THESIS

EFFICACY OF ANTIMICROBIAL COMPOUNDS AGAINST *SALMONELLA* SPP., *ESCHERICHIA COLI* O157:H7, NON-O157 *ESCHERICHIA COLI*, AND NON-PATHOGENIC *ESCHERICHIA COLI* ON BEEF AND POULTRY

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ABSTRACT

EFFICACY OF ANTIMICROBIAL COMPOUNDS AGAINST *SALMONELLA* SPP., *ESCHERICHIA COLI* O157:H7, NON-O157 *ESCHERICHIA COLI*, AND NON-PATHOGENIC *ESCHERICHIA COLI* ON BEEF AND POULTRY

Three experiments were conducted exploring efficacy of antimicrobial acids against pathogenic organisms on beef and poultry products. In experiment A, two studies were conducted to evaluate the antimicrobial efficacy of a commercially available blend of sulfuric acid and sodium sulfate (SSS) against *Salmonella* (i) at different immersion times, using differing rinse methods, (ii) and to compare efficacy against other common antimicrobial chemicals applied on the surface of whole chicken wings. Chicken wings were spot inoculated with 200 µl of inoculum; (5-strain mixture of novobiocin and nalidixic acid-resistant *Salmonella*) left for 20 min for cell attachment. For each treatment, wings were immersed in 350 ml of chemical and allowed to drip for 5 min before being placed in 150 ml of designated rinse buffers (buffered peptone water [BPW] or Dey/Engley [D/E] neutralizing buffer) and surface plated, incubated, and counted. For the first study, wings were randomly assigned to one of four treatment categories: (i) 10 s immersion treated + buffered with D/E, (ii) 10 s + BPW, (iii) 20 s + D/E, and (iv) 20 s + BPW. In the second study, wings were assigned in a random order to one of six treatments: (i) SSS at pH 1.1 via immersion for 20 s and stored 0 h, (ii) SSS at pH 1.1 via immersion for 20 s and stored 24 h, (iii) cetylpyridinium chloride CPC at 4000 ppm via immersion for 10 s and stored at 0 h, (iv) CPC 4000 at ppm via immersion for 10 s and stored 24 h, (v) peroxyacetic acid PAA at 700 ppm via immersion for 20 s and stored 0 h, and (vi) PAA at

700 ppm via immersion for 20 s and stored 24 h. In the first study, plate counts did not differ ($P > 0.05$) based on rinse type, but were influenced ($P < 0.05$) by treatment time. Immersion treatment of chicken wings for 10 or 20 s in SSS resulted in a reduction ($P < 0.05$) of *Salmonella* populations on all agars. In the second study, an interaction was detected between chemical and length of storage time ($P < 0.05$). All treated chicken wing *Salmonella* plate counts were less ($P < 0.05$) than the untreated control wing *Salmonella* counts for both agars. Results indicated that pH 1.1 SSS used as an immersion treatment for 20 s was an effective antimicrobial intervention to reduce *Salmonella* on inoculated chicken wings.

Experiment B, was conducted to: (i) validate that inoculants of non-pathogenic *Escherichia coli* effectively serve as surrogates for pathogenic *Escherichia coli* O157:H7, non-O157 shiga toxin-producing *Escherichia coli* (STEC), and *Salmonella* spp. when warm beef carcass tissue was treated with SSS, and (ii) validate that SSS applied between low and high pH and pressure levels was an effective intervention when applied to warm beef carcass surface tissue. The study was repeated over three days with two samples analyzed per treatment on each day (n = 6). This study utilized four inoculum, including: a 5-strain mixture of *E. coli* O157:H7, a 12-strain mixture of non-O157 STEC (two strains per serogroup), a 6-strain mixture of *Salmonella*, and a 5-strain mixture of non-pathogenic *E. coli*. Treatment application parameters included: (i) two SSS pH levels (pH 1.5 [high] and pH 1.0 [low]), and (ii) two application pressures (13 psi [low] and 22 psi [high]) for a total of four treatments. Samples were assigned randomly to one of the four possible SSS treatments which were administered in a custom-built spray cabinet (Chad Co., Olathe, KS) designed to simulate commercial beef slaughter floor operations. The surrogate and pathogen inocula responded similarly ($P > 0.05$), to application of SSS at high and low parameters on warm beef carcass surface tissue, making the surrogate

inoculum a viable option to use in a plant for validation purposes. Treatments at all parameter combinations reduced ($P < 0.05$) bacterial contamination from 6.3 log CFU/cm^2 to 4.7 to 5.7 log CFU/ cm^2. Based on the findings of this study, SSS was an effective ($P < 0.05$) antimicrobial intervention for the surrogate and pathogen inocula when applied between the high and low pH and pressure parameters on warm beef carcass surface tissue.

Experiment C, consisted of two studies that were designed to (i) determine if inoculants of non-pathogenic *Escherichia coli* effectively served as surrogates for pathogenic *Escherichia coli* O157:H7, non-O157 shiga toxin-producing *Escherichia coli* (STEC), and *Salmonella* spp. when warm beef carcass tissue was treated with a commercially available blend of lactic acid and citric acid (LCA) and (ii) investigate the efficacy of LCA between low and high application parameters (pH, pressure, temperature) when applied to warm beef carcass surface tissue, and (iii) utilize *E. coli* surrogate organisms to validate the use of hot water pasteurization in combination with LCA as a carcass antimicrobial spray intervention in a commercial beef harvest facility. Beef tissue (10 x 10 cm^2) was randomly assigned to either a (i) before treatment or (ii) after treatment group for each inoculum mixture. Four inoculum mixtures were used: a 5-strain mixture of *E. coli* O157:H7, 12-strain mixture of non-O157 STEC, a 6-strain mixture of *Salmonella*, and a 5-strain mixture of non-pathogenic *E. coli* (6.0 log CFU/cm^2). The solution of LCA was applied onto inoculated beef tissue in a spray cabinet between low and high application parameters of temperature (43, 60 ^{o}C), concentration (1.9, 2.5%), and application pressure (15, 30 psi). In-plant surrogate organisms were inoculated onto beef carcasses at a commercial beef harvest facility to evaluate the efficacy of the use of hot water pasteurization (92.2 to 92.7^{o}C at 13 to 15 psi) combined with a LCA spray intervention (1.9%; 13 to 15 psi; 50 to 51.7°C). Surrogate performance under these conditions were compared to the performance of each

pathogen; they responded similarly ($P > 0.05$) to the application of LCA. All treatment combinations effectively reduced ($P < 0.05$) the surrogate bacteria. A hot water pasteurization cabinet and a LCA spray cabinet used at these parameters ($P < 0.05$) reduced bacterial contamination on inoculated beef carcasses, thus validating effectiveness of the intervention as a system.

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DEDICATION

This thesis is dedicated to Dr. Robert J. Delmore. Your inspiration, guidance, and support as my advisor and mentor has helped me achieve my goals and taught me to believe that dreams are attainable if you work hard.

TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGEMENTS vi

DEDICATION viii

LIST OF TABLES xi

Chapters

1. Introduction 1
2. Review of Literature 5
   - 2.1. Meat safety 5
   - 2.2. *Escherichia coli* O157:H7/non-O157 STEC 10
   - 2.3. *Salmonella* spp. 15
   - 2.4. Pathogen contamination of beef 20
   - 2.5. Pathogen contamination of poultry 27
   - 2.6. Common chemical treatments of beef 31
   - 2.7. Common chemical treatments of poultry 37
   - 2.8. Process control and validation 42
3. Experiment A 45
   - Summary 45
   - Introduction 46
   - Materials and Methods 47
   - Results and Discussion 52

4. Experiment B 64

Summary 64

Introduction 65

Materials and Methods 67

Results and Discussion 70

5. Experiment C 81

Summary 81

Introduction 82

Materials and Methods 84

Results and Discussion 89

References 101

LIST OF TABLES

Table **Page**

3.1. Least squares mean (SE) plate counts (log CFU/ml) obtained from xylose lysine deoxycholate agar with novobiocin and nalidixic acid (XLDNN), tryptic soy agar with NN (TSANN), and tryptic soy agar (TSA) obtained from inoculated chicken wing samples after each treatment compared to the untreated controls. **60**

3.2. Least squares mean plate counts (SE) obtained from tryptic soy agar (TSA) and xylose lysine deoxycholate agar supplemented with nalidixic acid and novobiocin (XLDNN) of inoculated chicken wings treated with different antimicrobial chemicals and stored at different times compared to untreated control chicken wings. **61**

3.3. Least squares means for L* (white/black; SE), a* (red/green; SE) and b* (yellow/blue; SE) measurements of chicken wings at different storage times. **62**

3.4. Least squares mean (SE) pH measurements of chicken wings treated with different antimicrobial chemicals and stored at different times. **63**

4.1. For each treatment combination, P-values express the direct comparison of each pathogen to the surrogates for adjusted treated plate count results (initial plate counts used as a covariate) obtained with selective media (tryptic soy agar with rifampicin [TSA + rif] and xylose lysine deoxycholate [XLD] agar) from treated beef samples..**75**

4.2. For each treatment combination, P-values express the direct comparison of each pathogen to the surrogates for adjusted treated plate count results (initial plate counts used as a covariate) obtained with tryptic soy agar from treated beef samples.. ....**76**

4.3. Adjusted least squares mean (SE) plate counts (log CFU/cm^2) obtained with tryptic soy agar with rifampicin [TSA + rif] and xylose lysine deoxycholate [XLD] agar from warm carcass surface tissue after treatment of sulfuric acid and sodium sulfate applied at high and low pH compared to the untreated plate counts for each inoculum type..**77**

4.4. Adjusted least squares mean (SE) plate counts (log CFU/cm^2) obtained with tryptic soy agar from warm beef carcass surface tissue with an application of sulfuric acid and sodium sulfate at high and low pH compared to the untreated before plate counts for each inoculum type.............**78**

4.5. Adjusted least squares mean (SE) treated plate counts (log CFU/cm^2) obtained with tryptic soy agar (TSA) and tryptic soy agar with rifampicin (TSA + rif) from warm beef carcass surface tissue with an application of sulfuric acid and sodium sulfate at high and low pH, compared to the untreated before plate counts for the surrogate inoculum; P-values < 0.05 are significant...........**79**

4.6. Least squares means for pH (SE) of warm beef carcass tissue treated with sulfuric acid and sodium sulfate at different treatment parameters, high (pH 1.5 and 22 psi) and low (pH 1.0 and 13 psi), compared to the untreated control pH samples; P-values < 0.05 are significant...................**80**

5.1. For each treatment combination, P-values express the direct comparison of each pathogen to the surrogates for plate count results obtained with Tryptic Soy Agar (TSA) from treated beef samples..**95**

5.2. Adjusted least squares mean (SE) plate counts (log CFU/cm^2) on tryptic soy agar from warm beef carcass surface tissue with an application of lactic acid and citric acid (LCA) spray treatment, inoculated with different pathogen groups. **96**

5.3. Adjusted least squares mean (SE) plate counts (log CFU/cm^2) on selective media from warm beef carcass surface tissue with an application of a blend of lactic acid and citric acid (LCA) spray treatment, inoculated with different pathogen groups; P-values < 0.05 are significant. **97**

5.4. Adjusted least squares mean (SE) plate counts (log CFU/cm^2) on tryptic soy agar and tryptic soy agar with rifampicin from warm carcass surface tissue with an application of a blend of lactic acid and citric acid (LCA) spray for the surrogate inoculum; P-values < 0.05 are significant. **98**

5.5. Adjusted least squares mean (SE) *Enterobacteriaceae* counts (log CFU/cm^2) from warm beef carcass surface tissue before and after treatments of hot water pasteurization (H.P.; ≥ 70°C [carcass surface water], cabinet water temperature 92.2 -92.7ºC, 13-15 psi) and lactic acid and citric acid (LCA) spray cabinet (1.9% LCA, 13-15 psi, 50 – 51.7°C); P-values < 0.05 are significant. **99**

5.6. Adjusted least squares mean (SE) aerobic plate counts (log CFU/cm^2) from warm beef carcass surface tissue before and after treatments of hot water pasteurization (H.P.; ≥ 70°C [carcass surface water], cabinet water temperature 92.2 – 92.7ºC, 13-15 psi) and lactic acid and citric acid (LCA) spray cabinet (1.9% LCA, 13 - 15 psi, 50 - 51.7°C); P-values < 0.05 are significant.................**100**

CHAPTER 1

Introduction

Food safety is a concern for consumers as they become more worried and curious about where their food is coming from (Cross, 2004). With the increasing concern and demands from the consumer, producers and processers have increased efforts to control pathogens that are responsible for foodborne illness. After the *Escherichia coli* O157:H7 outbreak in 1993 associated with consumption of undercooked ground beef patties at Jack-in-the-Box, the meat industry was identified as the problem by consumers and has responded by revolutionizing the control of many foodborne pathogens (CDC, 1994). Examples of pathogens causing concern in the meat industry are: pathogenic *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* spp. to name a few (Sofos, 2008). Both meat and poultry products are associated with major foodborne pathogen that frequently cause illness such as *E. coli* O157:H7, non-O157 shiga toxin-producing *E. coli*, and *Salmonella* spp. (CDC, 2014a; Sofos, 2008).

