2) achieved by 0.02% PAA were expected based on the minimal reductions of *E. coli* O157:H7 and *Salmonella* in postchill application reported by King et al. *(69)*. Although not directly comparable due to differences in experimental design, it was clear that peracetic acid may not be the most effective intervention tested in the present study.

Regarding the potential for use of ESS to apply interventions on carcasses, very little research has been published about benefits of this technology, especially using hand-held ESS in beef applications. Therefore, it was not possible to compare the present study to studies conducted in the industry and come to meaningful conclusions. The hand-held ESS used in the present study has the potential for success in a small scale beef processing facility, yielding less water and antimicrobial waste than conventional spray. Phebus et al. *(83)* reported the success potential of large-scale cabinetry with ESS installed within. More research within the industry is needed to optimize beef safety with this application method. An advantage of the large-scale system described above is its multiple (8) nozzles, resulting in potentially greater coverage and thus higher efficacy than what the hand-held single nozzle is capable of.

Table 2. *Least square mean reductions*[a] *(log CFU/cm^2) of Shiga toxin-producing* E. coli *by treatments applied with conventional or electrostatic spray*

	Spray Type	
Treatment[b]	Conventional	ESS
W	0.2_{EF}[c]	-0.1_{F}
LA	3.2_{A}	1.7_{BCD}
LAE	2.3_{AB}	2.2_{BC}
CPC	1.6_{BCD}	1.2_{CDE}
PAA	1.0_{DEF}	0.7_{DEF}

[a] Reduction = (log CFU/cm^2 pre-treatment) - (log CFU/cm^2 post-treatment)

[b] W, 25°C sterile water spray; LA, 4.5%, 55°C lactic acid spray; LAE, 3%, 25°C lauric arginate ester spray; CPC, 0.8%, 25°C, cetylpyridinium chloride spray; PAA, 0.02%, 43°C, peracetic acid spray

[c] Numbers with the same letters are not significantly different (P > 0.05); Root Mean Square Error = 0.5613.

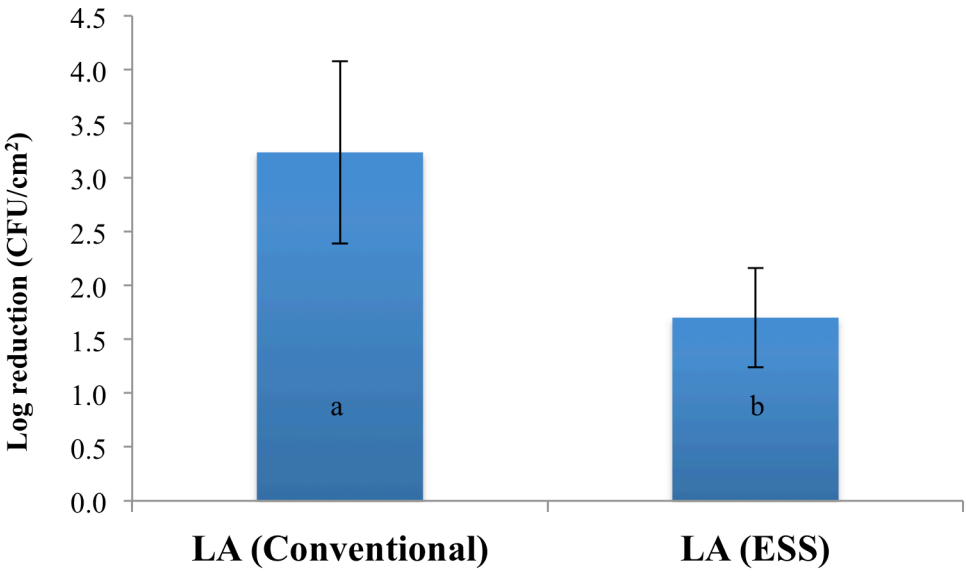

Figure 5. Log CFU/cm² reductions in Shiga toxin-producing *E. coli* by 4.5% lactic acid applied with conventional or electrostatic spray

[a,b] Columns with different letters differ statistically ($P < 0.05$)

Table 3. *Means of pre- and post-treatment pH taken from outside round surface regions treated with conventional or electrostatic spray*

		pH	
Treatment[a]	Spray type[b]	pre[e]	post[f]
25°C Water	CS[c]	6.0	6.6
	ESS[d]	5.9	6.3
4.5 %, 55°C LA	CS	6.2	2.8
	ESS	6.2	3.0
3%, 25°C LAE	CS	6.5	3.8
	ESS	6.2	4.1
0.8%, 25°C CPC	CS	6.2	6.5
	ESS	6.3	6.5
0.02%, 42°C PAA	CS	6.2	4.7
	ESS	6.1	4.9

[a]60 s spray with CS or ESS
[b]CS: Conventional spray, ESS: Electrostatic spray
[c]10 psi (69 kPa), 500 ml
[d]30 psi (207 kPa), 126 ml
[e]Mean of 3 surface pH readings pre-treatment
[f]Mean of 3 surface pH readings post-treatment

Table 4. *Means of pre- and post-treatment surface temperature from outside rounds treated with conventional or electrostatic spray*

Treatment[a]	Spray type[b]	Temperature	
		pre[e]	post[f]
25°C Water	CS[c]	8.2	18.3
	ESS[d]	7.9	17.9
4.5 %, 55°C LA	CS	8.4	22.7
	ESS	8.1	18.4
3%, 25°C LAE	CS	7.7	17.5
	ESS	7.9	16.4
0.8%, 25°C CPC	CS	7.4	17.5
	ESS	8.5	17.3
0.02%, 42°C PAA	CS	8.2	20.9
	ESS	7.6	17.8

[a]60 s spray with CS or ESS
[b]CS: Conventional spray, ESS: Electrostatic spray
[c]10 psi (69 kPa), 500 ml
[d]30 psi (207 kPa), 126 ml
[e]Mean of 3 surface temperature readings pre-treatment
[f]Mean of 3 surface temperature readings post-treatment

CONCLUSIONS

This work determined that chemical antimicrobials must be properly paired with spray technologies in order to optimize beef safety. Lactic acid worked most effectively when applied with CS instead of ESS, and was the most effective chemical antimicrobial tested. This antimicrobial was the only one of the treatments to cause statistically significant differences in STEC reduction between different spray technologies. Peracetic acid did not have any advantage over water alone, in any case. Lauric arginate ester produced numerically similar reductions of STEC8 between conventional and electrostatic spray, indicating that this application method may be efficacious for only specific antimicrobials. Although a novel and potentially efficacious technology, more research must be done with the hand-held ESS in order to optimize its use in small plants in the beef industry.

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