Physical and chemical decontamination treatments of meat and poultry products are used to prevent, eliminate and reduce pathogens to acceptable levels in U.S. processing plants to prevent foodborne infection (Bolder et al., 1998; Deumier, 2004; Huffman et al., 2002; Sofos, 2008). Years 2009 to 2010, *Salmonella* was responsible for 49% of the foodborne-related outbreaks, followed by shiga toxin-producing *Escherichia coli* contributing to 16% of the illnesses (CDC, 2013). Beef was responsible for 13% of foodborne illnesses in 2013 (CDC, 2013). Outbreaks of *Salmonella* causing human infection have been sourced to several further processed chicken products (CDC 2010, 2013, 2014). The recent serotype of interest associated with chicken products was identified as *Salmonella* Heidelberg causing human infections in 2013 and 2014 resulting in 33 hospitalizations (CDC 2013, 2014).

Preventing foodborne illness by applying antimicrobial interventions onto meat and poultry products is a priority for the industry and the United States Department of Agriculture's Food Safety Inspection Service (USDA-FSIS, 2011). To address the concern for meat safety, FSIS developed a Strategic Plan for 2011 to 2016 which included goals for control of foodborne pathogens for each fiscal year (USDA-FSIS, 2011). Some of the goals included maximizing compliance with food safety policies, improving public education in safe food handling, advancing employee training to maximize success in protecting public health, and effectively using scientific research to understand foodborne illness, pathogens of interest and emerging trends (USDA-FSIS, 2011).

Scientific investigation of antimicrobial intervention processes in meat and poultry must occur to achieve government and industry goals as well as consumer expectations (Sofos, 2008). Research, not only in the process but also on products that may be additionally contaminated, such as poultry parts or further processed beef, is a key factor in improving safety and meeting performance standards for *Salmonella* (Cosansu, 2010; Huffman, 2002; Sofos, 2005; USDA-FSIS, 2011). Antimicrobial intervention products should be easy to implement into existing systems, inexpensive, environmentally friendly, have no negative residual effects on the product, and perform to regulatory standards (Sinhamahapatra et al., 2004).

Since the 1990s, industry has increased efforts to control pathogen contamination in meat and poultry products (Wheeler et al., 2014). Progress for the control of pathogens in beef began after *E. coli* O157:H7 became an adulterant in ground beef in 1994 (USDA-FSIS, 2012; Wheeler et al., 2014). In 2011, six serotypes of shiga toxin-producing *E. coli* were declared an adulterant in addition to O157:H7 in ground beef products (USDA-FSIS, 2012; Wheeler et al., 2014). Industry and government agencies have invested in significant research efforts to reduce

pathogens in beef and have shown success. The United States Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) microbiological results for raw ground beef and raw ground beef components for *E. coli* O157:H7 and non-O157 STEC declined in positive samples from year 2001 to 2012 (0.77% positives to 0.24% positives; Wheeler et al., 2014).

Multiple hurdles technology is the most effective method for controlling risk of pathogens in meat and poultry products both pre- and post-harvest. These systems include multiple sequential interventions in a process to continually reduce risk of pathogens (Graves Delmore et al., 1998; Keeton et al., 2006; Koohmaraie et al., 2005; Pohlman et al., 2002; Sofos, 2008). Post-harvest interventions, both physical and chemical (i.e. immersion chilling, carcasses washes, steam pasteurization, hot water pasteurization, and chemical treatments) are the most effective interventions used in plants today (Koohmaraie et al., 2005; Sofos, 2008). There are a variety of chemical interventions, typically applied as a spray, that have been evaluated by the meat industry to determine most effective and economic applications for specific systems (Koohmaraie et al., 2005; Sofos, 2008). Examples of these chemicals include lactic acid, citric acid, sulfuric acid buffered with sodium sulfate, trisodium phosphate, peroxyacetic acid and others. All interventions used in meat processes to control hazards must be validated as effective or to prevent the hazards from being reasonably likely to occur

USDA-FSIS, as a part of HACCP, requires verification of process control in meat and poultry processing facilities (9 CFR 417, 2014). Processors accomplish this through verification procedures that support food safety interventions efficacy at controlling or preventing prevalent pathogen contamination in their products (9 CFR 417, 2014). Using pathogen testing or natural microflora to validate process control may be difficult due to the low number of bacteria cells to use as data to support effectiveness (Arthur et al., 2004). The use of high levels of surrogate

organisms may be a helpful mechanism to validate efficacy of a food safety intervention to provide evidence that a system is achieving the objective (Arthur et al., 2004).

Three experiments were conducted for this thesis. The objectives of these experiments were to determine efficacy of various antimicrobial compounds against pathogenic *Salmonella*, *Escherichia coli*, and non-pathogenic *Escherichia coli* on beef and poultry as surrogates for these pathogens at designated application parameter ranges for use in processing plants for validation purposes.

CHAPTER 2

Review of Literature

2.1. Meat Safety

The history of federal meat inspection began in the 1890s when European countries began to question the safety of meat they were importing from the United States (Hulebak and Schlosser, 2002). In 1891, congress gave the U.S. Department of Agriculture (USDA) the task of ensuring that meat exports would meet European requirements as well as begin ante-mortem and postmortem inspection of all livestock intended for consumption in the United States (Hulebak and Schlosser 2002; USDA-FSIS, 2014). In 1906 a novel written by Upton Sinclair, *The Jungle*, outraged American citizens with the graphic descriptions of unsanitary conditions of meat packing facilities that were portrayed in the novel. In response to this issue, Congress passed the Federal Meat Inspection Act of 1906 (FIMA), which become the first federal protection for meat safety (Hulebak and Schlosser 2002; USDA-FSIS, 2014).

The Federal Meat Inspection Act mandated ante- and postmortem inspection of all livestock (not poultry) and every carcass in both slaughter and processing facilities (Hulebak and Schlosser, 2002; USDA-FSIS, 2014). This also introduced the continued presence of USDA inspectors in meat processing plants along with veterinarians implemented by the USDA's Bureau of Animal Industry (Hulebak and Schlosser 2002; USDA-FSIS, 2014). Inspectors used primarily organoleptic techniques for inspecting meat products, with the main concern of presence of zoonotic disease from the live animal transmitting to humans through consumption of meat (Hulebak and Schlosser, 2002). Veterinarians helped USDA inspectors inspect both the livestock and carcasses for sign of disease; the sole purpose of meat inspection was to prevent

the diseased animals from entering the food supply (Hulebak and Schlosser 2002). The USDA inspectors also inspected the processing, operations and facilities of each plant ensuring sanitary dressing (Hulebak and Schlosser, 2002; USDA-FSIS, 2014). Inspectors viewed meat processing as an extension of slaughter and usually they did not inspect processed meat products individually, but more as a whole process; different from how they viewed every carcass in slaughter facilities (Hulebak and Schlosser, 2002).

After WWII, the meat industry began to change significantly with the growth of transportation (highways), the development of refrigerated trucks, and the growing population (USDA-FSIS, 2014). These changes allowed the meat industry to expand their business to urban areas and established a competition between different meat companies for customer business (USDA-FSIS, 2014). In 1926, the concern for safety of poultry was addressed when USDA began to offer voluntary poultry inspection to processors (USDA-FSIS, 2014). As demand for poultry products and ready-to-cook poultry increased, Congress passed the Poultry Products Inspection Act in 1957, which was very similar to the FMIA but addressed only inspection of poultry products with some notable differences (USDA-FSIS, 2011b).

In the 1950s and 1960s meat inspection began to focus not only on animal disease but also on visible contamination of products (USDA-FSIS, 2014b). Industry at this time was growing and new products were being created causing the processes to become more complex (USDA-FSIS, 2014b). Consumers developed concern with the invisible hazards that may be on meat products such as chemicals that could have been applied directly or indirectly to products (USDA-FSIS, 2014b). Therefore, in 1958, the Federal Drug and Cosmetic Act was amended with the Food Additive Amendment to include safety of ingredients that could be used in processed foods, which included animal drug residues in meat and poultry products (USDA-

FSIS, 2014b). Also in 1958, the Humane Methods of Slaughter Act was signed into law to require that livestock purchased by the government were to be harvested humanely; then, in 1978, this act was amended to include all livestock being slaughtered for human consumption to be harvested with humane methods (USDA-FSIS, 2014b). In 1967 and 1968, the Wholesome Meat Act and the Wholesome Poultry Act amended current federal meat inspection requirements (USDA-FSIS, 2014b). By 1965, federal meat and poultry inspection was merged into one program, reorganized again in 1971, and transferred to Animal and Plant Health Inspection Services (APHIS; USDA-FSIS, 2014b).

The Food Safety and Quality Services agency was created in 1977 to perform all the meat and poultry grading and inspection, removing responsibility from APHIS (USDA-FSIS, 2014b). The agency was renamed to the current Food Safety and Inspection Service (FSIS) in 1981 (USDA-FSIS, 2014b). Food safety, with a focus on microbiology, was a growing concern in the meat industry as addressed by FSIS (Hulebak and Schlosser, 2002; USDA-FSIS, 2014b). Issues such as *Salmonellosis*, visible fecal contamination or bruising, and other "invisible" hazards were addressed (Hulebak and Schlosser, 2002; USDA-FSS, 2014b). The FSIS developed a National Residue Program in 1967 as the regulatory mechanism oversee the level of potential residues such as pesticides and other chemical hazards that may be present in meat and poultry that are determined by Environmental Protection Agency (EPA) and FDA (Hulebak and Schlosser, 2002; USDA-FSS, 2014b).

Challenges increased as the meat and poultry industries grew and became more complex causing FSIS to explore ways to effectively and efficiently inspect the growing plants (Hulebak and Schlosser, 2002). A study conducted by a consulting firm, Allen and Hamilton, Inc., suggested to FSIS that they find a mechanism to move the responsibilities from the agency

inspectors and onto the processing plants; with this methodology, the inspectors would then play a verification role instead of acquiring responsibility and accountability for the establishments (Hulebak and Schlosser, 2002). The study conducted by the consulting company also suggested to FSIS that microbiological testing should be implemented on finished product from the plants to monitor their process control of microbial contamination (Hulebak and Schlosser, 2002). Due to the negative recommendations made to FSIS, suggestions were not widely accepted (Hulebak and Schlosser, 2002).

In 1993, there was a major outbreak of *E. coli* O157:H7 in the Pacific Northwest from undercooked ground beef patties served by a quick service restaurant, Jack-in-the-Box (Hulebak and Schlosser, 2002; USDA-FSIS, 2014b; Wheeler et al., 2014). Four hundred people became sick and 4 children died from this outbreak; this event was a turning point for meat safety in the United States (Hulebak and Schlosser, 2002; USDA-FSIS, 2014b; Wheeler et al., 2014). At the time of the outbreak, FSIS meat inspection was still primarily based on organoleptic evaluation. Later (circa 1994), FSIS pushed to make a change and asked for help from a "Blue Ribbon Task Force" to find what method would be best to prevent an outbreak such as this from occurring again (Hulebak and Schlosser, 2002; USDA-FSIS, 2014b; Wheeler et al., 2014).

Shortly after the outbreak in 1993, FSIS implemented what was referred to as the "Zero Tolerance Rule" to eliminate visible milk, ingesta or fecal material on beef carcasses to assist in control of *E. coli* O157:H7 in beef production (USDA-FSIS, 2006; Wheeler et al., 2014). Additionally, in 1994, *E. coli* O157:H7 was declared an adulterant in ground beef by FSIS (Wheeler et al., 2014). Based on recommendations from the Blue Ribbon Task Force, FSIS researched the benefits of perhaps implementing a Hazard Analysis and Critical Control Points (HACCP) regulatory system to assist in the control of foodborne pathogens in meat and poultry

products (Hulebak and Schlosser, 2002; USDA-FSIS, 2014b; Wheeler et al., 2014). Following publication of the Final Rule in July 1995, responsibility for food safety shifted from FSIS to the establishments (Hulebak and Schlosser, 2002; USDA-FSIS, 2014b; Wheeler et al., 2014). In 1996, FSIS developed a regulatory proposal that became the Pathogen Reduction and HACCP systems Final Rule (9 CFR 304, 1996). In this rule, FSIS established goals to reduce risk of foodborne illness associated with the consumption of meat and poultry products by ensuring that appropriate and reasonable measures were taken at each step in the process where potential hazards could enter the processing system and where procedures and technologies exist or could be developed to prevent or eliminate the hazard, or reduce the likelihood of its occurrence (9 CFR 304, 1996; Hulebak and Schlosser, 2002). The FSIS also stated in this rule that the enteric pathogens causing risk were primarily sourced at slaughter and could be controlled through proper implementation of HACCP systems throughout all the sectors of meat production (9 CFR 304, 1996; Hulebak and Schlosser, 2002). The FSIS's primary goal with HACCP was to improve the safety of meat and poultry products by requiring safety standards that became the responsibility of the plants (Hulebak and Schlosser, 2002).

Hazard Analysis and Critical Control Points is a systematic approach to risk management that forces plants to identify and control significant biological, chemical and physical hazards associated with meat and poultry products while also providing documentation towards proof of implementation (Hulebak and Schlosser, 2002; Motarjemi et al., 1996; USDA-FSIS, 2014b; Wheeler et al., 2014). The concept of HACCP was developed by the Pillsbury Company, along with NASA and the U.S. Army's Research, Development and Engineering Center at Natick with the purpose of ensuring the safety of food for astronauts during Apollo missions in the late 1960s/early 1970s (Hulebak and Schlosser, 2002; Motarjemi et al., 1996; USDA-FSIS, 2014b;

Wheeler et al., 2014). Due to a lack of medical help available for astronauts and a need for food that could be consumed in zero-gravity, NASA wanted a zero defect system implemented in the production of the food produced to be consumed in space (Hulebak and Schlosser, 2002). After researching several mechanisms for ensuring such safety, NASA recognized that HACCP was the best approach to ensure safe and wholesome food as well as reduce reliability on final product testing (Hulebak and Schlosser, 2002). While HACCP was originally developed to ensure microbiological safety of food, it has since evolved to also include management of chemical and physical hazards that may pose risk in food products (Hulebak and Schlosser, 2002; Motarjemi et al., 1996). The FSIS has further endeavored to control foodborne pathogens by increasing requirements associated with *Listeria monocytogenes*, creating strict performance standards for *Salmonella* and *Campylobacter* in poultry products, and adding non-O157 STEC (O21, O45, O103, O111, O121, O145) to the adulterant list for non-intact beef products (Hulebak and Schlosser, 2002; USDA-FSIS, 2014b). The Centers for Disease Control and Prevention recognized that implementation of HACCP into meat and poultry systems has contributed to a reduction in incidence of foodborne illness overall in the United States since 1996 (USDA-FSIS, 2014b).

2.2. *Escherichia coli* O157:H7/non-O157 STEC

In 1885, Theodor Escherich first characterized *Escherichia coli* as *Bacterium coli commune*, which he isolated from the feces of newborn babies (Todar, 2008). Later, this bacteria was renamed *Escherichia coli* and, for many years, the bacteria were simply considered to be a collaborative organism of the large intestine (Todar, 2008). In 1982, *Escherichia coli* O157 was first identified as a human pathogen, an addition to one of many other shiga toxin-producing serotypes known to cause human foodborne illness (FDA, 2012; Johnson et al., 2006; Mead and

Griffin, 1998; Souza et al., 2002). It was associated with two different outbreaks of hemorrhagic colitis which were characterized by abdominal cramps, bloody diarrhea, and no fever (Mead and Griffin, 1998). It is thought that *E. coli* O157 evolved through a horizontal acquisition of genes for shiga toxin production in conjunction with several virulence factors (Mead and Griffin, 1998). Karmali et al. (1983) reported an association between *E. coli* that produce shiga toxins (including *E coli* O157:H7) and post-diarrheal hemolytic uremic syndrome (HUS), which is a clinical condition defined by acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia (Brooks et al., 2005; CDC, 2014b; Johnson et al., 2006; Mead and Griffin, 1998).

Escherichia coli O157:H7 and non-O157 STEC (O26, O45, O103, O111, O121 and O145) are members of the *Enterobacteriaceae* family, as are several other major pathogens such as *Salmonella*, *Shigella*, *Vibrio*, and *Haemophilius* (CDC, 2014b; Souza et al., 2002). With this clinical connection, *E. coli* O157:H7 became the first of several strains referred to as enterohemorragic *E. coli* or EHEC, which are now characterized as a subgroup of shiga-like toxin producing *E. coli* (STEC); other non-O157 STEC strains also were added to this classification: O26, O45, O103, O111, O121, and O145 (Caprioli et al., 1996; Johnson et al., 2006; Karmali et al., 1983; Mead and Griffin, 1998).

Many human infections occur from the EHEC pathogen *E. coli* O157:H7, but additional infections caused by EHEC are associated with one of six major non-O157 STEC strains (Brooks et al., 2005; Caprioli et al., 1996; Johnson et al., 2006). In 1994, *E. coli* O157:H7 was declared an adulterant in raw ground beef products by FSIS as a result of *E. coli* O157:H7 being the causative agent in major outbreaks; O157 became the focus of EHEC infections while the other major non-O157 strains were overlooked for a time in public health attribution (Caprioli et al.,

1996; USDA-FSIS, 2012; Wheeler et al., 2014). With advancement in technology for outbreak investigation and more data, FSIS declared the "big six" non-O157 STECs adulterants in raw ground beef in 2011 (Brooks et al., 2005; USDA-FSIS, 2012).

The EHEC pathogens are gram negative facultative anaerobes, which allow them to colonize in open-air environments in both aerobic and anaerobic conditions such as the digestive tract of warm blooded animals (FDA, 2012; Mead and Griffin, 1998; Souza et al., 2002). *Escherichia coli* O157:H7 is named because it expresses the 157th somatic (O) antigen identified and the 7th flagella (H) antigen; all six of the non-O157 STEC strains are all named in relation to their specific O antigens (Caprioli et al., 1996; Mead and Griffin, 1998; Todar, 2008). *Escherichia coli* O157:H7 have different strains with various H antigen identifiers, but there is less information on which are common in outbreaks (Caprioli et al., 1996; Mead and Griffin, 1998; Todar, 2008). The shiga toxins produced by EHEC pathogens, both I and II, are encoded by *stxI* and *stxII* genes, and are essentially identical to the shiga toxins produced by *Shigella dysenteriae* Type I (Goldwater, 2007; FDA, 2012). Shiga toxin II, along with intimin (*eae* gene), is commonly associated with the cause of hemolytic uremic syndrome (HUS) subsequently causing acute renal failure (Brooks et al., 2005; FDA, 2012; Johnson et al., 2006; Todar, 2008). From the years 1983 to 2002, the virulence gene profiles causing sporadic illness cases from an STEC pathogen in the United States were as follows: 61% *stx1* only, 22% *stx2* only, 17% both *stx1* and *stx2,* 84% intimin, and 86% enterohemolysin (*E-hyl* gene; Brooks et al., 2005). Those whose infection from EHEC pathogens progressed to HUS had a mortality rate of approximately 3% to 5% (FDA, 2012).

The infective dose for STECs is considered to be low, as little as 10 to 100 cells to cause infection (CDC, 2014b; FDA, 2012). Once this pathogen is ingested, the onset of the infection

will occur between 3 and 4 d after exposure, but in some cases can range from 1 to 9 d (CDC, 2014b; FDA, 2012). Symptoms and implications from STEC infection can vary from asymptomatic, mild diarrhea or to severe complications (CDC, 2014b; FDA, 2012). The severe symptoms are classified as hemorrhagic colitis (HC), which is characterized by abdominal cramps and bloody diarrhea, which can progress to HUS or thrombotic thrombocytopenia purpura (TTP) causing blood clotting in small blood vessels in the body resulting in low platelets (Brooks et al., 2005; CDC, 2014b; FDA, 2012). Shiga toxin receptors are highly concentrated in kidney cells; therefore, the kidney is a common site of damage when the infection increases typically with an increase of *stx2*/ shiga toxin II production (Brooks et al., 2005; CDC, 2014b; FDA, 2012). Antibiotic treatment for STEC infection has had varied results and, in some cases, increased the infected patient's risk of developing HUS (CDC, 2014b; FDA, 2012). It is thought that antibiotics used to treat this infection lyse the cells, and therefore release more shiga toxins into the infected patient potentially increasing their risk for HUS (CDC, 2014b; FDA, 2012).

The FDA stated that the most common route of entry for *E. coli* O157:H7 and non-O157 strains is via ingestion of contaminated food or water. Foods most commonly associated with STEC have historically been ground beef, raw milk, and produce such as leafy greens and sprouts (CDC, 2013; FDA, 2012). The STEC pathogens have a pathogenicity island within their genome referred to as locus of enterocyte effacement (LEE) which contains the genetic material responsible for the interaction between the bacterial cells and the host (typically human) that it attaches to (Beauchamp and Sofos, 2010; Elliot et al., 1998; FDA, 2012; Jerse et al., 1990). The STECs use a specialized type III secretion system, encoded by the LEE, which is used for the initial attachment in the intestines and the transfer of the effector proteins into the intestinal enterocytes (Knutton et al., 1998). Ebel et al. (1988) discussed the process of initial binding of

STEC strains to the intestinal epithelial cells, facilitated through the EspA protein of the type III secretion system. After pathogen cells attach to the intestinal epithelial cells, they insert the intimin receptor, Tir, into the host (human) cell wall (Hayward et al., 2006). The adhesion protein intimin (*eaeA*) binds Tir thus initiating the degradation of the host cell microvilli and cause effacing lesions (Torres et al., 2005). Lesions, along with the hemorrhaging caused by shiga toxins in the intestines, are characteristic to the infection caused by STEC pathogens and hence cause bloody diarrhea, a sign for increasing risk of HUS (Kapar and O'Brien, 1998; Griffin, 1995; Mead and Griffin, 1998; Tarr et al., 2005). In summary, after a person has ingested STEC pathogen cells, the cells attach to intestinal epithelial cells using the LEE-encoded factors and produce shiga toxins (*stx1* or *stx2*) that are internalized, activated, and therefore can be transferred into the bloodstream causing the infection to become septic (Beauchamp and Sofos, 2012; FDA, 2012).

The Centers for Disease Control and Prevention described *E. coli* O157:H7 as one of the most common foodborne pathogens causing human illness (CDC, 2013; Mead and Griffin, 1998). *Escherichia coli* O157:H7 causes an estimated 73,000 infections and 61 deaths in the United States every year and accounts for 75% of the EHEC infections worldwide (CDC, 2013; FDA, 2012). The other 25% of EHEC infections are associated with one of the six non-O157 pathogens (Brooks et al., 2005). Brooks et al. (2005) collected data from 43 public health laboratories between 1983 and 2002 with 940 human non-O157 STEC isolates from persons who had sporadic illness and had isolates confirmed and serotyped by the CDC. From those isolates, the most common serogroups confirmed were: O26 (22%), O111 (11%), O103 (12%), O121 (8%), O45 (7%) and O145 (5%; Brooks et al., 2005).

Those infected with foodborne STEC pathogens, in most cases, are immunocompromised (Brooks et al., 2005; FDA, 2012; Souza et al., 2002). In attempts to prevent products from contributing to illness, suspect products are often recalled for potential pathogen contamination. On May 19, 2014, Wolverine packing company recalled 1.8 million pounds of ground beef products that they thought may have been contaminated with *E. coli* O157:H7 (CDC, 2014a). This ground beef product was suspected to have caused a multistate outbreak with 12 persons reported with the infection (CDC, 2014a). In December 2013, there was a multistate *E. coli* O157:H7 outbreak involving contaminated ready-to-eat salads that were sold at Trader Joe's grocery stores and that resulted in 33 cases and 7 hospitalizations (CDC, 2013b). A multistate outbreak from non-O157 STEC O121 linked to raw clover sprouts occurred in August 2014, infecting 19 persons and causing 44% hospitalizations (CDC, 2014a). Additionally, in 2012, there was a multistate outbreak of non-O157 STEC O26 associated with clover sprouts served at Jimmy John's restaurants, which affected 29 persons in 11 states and caused 7 hospitalizations (CDC, 2012). *Escherichia coli* O157:H7 and the big six non-O157 STECs continue to cause illness associated with beef products as well as fresh produce; STEC need to be addressed as hazards for these products and increased confirmation of the non-O157 strains associated with outbreaks needs to occur to assist in controlling foodborne illness.

2.3. *Salmonella* spp.

In 1885, Dr. Daniel E. Salmon identified *Salmonella* as a pathogen in his efforts to improve public health and disease (Cooke et al., 2007; USDA-APHIS, 2014). The FDA and others described *Salmonella* species (spp.) as non-spore forming, gram-negative, facultative anaerobes that are classified in the enterobacteriaceae family and that are able to thrive in environments such as the gastrointestinal system in humans or animals (Coburn et al., 2007).

Salmonella are very similar to pathogenic *E. coli* bacteria which result in similar infection symptoms; they are so similar that they cannot be differentiated on non-selective media (Coburn et al., 2007; Jay et al., 2005). *Salmonella* is divided into two species, *S. enterica* and *S. bongori* (Coburn et al., 2007; FDA, 2012). *Salmonella enterica* has the greatest risk for public health (Chan et al., 2002; Coburn et al., 2007; FDA, 2012). *Salmonella enterica* has six subspecies: *S. enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI; Coburn et al., 2007; FDA, 2012). Approximately 60% of all *Salmonella* identified and 99% of the serovars responsible for infection and disease in warm blooded animals are typically members of the *S. enterica* (subspecies I; Chan et al., 2002). Serovars from *Salmonella* subspecies I have a vast ability to infect various animals as well as cause disease with hosts and varying severity (Chan et al., 2002). The serovar Typhi (*Salmonella* Typhi) can cause a systemic disease called typhoid fever only in humans, whereas serovar Enteritidis can cause a self-limiting gastrointestinal disease in many different species of animals (Chan et al., 2002; Coburn et al., 2007). *Salmonella* strains are named based on the different serovars or serotypes that are based on the Kaufmann-White typing scheme published in 1934 (FDA, 2012). This naming scheme differentiates *Salmonella* strains according to the expression of somatic lipopolysaccharide (LPS) O antigens and flagellar H (may have multiple in different phases) antigens (Cooke et al., 2007; FDA, 2012). According to the FDA and others, there are approximately 2,600 or more different *Salmonella* serotypes identified at this time (Cooke et al., 2007).

Some of the most popular strains of *Salmonella* in terms of association with outbreaks in foodborne illness are: *Salmonella* Typhimurium, Enteritidis, Newport, Montevideo, Heidelberg, Saint Paul, Reading, Agona, Anantum, Dublin and Kentucky (CDC, 2013; FDA, 2012). Depending on the serotype that causes the infection, *Salmonella* can cause two types of illness

(FDA, 2012). First of the illnesses is non-typhoidal salmonellosis, the second is typhoid fever which is caused by infection from *Salmonella* Typhi and *Salmonella* Paratyphi A (FDA, 2012). Symptoms associated with typhoid fever are: high fever, 39ºC to 40ºC, gastrointestinal symptoms, headaches, achiness, loss of appetite, red colored spotted rash, and in some cases can lead to septicemia (FDA, 2012). Also in some complications with typhoid fever, a chronic infection of the gallbladder may occur which results in the infected person becoming a carrier (FDA, 2012).

Non-typhoidal salmonellosis is caused by all other strains of *Salmonella* except *S.* Typhi and *S.* Paratyphi A (Morgan, 2007; FDA, 2012). The most typical route of entry for non-typhoidal *Salmonella* is by ingesting contaminated water or food; foods can include raw poultry or ground beef, pork, most recently nuts, vegetables, and bean sprouts (CDC, 2014a; Morgan, 2007). As few as one cell of *Salmonella*, depending on age of person and strain, can be an infective dose to cause symptoms (FDA, 2012). Once the cells have been ingested, it may take from 6 to 72 h before symptoms occur and they can last between 4 to 7 d depending on the host factors, dose, and strain characteristics (FDA, 2012; Morgan, 2007). Salmonellosis symptoms range from nausea, vomiting, abdominal cramps, diarrhea, fever and headaches; some infections may warrant hospitalization (FDA, 2012; Morgan, 2007). Complications from this illness include dehydration from the vomiting and diarrhea which can cause imbalance of electrolytes in the body and, in some cases, can cause death in young children, elderly and the immunocompromised (FDA, 2012; Morgan, 2007). In 2% of confirmed *Salmonella* cases, arthritis may develop 3 to 4 weeks following the symptoms from salmonellosis; this type of arthritis is called reactive arthritis and is developed as an autoimmune response to an infection (FDA, 2012). Also from salmonellosis, *Salmonella* cells may escape the gastrointestinal system

and enter the blood stream causing septicemia or infect internal organs and joints causing bacteremia (FDA, 2012).

Non-typhoidal *Salmonella enterica* require several components to cause infection, which are carried on discrete regions of the chromosome called *Salmonella* pathogenicity island (SPI; Baumler et al., 1998; Morgan, 2007). It is thought that SPI was developed by a lineage ancestral to all *Salmonella* serotypes since it is present in all lineages of the genus *Salmonella,* but not found in *E. coli* species and other closely related pathogens (Baumler et al., 1998). Currently, there are 14 SPIs (SPI-2 only present in *S. enterica* and not *S. bongori*); they all vary in structure, function and distribution of the different subspecies and serovars which may contribute to the host-specificity of *Salmonella* (Baumler et al., 1998; Morgan, 2007). Once inside the host's intestinal environment, *Salmonella* SPI-1 and SPI-2 encode a molecular apparatus called type III secretion system (similar to STEC) that is capable of injecting proteins called effectors into the host cell membranes (Baumler et al., 1998; Coburn et al., 2007; Raffatellu et al., 2007). Penetration and passage of *Salmonella enterica* from the gut lumen of the intestines into epithelium of small intestine is its pathway for entry (FDA, 2012).

Salmonella induce dramatic cytoskeletal changes at the membrane surface of the host epithelial cells as part of their entry mechanism (Chopra et al., 1999). In the small intestines, inflammation occurs which is thought to be caused by *Salmonella* flagellin intercellularly, and is critical for initiation of the immune response (Coburn et al., 2007; FDA, 2012). The *Salmonella* invasive cells are engulfed by macrophages in the lamina propria of the host, and then delivered to the mesenteric lymph follicles where they increase and disperse to other organs (Chopra et al., 1999). It is thought that once attached, *Salmonella* may produce enterotoxins (*stn* gene), perhaps within enterocytes (FDA, 2012). Chopra et al. (1999) investigated the role of the enterotoxin

(*stn*) in the pathogenicity of *Salmonella* and they found that the *stn* gene contributed significantly to the overall virulence of *Salmonella* Typhimurium. The varying dynamics of *Salmonella* and the different number of subspecies and serovars make *Salmonella* a complicated and continual public health hazard as the host specificity changes it will remain a growing issue related to foodborne illness.

In 2011, non-typhoidal *Salmonella* contributed to 11% of the total domestic foodborne illness cases, and was listed as the second most common pathogen causing infection in people, the first being Norovirus (CDC, 2011). Although *Salmonella* was listed as the second most common agent causing foodborne infection, it was the number one pathogen resulting in hospitalizations (35%) and death in the United States (CDC, 2011). Of the top five pathogens that caused the most foodborne illness related deaths in 2011, *Salmonella* was listed first again, contributing to 28% of deaths (CDC, 2011). *Salmonella* outbreaks seem to be associated with people of any age; however, the typical vulnerable persons develop the most complications (young, elderly, and immunocompromised; FDA, 2012).

Beginning in November 2014 through January 2015, there was a multistate outbreak of *Salmonella* Enteritidis infection linked to bean sprouts that caused 115 illnesses in 12 states with 25% requiring hospitalization (CDC, 2014c). In 2013 and 2014, two large poultry processors, Foster Farms and Tyson Foods Inc., were involved in a *Salmonella* Heidelberg outbreak that infected 643 people (634 Foster Farms, 9 Tyson; CDC 2014d). Foster Farms branded fresh chicken products were responsible for the multistate outbreak, while Tyson's outbreak in 2014 infected 9 people in a correctional facility that consumed their mechanically separated chicken (CDC, 2014d). The outbreaks and estimates reported by the CDC conclude that *Salmonella* is a current and growing public health hazard related to foodborne illness.

2.4. Pathogen contamination of Beef

The outbreak of *E. coli* O157:H7 in 1993, caused by undercooked ground beef products, was the turning point for the improvement of meat safety and concern for pathogen contamination in meat products across the industry (Bosilevac et al., 2006; Koohmaraie et al., 2005; Wheeler et al., 2014). Skeletal muscle from healthy animals has generally been considered sterile prior to slaughter, with the exception of the lymphatic system (Huffman et al., 2002). Harvest of livestock and processing of the meat can produce consistently safe products when best practices are used in handling the product. However, history shows that pathogens may evade even the best antimicrobial intervention systems (Huffman et al., 2002). *Escherichia coli* O157:H7, non-O157 shiga toxin-producing *E. coli*, and *Salmonella* spp. are examples of prevalent pathogens associated with beef products today that are first controlled at the slaughter process (Arthur et al., 2004; Huffman, 2002; Koohmaraie et al., 2005; Sofos, 2008; USDA, 2012).

Post-harvest food safety practices begin with the beef slaughter process (Elder et al., 2000; Huffman et al., 2002). Beef carcasses may become contaminated during slaughter and, subsequently, contaminated surfaces of the carcasses create contamination of contact surfaces later in the processing system (Huffman et al., 2002). The origins of pathogen contamination on beef carcasses, and subsequently further processed beef products, are fecal material derived from contaminated cattle hides and ingesta from gastrointestinal systems (Arthur et al., 2004; Elder et al., 2000; Loneragan and Brashears, 2005; McEvoy et al., 2000). The USDA-FSIS has a “zero tolerance” policy for visible carcass contamination with fecal, milk, and ingesta material on carcasses; if visible contamination exists, it must be removed by cutting the contaminated portion away from the carcasses (USDA-FSIS, 1996).

Elder et al. (2000) surveyed lots of cattle presented for slaughter to determine the frequency of *E. coli* O157 in feces and on hides, as well as the frequency of carcass contamination during the processing of cattle within the same lots. They found that 72% of the cattle lots had at least one positive fecal sample and 38% of the cattle had at least one positive hide sample (Elder et al., 2000). Arthur et al. (2004) reported similar results with 76% positive for *E. coli* O157 when hides of beef cattle were sampled at slaughter. Overall, prevalence of *E. coli* O157 in feces were approximately 28% (91 out of 327) and on hides were 11% (38 out of 355), respectively (Elder et al., 2000). Elder et al. (2000) collected carcass samples at three different points in the slaughter process: pre-evisceration, post-evisceration before antimicrobial interventions, and post-processing after carcasses were in the cooler. Of 30 lots sampled, 87% had at least one positive *E. coli* O157 pre-evisceration sample, while 57% were positive post-evisceration, and 17% were positive post-processing (Elder et al., 2000). Arthur et al. (2004) found no positive samples on carcasses in the cooler, while Elder et al. (2000) found 17% positives; these results may suggest that improvements in beef slaughter antimicrobial interventions and sanitary dressing procedures occurred between the times that these studies were conducted. Results showed that hide contamination were highly correlated with carcass contamination, indicating that live cattle were the source for contamination with EHEC pathogens (Elder et al., 2000).

To evaluate that hides were the primary source for *E. coli* O157:H7, PFGE (pulse-field gel electrophoresis) fingerprinting of bacterial isolates was used to determine whether those found on carcasses came from the hides (Barkocy-Gallagher et al., 2001; Koohmaraie et al., 2005). Barkocy-Gallagher et al. (2001) determined that over 66% of *E. coli* O157:H7 detected on carcasses were traceable to the same animals pre-harvest. With improved methods for

detection and isolation of foodborne pathogens, additional research surveyed beef processing plants through four different seasons (spring 2001 through winter 2002; Barkocy-Gallagher et al., 2002). This study found that *E. coli* O157:H7 prevalence in fecal samples was highest in the summer and lowest in the winter; hides were highest in the spring and summer (74%) and slightly lower in the fall, and lowest in the winter (29.4%; Barkocy-Gallagher et al., 2002; Koohmaraie et al., 2005). *Escherichia coli* O157:H7 on pre-evisceration carcasses were highest in spring and summer (39%) and lowest in the winter (1%); across all four seasons, only 1.2% of the carcasses sampled post-intervention were positive for *E. coli* O157:H7 (Barkocy-Gallagher et al., 2002; Koohmaraie et al., 2005). These studies and others contributed to the conclusion that cattle hides were the primary source of *E. coli* O157:H7 on beef carcasses. Further support was added when evidence from chemical dehairing of carcasses suggested a reduction of 50% positive hides to less than 1% positive hides, concluding the source for *E. coli* O157:H7 contamination to help develop and improve processing strategies to control this pathogen in harvest (Graves-Delmore et al., 1997; Koohmaraie et al., 2005).

According to baseline data from FSIS, 0.2% of beef steer/heifer carcass samples were positive for *E. coli* O157:H7 (Huffman et al., 2002). The baseline data for domestic beef trim was updated between 2005 and 2007 from data collected by FSIS (USDA-FSIS, 2011a). The USDA-FSIS detected 13% *E. coli* O157:H7 positive samples out of 1900 collected in raw ground beef trim. As previously determined, the primary source of contamination in beef products is assumed to mainly occur during slaughter (Gill and McGinnis et al., 2000). Beef carcasses are fabricated into retail cuts; therefore, any microbial contamination present on carcasses is inoculated onto newly exposed surfaces during this process (Emswiler et al., 1976; Pohlman et al., 2002). Dorsa et al. (1998) agreed that the most important factor contributing to

the level of microbial contamination in ground beef was the microbial quality of raw materials used for grinding (Pohlman et al., 2002). Consequently, research shows that the numbers of *E. coli* recovered from beef primals were higher than the levels obtained from the carcasses entering fabrication (Gill and McGinnis et al., 2000). This has suggested that possible cross contamination from employees and improperly cleaned equipment could be causing the increase in risk of *E. coli* contamination on beef fabricated products compared to the carcasses (Gill and McGinnis et al., 2000). Bosilevac et al. (2007) reported similar results for boneless beef trim destined for use in ground beef; they found that 30% of the U.S. beef trim contained STEC positives (PCR positive; Bosilevac et al., 2007). Prevalence of *E. coli* O157:H7 ranges from 0.1% to 54% in ground beef, while corresponding prevalence for non-O157 STEC (O21, O45, O103, O111, O121, and O145) ranges from 2.4% to 30.0%, respectively (Hussein, 2006).

Barkocy-Gallagher et al. (2003) studied prevalence of non-O157 STEC in cattle feces compared to that for *E. coli* O157:H7. They detected that non-O157 on 13.9% to 27.1% of fed carcasses depending on the season (Barkocy-Gallagher et al., 2003). In this study, they also determined prevalence of non-O157 STEC on cattle hides at slaughter (Barkocy-Gallagher et al., 2003). Non-O157 STEC prevalence ranged from 43% in the spring to a high of 78% in the fall on cattle hides presented at slaughter; they concluded that no major differences occured between O157 and non-O157 contamination (Barkocy-Gallagher et al., 2003).

After non-O157 STEC were declared adulterants in ground beef, a question of whether or not current antimicrobial systems would effectively control the additional six non-O157 STECs evolved. A total of 53.9% of beef carcasses sampled in large U.S. beef processing plants were positive for at least one strain of non-O157 STEC before evisceration; but the prevalence was reduced to 8.3% of carcasses at post-processing with the use of various intervention systems

(including, but not limited to: steam vacuum, hot water, organic acids, and steam pasteurization) indicating effectiveness against these pathogens as well (Arthur et al., 2002; Kalchayanand et al., 2012; Koohmaraie et al., 2005). Intervention systems in large U.S. beef processing plants are effective for controlling both *E. coli* O157:H7 and the big six non-O157 STECs (Arthur et al., 2002; Kalchayanand et al., 2012; Koohmaraie et al., 2005).

Bosilevac and Koohmaraie (2011) conducted a study to determine prevalence of non-O157 STEC in commercial ground beef produced in the U.S. Their results suggested that STEC in ground beef based on the detection of either *stx1* or *stx2* was approximately 24.3% positive (Bosilevac and Koohmaraie, 2011). Samples were tested during different parts of the year in numerous locations; they found prevalence as low as 5.5% in May and as high as 38.4% in March (Bosilevac and Koohmaraie et al., 2011). After a positive detection, they confirmed all isolates and, of the 24.3% positive STEC ground beef samples, they were able to confirm 7.3% of the isolates using molecular confirmation methodologies (Bosilevac and Koohmaraie et al., 2011). The STEC pathogens conclusively are a major pathogen in beef products, along with others such as *Salmonella*.

Salmonella is one of the most common foodborne pathogens and it is frequently detected in cattle feces (Barkocy-Gallagher et al., 2003; Koohmaraie et al., 2005). According to USDA-FSIS (1998) baseline data, 1.2% of beef carcass samples were positive for *Salmonella*. Cattle feces samples collected at slaughter, over an entire year, were found to be *Salmonella* positive 2.1% to 9.1% of the time depending on season (Barkocy-Gallagher et al., 2003). The Barkocy-Gallagher et al. (2003) study was similar to the USDA's National Animal Health Monitoring System (NAHMS) Feedlot study where *Salmonella* was found in 2.8% to 11.2% of feces from feedlot cattle; the greatest prevalence occurred during the summer months (Koohmaraie et al.,

2005; USDA, APHIS, 2001). *Salmonella* also is found on cattle hides and the prevalence is higher on hides than in feces (Barkocy-gallagher et al., 2003).

Prevalence of *Salmonella* in feces has a similar seasonal trend to hide samples; Barkocy-Gallagher et al. (2003) found that *Salmonella* was present 27% of the time on hides during the winter, 91.6% in the summer, and 97.7% positive in the fall. A different study conducted by Rivera-Betancourt et al. (2004) also found between 50.3% to 91.8% prevalence of *Salmonella* on hides of cattle at two different beef plants. Due to the frequency of *Salmonella* contamination on cattle hides and in feces, the potential for transmission onto the carcasses is possible. Carcasses sampled after hide removal and before intervention systems had *Salmonella* positives from 3.0% to 25% in fed beef cattle (Bacon et al., 2000; Barkocy-Gallagher et al., 2003; Koohmaraie et al., 2005; Rivera-Betancourt et al., 2004). *Salmonella* was reduced to a low prevalence after the application of antimicrobial interventions (Bacon et al., 2000; Barkocy-Gallagher et al., 2003; Koohmaraie et al., 2005; Rivera-Betancourt et al., 2004).

In 2007, USDA-FSIS reported that *Salmonella* was detected in 22 out of 1719 samples of beef trim (1.28% prevalence). Several outbreaks of *Salmonella* associated with ground beef have occurred in the United States (Talbot et al., 2006; CDC, 2014a). In 1998, *Salmonella* was detected in 3.5% of retail samples of ground beef collected in various states in the U.S. (Zhao et al., 2002). Scanga et al. (2000) collected beef trim in eight plants in the U.S. and Canada and found positive *Salmonella* samples in 2.0% and 2.2% of the beef trim, respectively. Scanga et al. (2000) also found that *Salmonella* was present in approximately 1.4% of the final ground beef product blends that were at least 25% fat. An additional potential source for *Salmonella* contamination in beef trimmings and ground beef products has been identified in the lymph nodes (Arthur et al., 2008).

Previous studies have investigated contamination of mesenteric lymph nodes; but mesenteric lymph nodes typically would not be included in ground beef trimmings (Arthur et al., 2008). Arthur et al. (2008) conducted a study to determine prevalence of *Salmonella* in beef lymph nodes collected from commercial beef plants (cull cow and bull; fed beef plants) associated with lean and fat trimmings that would be incorporated into ground beef products. Results showed a low prevalence of *Salmonella* in the lymph nodes, 1.65% to 2.3% (Arthur et al., 2008). *Salmonella* was detected more in lymph nodes collected from the flanks of culled cows and bull carcasses (3.86%), while lymph nodes from the chuck region of the fed beef cattle had the lowest prevalence (0.35%; Arthur et al., 2008). Another study examined *Salmonella* prevalence in subiliac lymph nodes of cull and feedlot cattle destined for slaughter and found similar results (Gragg et al., 2013). They collected 3327 subiliac lymph nodes from seven different plants that were located in three different U.S. regions over three different seasons to examine the factors that may affect prevalence of *Salmonella* (Gragg et al., 2013). Median numbers of positive samples for all the lymph nodes collected were considered low (1.3%), but the prevalence was greater in feedlot cattle (11.8%) than in cull cows (0.65%); contrary to what Arthur et al. (2008) found (Gragg et al., 2013). They found 24 different *Salmonella* serotypes; the majority were *S.* Montevideo (44.0%) and Anantum (24.8%; Gragg et al., 2013). Unfortunately, some lymph nodes are surrounded by muscle and fat tissue of beef carcasses, leaving them untouched by slaughter interventions. Additional research needs to be conducted to determine the optimal method to control *Salmonella* in lymph nodes, either pre-harvest or post-harvest.

Escherichia coli O157:H7, non-O157 STEC, and *Salmonella* spp. are prevalent in beef production systems and should be controlled in the slaughter process to improve microbiological

quality of further processed beef products, including ground beef. With additional sources of contamination such as lymph nodes, intervention techniques should be implemented pre- and post-harvest to provide additional control of these pathogens (Arthur et al., 2008). Continued research to control these pathogens in beef products is imperative as the dynamics of these organisms advance.

2.5. Pathogen contamination of poultry

Salmonella spp. and *Campylobacter jejuni* are the most prevalent pathogens associated with raw poultry (Bryan and Doyle, 1995; Jones et al., 1991; Oyarzabal, 2005). Poultry are contaminated with pathogens pre-harvest in a variety of vehicles including the environments where broilers are housed (Bryan and Doyle, 1995; Jones et al., 1991). Research has shown that pathogens need to be controlled both during pre- and post-harvest to reduce incidence of contamination in the final poultry products (Corrier et al., 1999; Roy et al., 2002). A study was conducted to determine prevalence of *Salmonella* in broiler houses; two *Salmonella* serotypes were the most prevalent - *S.* Heidelberg (50.0%) and *S.* Kentucky (59.6%; Roy et al., 2002). Contaminated live birds pose risk for further contaminating carcasses through other processes such as scalding, defeathering, evisceration and giblet removal, common points in the process for contamination (Bautista et al., 1997; Bryan and Doyle, 1995).

There are steps in the process for processing poultry that may cause cross-contamination and introduce contamination onto poultry carcasses (Bautista et al., 1997; Jones et al., 1991; Lillard, 1990; Oyarzabal, 2005; Roy et al., 2002). Oyarzabal (2005) conducted a study to determine the prevalence of *Campylobacter* at various processing steps for broiler chickens. After exsanguination, up to 7.5 log CFU/g of *Campylobacter* were detected before the scalding

process. After scalding, counts were reduced to under 2 log CFU/g (Oyarzabal, 2005). However, with the scalding process and the subsequent defeathering step, cross-contamination of birds with *Campylobacter* can occur between birds with additional fecal material released from the cloaca due to the pressure of the defeathering process (Oyarzabal, 2005). Production practices during bird rearing, as well as feed withdrawal, play key roles in reducing prevalence of contamination during evisceration (Oyarzabal, 2005). Post-evisceration bird carcass washes are commonly used, but effectiveness varies by washer type, water temperature, chemical concentration, etc. Although some studies have shown that carcass washers reduce risk from *Campylobacter,* the reductions are variable and often times the wash water, if reused, can cause cross-contamination (Oyarzabal, 2005). Use of antimicrobial chemicals, such as chlorine or peroxyacetic acid in immersion chilling solution has proven to assist in reduction of pathogens, but chiller water still poses a risk for cross-contamination (Lillard, 1990; Oyarzabal, 2005). Some processing plants are now air or dry chilling poultry carcasses to avoid using chilling water in an effort to reduce contamination, but it does not chill the carcasses as rapidly as immersion chilling. Further research, implementation of technology, and interventions need to occur pre- and post-harvest poultry processing to increase control of pathogen cross-contamination. The safety of poultry products is a concern for today's consumer.

Consumers that are concerned with safety of poultry products look to alternatively marketed products such as organic chicken and turkey. A study was conducted comparing organic and conventional chicken sold at retail markets in Maryland (Cui et al., 2005). Results showed that organic chicken was 76% positive for *Campylobacter* while conventional chicken was 74% positive (Cui et al., 2005). *Salmonella* was found in 61% of the organic chicken and 44% of conventional chicken; this research concluded that organic poultry may need to be

monitored as a separate entity in FSIS testing programs as organic sales have increased over the years (Cui et al., 2005). Independent of production system, consumers that undercook poultry products are at a high risk for contamination from *Salmonella* or *Campylobacter,* making these two pathogens important biological hazards to control during production (Bryan and Doyle, 1995).

In 1995, the USDA-FSIS showed that broiler chicken carcasses are positive for *Campylobacter jejuni* 88.2% of the time, and for *Salmonella* 20.0% of the time based according to baseline data (USDA-FSIS. 1995). In 2000, 10 serotypes of *Salmonella* were identified in broiler chickens and the two most prevalent serotypes were *Salmonella* Heidelberg (31.8%) and *Salmonella* Kentucky (16.8%; Roy et al., 2000). Furthermore, USDA-FSIS reported in 2008 that young chicken carcasses, rehung, were 45.80% positive for *Salmonella* and 8.15% post-chill. Young chicken carcasses, post-chill, were 40.23% positive for *Campylobacter jejuni* (USDA-FSIS, 2008). A study by Logue et al. (2003) concluded that 34.9% of poultry carcasses in the Midwest were positive for *Campylobacter,* observing differences between pre-chill and post-chill samples (41.8% and 19.8%). Contamination from the live chickens may occur during slaughter, but the primary source of contamination has been sourced from the fecal material in the lower intestinal tract and the ceca of the chickens (Oyarzabal, 2005).

USDA-FSIS has a *Salmonella* Testing Program in place for poultry processors. The FSIS collects data from poultry establishments to determine compliance based on a performance standard for *Salmonella* (Altekruse et al., 2006; USDA-FSIS, 2015). Samples are tested for presence of both *Salmonella* and *Campylobacter* and findings are reported on a quarterly basis (Altekruse et al., 2006). Current performance standards for *Salmonella* consists of: young chicken 7.5% allowable positives, ground chicken 44.6%, ground turkey 49.9%, and turkeys

1.7% (USDA-FSIS, 2015). Additionally there is a performance standard for *Campylobacter* in poultry products that includes: 10.4% positives for young chickens and 0.79% positive for turkey (USDA-FSIS, 2015). The FSIS categorizes poultry plants based on their level of compliance with a Category assignment. For example, in a young chicken plant, a Category 1 establishment fulfills two sets of testing with only at or below 4% positives, a Category 2 establishment fulfills at least 4% but below the standard, and a Category 3 establishment exceed the performance standard with more than 7.5% positives (USDA-FSIS, 2015).

The quarterly progress report on *Salmonella* and *Campylobacter* was released by FSIS for January 2014 through March 2014 (USDA-FSIS, 2014). Samples were not collected from category 1 and 2 establishments for ground poultry products due to the start of the Not Ready to Eat Comminuted Poultry Sampling project (USDA-FSIS, 2014). From the previous quarter, young chicken Category 3 establishments decreased from 4.5% of the plants to 3.4% of the plants (USDA-FSIS, 2014). The establishments for ground chicken that were Category 3 facilities, decreased from 1 out of 7 plants to 0 plants (USDA-FSIS, 2014). There were approximately 3.6% positive *Salmonella* samples reported from all young chickens while there were 1.4% positive samples for turkeys (USDA-FSIS, 2014). *Campylobacter* positives for young chickens were 5.9% and turkeys were 1.6% positive (USDA-FSIS, 2014). The updated report from the Not Ready to Eat Comminuted Poultry products reported 47.57% positive *Salmonella* samples and 7.28% positive *Campylobacter* samples (USDA-FSIS, 2014). Consumers continue to be concerned with the risk of consuming poultry products as foodborne infections from *Salmonella* and *Campylobacter* continue to pose as issues. Both government and industry food safety professionals continue to invest in research to control foodborne pathogens in poultry products.

2.6. Common chemical treatments of beef

Considerable effort since the 1990s to control pathogens in beef products was expended (Wheeler et al., 2014). Multiple hurdles technology is the most common and most effective method for controlling the risk of pathogens (Graves Delmore et al., 1998; Huffman, 2002; Koohmaraie et al., 2005; Pohlman et al., 2002; Sofos, 2008). Consecutive physical and chemical interventions such as steam pasteurization, hot water pasteurization and chemical treatments are the most effective interventions used in the multiple hurdles system on a beef slaughter floor today (Koohmaraie et al., 2005; Sofos, 2008). There are various chemicals that are implemented into beef systems; they are typically used as a spray or immersion treatment and can be utilized on carcasses, primals, and trim and often managed as critical control points for hazard analysis and critical control point plans (Belk, 2001).

Lactic Acid

Lactic acid is the most commonly used organic acid treatment on beef carcasses and fabricated products (Belk, 2001; Huffman, 2002; Koohmaraie et al., 2005; Wheeler et al., 2014). Lactic acid is generally recognized as safe (GRAS) and is approved to be used at 2.0% to 5.0% concentration at up to 55°C as a processing aid on carcasses, primals, variety meats and trimmings (USDA-FSIS, 2013). Extensive research has shown lactic acid effectively reduced *E. coli* O157:H7, non-O157 STECs and *Salmonella* spp on hot and cold beef products (Belk, 2001; Koohmaraie et al., 2005; Sofos, 2008).

It is widely accepted that lactic acid spray should be applied to hot beef carcasses shortly after hide removal; it is commonly applied pre-evisceration and as a final carcass spray (Huffman, 2002). Multiple studies have explored the effects of lactic acid and have shown it to

be an effective beef carcass intervention against both natural microflora and pathogen contamination (Huffman, 2002). An early study conducted by Cutter and Siragusa (1994) determined that lactic acid sprayed at 5% concentration was able to reduce inoculated *E. coli* O157:H7 by approximately 4 log CFU/cm^2 on warm beef carcasses tissue. Another study used lactic acid at 2.0% concentration applied to hot beef carcass tissue inoculated with *E. coli* O157:H7 and showed approximately a 1 log CFU/cm^2 reduction; a microbiologically significant improvement to the product (Gill, 2009). A different study found similar results; lactic (2%) was shown to reduce E. coli O157:H7 on beef carcass tissue by 3.3 log CFU/cm^2 (Huffman et al., 2006). Hardin et al. (1995) conducted a study that a beef carcass wash in combination with 2.0% acid spray was more effective than trimming or washing with water alone, against *E. coli* O157:H7 and *Salmonella* Typhimurium.

Castillo et al. (2001) reported reductions in APC, coliforms and *E. coli* counts obtained from cold carcass surfaces with a treatment of lactic acid solution (4% concentration; 55°C); data from this study indicated that the cold carcass application was effective when lactic acid was applied at higher temperatures. Generally, research shows that when beef carcasses are treated with lactic acid between 2.0 and 5.0%, they obtained effective results for reducing bacterial contamination (Huffman, 2002; Youssef et al., 2012). Results vary based on multiple factors when applying lactic acid such as acid concentration, temperature, pressure, product type, etc. (Gill and Badoni, 2003; Youssef et al., 2012). Lactic acid was investigated for use on processed beef products due to the conclusive efficacy as an antimicrobial when applied on beef carcasses (Gill and Badoni, 2003). Lactic acid, an organic acid, inhibits bacterial cells by entering the cell in the non-dissociated form and dissociating in the interior of the cell (more alkaline), causing acidification of the cytoplasm and inhibition of cell metabolism thus causing it to die

(Knarreborg et al., 2002). This mode of action is seen in many research studies determining effectiveness of lactic acid against bacteria on beef products.

Effectiveness of lactic acid against pathogen strains inoculated onto surfaces of fresh beef, including *E. coli* O157:H7 and non-O157 STEC, have been demonstrated through many studies (Wheeler et al., 2014). Lactic acid is commonly implemented into a multiple hurdles system for fresh beef products including beef trim. Ransom et al. (2003) conducted a study comparing different antimicrobial treatments on the effectiveness of reducing *E. coli* O157:H7 on inoculated beef trimmings. They found that lactic acid applied at 2.0% onto inoculated beef trimmings reduced 4.7 log CFU/g of contamination by 1.1 log CFU/g (Ransom et al., 2003). Additionally, they applied lactic acid at 2.0% with an application temperature of 55°C and were able to achieve a 1.3 log CFU/g reduction (Ransom et al., 2003). Lactic acid is an effective organic acid to use as an intervention in various areas of the beef production chain to achieve control of microflora and pathogenic contamination in combination with other methods.

Acetic Acid

Acetic acid is a common organic acid used as an antimicrobial acid treatment in beef processing systems (Bell et al., 1997; Huffman, 2002; Wheeler et al., 2014). Acetic Acid is GRAS and can be used as a spray up to 4.0% in concentration on carcasses, primals, variety meats and trimmings (Bell et al., 1997; USDA-FSIS, 2013). Acetic acid's effectiveness has been validated against STEC, *Salmonella*, and *Listeria monocytogenes* contamination associated with beef products (Bell et al., 1997). Acetic acid has been widely accepted as an effective antimicrobial, particularly in combination with multiple hurdle interventions (Wheeler et al., 2014). Acetic acid has the same mode of action as lactic acid as it is an organic acid, utilizing

pH to disassociate in the cytoplasm of the bacteria cell ceasing cell metabolism; this process is what makes this chemical effective against pathogens.

Ransom et al. (2003) conducted a study treating beef carcass tissue with various antimicrobial acids to compare the effects against *E. coli* O157:H7. Acetic acid was applied at 2.0% concentration and reduced inoculated *E. coli* O157:H7 (initial 4.3 log CFU/cm^2) counts by 1.6 log CFU/cm^2 on beef carcass tissue (Ransom et al., 2003). Berry and Cutter (2002) conducted a study and reported similar initial results when they treated inoculated beef carcass tissue with 2.0% acetic acid spray against *E. coli* O157:H7; and achieved approximately > 1.0 log CFU/cm^2 reduction, respectively. A study that resulted in greater reductions used acetic acid at 1.0% against non-pathogenic *E. coli* and resulted in approximately 2.5 log CFU/cm^2 reduction; this greater reduction may have resulted because of the type of inoculum as well as a different spraying mechanism compared to the other studies (Bell et al., 1997). Bell et al. (1997) also reported effects of acetic acid against *Salmonella* and saw initial reductions of approximately 3.5 log CFU/cm^2. Acetic acid is an effective antimicrobial intervention for beef carcasses and may result in various reductions of contamination based on the parameters of acetic acid used.

Acetic acid also is commonly used on beef trimmings as part of a multiple hurdles approach for further processed products. When acetic acid was applied at 2.0% onto inoculated beef trimmings, it was able to achieve a 1.1 log CFU/g reduction in *E. coli* O157:H7; similar to its effectiveness on warm beef carcass tissue (Ransom et al., 2003; Wheeler et al., 2014). Similar results ensued using acetic acid at 2.0% on beef cheek meat to achieve approximately 1.0 log CFU/cm^2 reduction in *E. coli* O157:H7 (Wheeler et al., 2014). Acetic acid is a versatile organic acid that can be effectively applied as an intervention for any point in the beef processing chain to control bacterial contamination.

Peroxyacetic Acid (PAA)

Peroxyacetic acid (PAA), also referred to as peracetic acid, is a commonly used antimicrobial acid in the beef industry (Huffman, 2002; Koohmaraie et al., 2005; Sofos, 2008; Wheeler et al., 2014). Peroxyacetic acid, and mixtures with other acids in minor quantities, is a strong disinfectant with a wide spectrum of antimicrobial activity (Kitis, 2004). Peroxyacetic acid is GRAS and approved for use up to 2000 ppm depending on application but is typically used at 200 ppm on beef carcasses (USDA-FSIS, 2013; Wheeler et al., 2014). The mode of action has not been critically researched for PAA, but it is thought to act similarly to other peroxides and oxidizing agents (Kitis, 2004). It is able to disinfect based on the release of active oxygen which disrupts the chemiosmotic function of the lipoprotein cytoplasmic membrane and transport through dislocation or rupture of bacteria cell walls (Kitis, 2004). In industry, PAA is often used as a carcass wash, spray, spray chill, primal and trim rinses (King et al., 2005; Wheeler et al., 2014).

As a carcass spray PAA (200 ppm) has been shown to reduce inoculated *E. coli* O157:H7 by 1.4 log CFU/cm^2 on beef carcass tissue (Ransom et al., 2003). King et al. (2005) found that PAA was less effective against inoculants of *E. coli* O157:H7 and *Salmonella* Typhimurium on hot beef carcasses, with only a 0.7 log CFU/cm^2 reduction; however, treatment parameters, inoculants and tissue types may have varied causing the difference in reductions (Wheeler et al., 2014). Gill and Badoni et al. (2004) found that when PAA was used at 200 ppm in a commercial beef harvest facility, it was less effective at reducing natural microflora than lactic acid when used as a carcasses spray on chilled beef carcasses. King et al. (2005) found similar results when treating chilled beef carcasses with 200 ppm PAA and found it to be less effective than lactic

acid. Further research looking at PAA as an acid treatment incorporated into the spray chill system have been recently investigated along with its application to treat beef trim.

The need for controlling pathogens in beef trim has increased because risk of contamination in finished ground beef products has become of paramount importance (Ellebracht et al., 2005; Sofos, 2008). The effectiveness of PAA as a pre-grinding intervention on beef trim has been researched (Ellebracht et al., 2005; Geornaras et al., 2012b; Ransom et al., 2003). Geornaras et al. (2012b) conducted a study to compare effectiveness of various antimicrobials against *E. coli* O157:H7 and non-O157 STEC on inoculated beef trimmings. They utilized PAA (200 ppm) as an acid immersion treatment, and observed approximately 1.0 log CFU/g reduction of all STEC inoculants, concluding that PAA was an effective treatment (Gerornaras et al., 2012b). Another study utilized PAA (200 ppm) as a spray treatment on inoculated (*E. coli* O157:H7) beef trimmings and resulted in a 1.0 log CFU/g reduction; similar results were observed with both immersion and spray treatments. In conclusion, PAA is an effective antimicrobial acid commonly used in the beef industry at various interventions in the processing system.

Additional Chemicals

Additional chemicals have been used to treat beef products in an attempt to utilize the best chemical for specific processing systems with the least environmental and economic costs. Chemicals such as chlorine, SSS (sulfuric acid and sodium sulfate), LCA (lactic acid and citric acid blend), citric acid, and trisodium phosphate all have been investigated and utilized in beef processing systems and have been proven to be effective as antimicrobial interventions when implemented into multiple hurdle methods (Geornaras et al., 2012b; Laurey et al., 2009;

Pohlman et al., 2002; Ransom et al., 2003; Wheeler et al., 2014). The need for an effective inexpensive antimicrobial solution has become increasingly more important as costs of beef production systems increase.

2.7. Common chemical treatments of Poultry

There are multiple chemical treatments used in the poultry industry to reduce pathogenic contamination on poultry carcasses and fabricated parts (Bauermeister et al., 2008; Breen et al., 1997). Chemicals such as chlorine or chlorine dioxide, ozone, hydrogen peroxide, lactic acid, sodium carbonate, peroxyacetic acid and cetylpyridinium chloride have all been investigated and utilized at various steps in the poultry production chain (Bauermeister et al., 2008; Breen et al., 1997; Loretz et al., 2010; Waldrup et al., 2010). The four most common chemical treatments being utilized to treat poultry for pathogenic contamination are chlorine, cetylpyridinium chloride (CPC), peroxyacetic acid (PAA), and lactic acid (Bauermeister et al., 2008; Breen et al., 1997; Loretz et al., 2010; Waldrup et al., 2010).

Chlorine

Chlorine as a spray treatment or as an immersion is one of the longest used chemicals in the poultry industry (Bautista et al., 1997; Bolder, 1997; Breen et al., 1997; Loretz et al., 2010; Nagel et al., 2013; Thompson et al., 1975; Whyte et al., 2001). Chlorine used in potable water is GRAS, and FSIS allows its use as a spray and in immersion chiller water not exceeding 50 ppm. The mode of action by chlorine can result from a number of factors: oxidation of sulfhydryl enzymes and amino acids; ring chlorination of amino acids; loss of bacterial intracellular contents; decreased uptake of nutrients; inhibition of protein synthesis; decreased oxygen uptake; and oxidation of respiratory components (CDC, 2009). Chlorine can be utilized at various steps

in the processing of poultry and historically has been effective when implemented into a multiple hurdle system to control pathogenic organisms on poultry products. Chlorine often is used in poultry immersion chillers to help control *Salmonella* spp. and *Campylobacter* spp. contamination and prevent cross-contamination during the chilling process (Bautista et al., 1997; Whyte et al., 2001).

Chlorine as an antimicrobial agent in poultry products has been heavily research from the 1970s to the present. Chlorine has typically demonstrated a 1 log CFU/ml reduction of bacterial contamination on poultry carcasses when processed during slaughter (Bolder et al., 1997). A study designed to determine efficacy of various antimicrobial agents against *Salmonella* on turkey carcasses used chlorine (50 ppm) as a spray treatment for approximately 10 s before sampling the carcasses for microbial counts (Bautista et al., 1997). In this study, chlorine did not achieve a 1 log reduction; they concluded that the ineffectiveness when compared to other research literature was due to the use as a spray and possible evaporation of the chlorine from the carcasses (Bautista et al., 1997).

Another study was conducted to investigate efficacy of chemical decontamination procedures on broiler carcasses during processing; the researchers utilized chlorine at 25 ppm as a final carcass wash post-evisceration and observed reductions in aerobic plate counts (Whyte et al., 2001). Nagel et al. (2013) conducted a study looking at the effectiveness of several antimicrobial agents when used as a post-chill immersion treatment against *Salmonella* and *Campylobacter* on inoculated broiler chicken carcasses. They used chlorine as a post-chill immersion at 40 ppm and found it effective against *Samonella* Typhimurium and *Campylobacter jejuni*, producing approximately less than 1 log CFU/ml compared to the positive control (Nagel et al., 2013). Chlorine was most effective when used as a spray in combination with a

chlorinated immersion chiller (Loretz et al., 2010). Chlorine has been used for many years and is still being implemented into food safety systems today, but other antimicrobial solutions are being utilized for greater effectiveness and to avoid chlorine use due to export specifications; the European Union does not accept chlorine treated meat and poultry products (Johnson, 2012).

Cetylpyridinium chloride (CPC)

Cetylpyridinium chloride is a quaternary ammonia is found in commercial mouth washes, which has caused some discrepancy in its use as an antimicrobial treatment on poultry products by some consumers as they don't like to associate mouth wash being used on their food (Kim and Slavik, 1995). USDA-FSIS has approved CPC use on poultry products on the safe and suitable ingredients list used in the production of meat, poultry, and eggs and is GRAS (USDA-FSIS, 2013). It is approved for use on poultry carcasses, giblets, and parts (skin on or skinless) applied as a spray (max 0.8% by weight) or immersion (USDA-FSIS, 2013). USDA-FSIS states that if CPC is used as an immersion treatment, it cannot be immersed longer than 10 s and if immersion chill does not follow, it must be followed with a rinse of potable water (USDA-FSIS, 2013). Cetylpyridinium chloride has a positively charged hydrophilic region and a hydrophobic region (FDA, 2003). The mode of action of CPC is dependent upon the ability of the positively charged molecule to interact with negatively charged anionic sites on the cell walls of bacteria, which will cause leakage of cellular components, disruption of bacterial metabolism, inhibition of cell growth, and cell death (FDA, 2003). The effects of CPC have been extensively investigated to determine its ability to decontaminate poultry products at various stages in processing (Kim and Slavik, 1995).

Initial investigation of CPC was conducted in laboratory settings to determine efficacy on poultry skin surfaces as a model for poultry carcasses in slaughter (Kim and Slavik, 1995). Kim and Slavik (1995) conducted a study to explore the effect of CPC against *Salmonella* inoculated on poultry skin surfaces before its approval by FDA and FSIS. Two treatments were used in this study, (i) spray treatment of CPC (0.1%) at 15ºC or 50ºC, (ii) and immersion treatments of CPC at 1 min, 1 min with 2 min dwell, and 3 min (Kim and Slavik, 1995). Results were compared to poultry skin treated with water only at the same parameters; they found CPC reduced *Salmonella* by 1 log CFU/cm^2 when used as a spray and immersion treatment, with no differences detected in time immersed (Kim and Slavik, 1995). Breen et al. (1997) found greater reductions of *Salmonella* when treating inoculated poultry skin with the use of CPC; different concentration and immersion times were used. The application of CPC (0.1%) as a post-chill spray on chicken carcasses reduced *Salmonella* Typhimurium and reduced contamination by 0.59 log CFU/bird to 1.2 log CFU/bird depending on spray time (30 s to 90 s); similar reductions were observed in other previous studies (Li et al., 1997).

Xiong et al. (1998) explored effects of CPC (0.1%) as a spray at various parameters of temperature, pressure, and time; they reported reductions of 1 to 2.5 log CFU/skin depending on parameter combination. Yang et al. (1998) used CPC at a higher concentration (0.5%) than previous studies as a wash and reported approximately 2.0 log CFU/carcass reduction of *Salmonella* on inoculated chicken carcasses. A study investigating the capabilities of Cecure (CPC), a commercially available product, against *Campylobacter* spp. on poultry carcasses reported that it will reduce *Campylobacter* levels by 1 to 2.5 logs as a pre-chill treatment. This product additionally reduced *Campylobacter* by 2 to 3 logs as a post-chill treatment which decreased incidence to 3 to 5% positive (from 90%; Waldroup et al., 2010). Cetylpyridinium

chloride is an effective antimicrobial agent when used between 0.1% and 0.5% as a spray or immersion treatment in poultry production to control *Salmonella* and *Campylobacter* contamination.

Peroxyacetic acid (PAA)

Organic acids traditionally have adverse effects on flavor and color when utilized as a control method for pathogens in poultry products (Bauermeister et al., 2010). Peroxyacetic acid (PAA or peracetic acid) blended with hydrogen peroxide is an alternative that can be used at lower concentrations with the same effectiveness and little effect on the quality of poultry products (Bauermeister et al., 2010; Nagel et al., 2013). Peroxyacetic acid is GRAS and permitted for use at 220 ppm peroxyacetic acid and 120 ppm hydrogen peroxide when treating poultry products in immersion chill and allowed up to 2000 ppm PAA in a post-chill immersion treatment (Bauermeister et al., 2010; Nagel et al., 2013; USDA-FSIS, 2013). Peroxyacetic acid treated on poultry products is effective against bacteria, bacterial spores, fungi, and yeasts at various treatment parameters (Bauermeister et al., 2010).

Peroxyacetic acid is effective at controlling *Salmonella* spp. and *Campylobacter* spp. on poultry carcasses, giblets, and parts (Bauermeister et al., 2010; Nagel et al., 2013). Bauermeister et al. (2010) reported that PAA (25 ppm, 100 ppm, and 200 ppm) used in immersion chillers was effective at reducing populations of *Salmonella* and *Campylobacter* by 1.7 to 2.6 log CFU/sample on inoculated poultry carcasses. Nagel et al. (2013) utilized PAA (400 ppm and 1000 ppm) alternatively as a post-chill immersion treatment rather than an immersion chill treatment and found favorable results. They reported approximately 2 log CFU/ml reductions of *Salmonella* and *Campylobacter* populations after treatment with PAA on inoculated poultry

carcasses (Nagel et al., 2013). Greater reductions of pathogenic contamination were reported in this study than in previous studies due to the increased allowable concentration of PAA as a post-chill immersion treatment as opposed to a chiller immersion treatment (Bauermeister et al., 2010; Nagel et al., 2013). Peroxyacetic acid is an effective antimicrobial treatment to control pathogenic contamination as well as maintain integrity of quality of the product and is best used in a multiple hurdle system.

2.8. Process control and validation

The USDA-FSIS requires verification of process control by validating that food safety interventions and critical control points are effective for controlling prevalent pathogens (USDA-FSIS 9 CFR 416, 417, 430). Chemicals used as interventions must be validated as they are intended to be used, including data showing effectiveness of the chemical at specific parameters including but not limited to: temperature, concentration, pressure, volume, treatment time and target pathogens/microorganisms. Using pathogen testing or natural microflora plate counts to verify process control may be difficult due to the low number of bacteria cells typically found on products to support effectiveness of the intervention (Arthur et al., 2004; Kim and Linton et al., 2008). Use of high levels of surrogate organisms may be a helpful mechanism to validate efficacy of a food safety intervention to provide evidence that a system is working properly in a worst case scenario environment (Arthur et al., 2004; Kim and Linton, 2008).

Surrogate organisms are a viable option for use in validating process control, but the surrogate organisms must be validated to behave similarly, and or properly represent, target organisms, most commonly pathogens. Cabrera-Diaz et al. (2009) compared fluorescent protein marked non-pathogenic *E. coli* to pathogenic *Salmonella* strains and *E. coli* O157:H7 to serve as

surrogates for validation purposes in commercial beef facilities. To evaluate the behavior of both potential surrogates and pathogens, they evaluated resistance and growth curves on TSA and found no difference in behavior (Cabrera-Diaz et al., 2009). Additionally, the research team evaluated D-values in heated phosphate buffered saline (PBS) of the bacteria and found no difference ($P > 0.05$) in thermal resistance between the target pathogens and the surrogates (Cabrera-Diaz et al., 2009). Lastly, they compared acid resistant properties using lactic acid acidified PBS and determined that log reductions were the same for the surrogates, *E. coli* O157:H7, and most of the *Salmonella* strains; however, some *Salmonella* serotypes were less resistance than the surrogate bacteria (Cabrera-Diaz et al., 2009). After evaluation of the characteristics, they concluded that the surrogates appropriately represented the target pathogens for use in validation purposes (Cabrera-Diaz et al., 2009).

A different study evaluated use of non-pathogenic *E. coli* as appropriate surrogates for *E. coli* O157:H7 for validation of pathogen control in dry-aged beef products (Ingham et al., 2010). They subjected both surrogates and *E. coli* O157:H7 to acid treatments and dry aging environment to ensure similar behavior (Ingham et al., 2010). Similar to Cabrera-Diaz et al. (2009), this study was able to conclude that the use of non-pathogenic *E. coli* as an appropriate surrogate for use in validation purposes in their specific intervention system.

Another common non-pathogenic surrogate extensively used for *Salmonella* are *Lactobacillus* spp. (Gurtler et al., 2010; Kim and Linton, 2008). *Lactobacillus* has been used as an indicator organism for *Salmonella* in multiple food processing settings such as juices and beef jerky systems to validate thermal processing procedures to control *Salmonella* (Kim and Linton, 2008). It is imperative to use appropriate indicator organisms to not only fulfill regulatory

requirement for validation of process control, but also to ensure that food safety systems are appropriately designed and effective for target pathogen control.

CHAPTER 3

Experiment A

Summary

Two studies were conducted to evaluate the antimicrobial efficacy of sulfuric acid and sodium sulfate against *Salmonella* (i) at different immersion times, using differing rinse methods, (ii) and compare efficacy to other common chemicals on the surface of chilled whole chicken wings. Wings were spot inoculated with 200 µl of inoculum (5-strain mixture of novobiocin and nalidixic acid-resistant *Salmonella*) and allowed 20 min for cell attachment. For each treatment, wings were immersed in 350 ml of designated chemical and allowed to drip for 5 min before placing in 150 ml of designated rinse buffers (buffered peptone water [BPW] or Dey/Engley [D/E] neutralizing buffer) and surface plated, incubated, and counted. For the first study, wings were randomly assigned to one of four treatment categories: (i) 10 s immersed + buffered with D/E, (ii) 10 s + BPW, (iii) 20 s + D/E, and (iv) 20 s + BPW. In the second study, wings were randomly assigned to one of six treatments: (i) treated with SSS pH 1.1; immersed 20 s stored 0 h, (ii) SSS pH 1.1; immersed 20 s stored 24 h, (iii) Cetylpyridinium chloride (CPC) 4000 ppm; immersed 10 s stored 0 h, (iv) CPC 4000 ppm; immersed 10 s stored 24 h, (v) peroxyacetic acid (PAA) 700 ppm; immersed 20 s stored 0 h, and (vi) PAA 700 ppm; immersed 20 s stored 24 h. In the first study, no differences ($P > 0.05$) in plate counts occurred due to rinse type, but there was an effect ($P < 0.05$) of treatment time. Immersion of chicken wings in 10 and 20 s in SSS resulted in a reduction ($P < 0.05$) on all agars evaluated. In the second study, there was an interaction ($P < 0.05$) between chemical and storage time. All treated plate counts were less ($P < 0.05$) than the untreated control plate counts for both agars. Results indicated that pH 1.1 SSS

used as an immersion treatment for 20 s was an effective antimicrobial intervention to reduce *Salmonella* on inoculated chicken wings.

Introduction

Antimicrobial interventions are applied to poultry products to both physically and chemically reduce risk of pathogens such as *Salmonella* spp. in U.S. processing plants to prevent human foodborne infection (Bolder et al., 1998; Deumier, 2004; Huffman et al., 2002). Outbreaks of *Salmonella* causing human infection have been sourced from several further processed chicken products (CDC 2010, 2013, 2014). The recent serotype of interest associated with chicken products has been identified as *Salmonella* Heidelberg causing human infections in 2013 and 2014 that resulted in 33 hospitalizations (CDC 2013, 2014).

Preventing foodborne illness by the use of antimicrobial interventions for meat and poultry products is a priority for the industry and the United States Department of Agriculture's Food Safety Inspection Service (USDA-FSIS, 2011a). To address the concern for meat safety, FSIS developed a Strategic Plan for 2011-2016 including goals addressing strategies to improve control of foodborne pathogens for each fiscal year (USDA-FSIS, 2011a). Some of the goals include maximizing compliance of food safety policies, improving public education in safe food handling, advancing employee training to maximize success in protecting public health, and effectively using scientific research to understand foodborne illness, pathogens of interest and emerging trends (USDA-FSIS, 2011a).

In order to move forward with both regulatory and industry goals, continued scientific investigation of bacterial antimicrobial intervention processes in poultry must occur. Research, not only in the harvest process but also in products that may be contaminated such as poultry

parts, is a key factor in improving safety of poultry products and meeting performance standard goals for *Salmonella* (Cosansu, 2010; Huffman, 2002; Sofos, 2005; USDA-FSIS, 2011a). The antimicrobial interventions used for poultry products should be easy to implement into existing systems, inexpensive, environmentally friendly, have no negative residual effects on the product, and perform to regulatory standards (Sinhamahapatra et al., 2004). Sulfuric acid and sodium sulfate as an antimicrobial treatment may contribute to reducing bacterial contamination on poultry products, and may provide financial and environmental benefits; however, little data has been published exploring the capabilities of this product. Therefore, objectives of these studies were: (i) to explore the antimicrobial efficacy of sulfuric acid and sodium sulfate (SSS) against *Salmonella* resistant to nalidixic acid and novobiocin at different immersion times and rinsing solutions on inoculated chicken wings, and (ii) compare to the efficacy of other common used chemicals at different treatment times and storage periods.

Materials and Methods

Preparation of *Salmonella* inoculum. Five strains of pathogenic *Salmonella,* resistant to nalidixic acid and novobiocin and obtained from poultry origin, were used. The strains included: *Salmonella* Montevideo, Typhimurium, Heidelberg, Enteritidis, and Newport (generously provided by Dr. Thomas Edrington; Research animal scientist; USDA). The strains were hydrogen sulfide producing strains, indicated by the formation of black colonies on xylose lysine deoxycholate (XLD; Acumedia, Lansing, MI) agar supplemented with nalidixic acid and novobiocin (for selective purposes) to isolate the colonies of each strain on selective media (XLDNN). Working cultures of the five *Salmonella* strains were maintained on XLDNN. Before the start of each experiment day, a single isolated colony from each strain of *Salmonella* was individually inoculated into 10 ml of tryptic soy broth (TSB; Difco, Becton Dickinson)

supplemented with 20 µl/ml of nalidixic acid and 26 µl/ml of novobiocin (TSBNN) and incubated for 22 to 24 h at 35ºC. After incubation, broth cultures were subcultured (0.1 ml) by transferring into 10 ml of new TSBNN and incubated for 22 to 24 h at 35ºC. At the completion of the second incubation, the cultured cells were combined and harvested using a centrifuge (20 min, 4ºC, 3220 x *g*, Eppendorf model 5810 R, Brinkman Instruments Inc.; Hamburg, Germany). Cell pellets were rinsed with 10 ml of phosphate-buffered saline (PBS, pH 7.4; Sigma, St. Louis, MO) and vortexed to be resuspended and washed, then centrifuged an additional time and resuspended to the original volume (*Salmonella* [50 ml]) with PBS for a target inoculum concentration level of approximately 7 to 8 log CFU/ml.

Inoculation of chicken wings. For study 1 and study 2, whole skin-on broiler chicken wings (including the humerus, radius, ulna, and phalanges [wing tip]) of similar weight were collected after post-harvest chilling from a commercial poultry facility before application of acid treatments (after harvest) and shipped fresh and refrigerated to the Center for Meat Safety & Quality at Colorado State University. Chicken wings were randomly assigned to a treatment category and inoculated. Chicken wings were spot inoculated with 200 µl of the *Salmonella* mixture (100 µl on each side) (Geonaras et al., 2013). The droplets of inoculum were randomly dispersed over the surface of the chicken wing and then allowed to sit at refrigerated temperatures (4ºC) for 20 min to simulate cell attachment time in a commercial processing facility (Geonaras et al., 2013). The target inoculation level of the chicken wings before chemical treatment was approximately 6 log CFU/ml. After cell attachment, half of the chicken wings were randomly assigned to a treatment and the other half were left untreated to serve as control samples to obtain initial plate counts.

Chemical treatment of chicken wings. For the first study, chicken wings were immersed in 350 ml of SSS (sulfuric acid [H_2SO_4] + sodium sulfate [Na_2SO_4] + water [H_2O]) solution (pH 1.1; Zoetis Florham Park, NJ) for one of two immersion times (10 s and 20 s). Chicken wings were aseptically placed in a sterile Whirl-Pak (Nasco; Fort Atkinson, WI) bag containing the SSS solution and then removed aseptically after designated treatment time and allowed to drip on wire racks for 5 min. Each treated chicken wing was immersed in a new Whirl-Pak bag with fresh solution. After the completion of the drip time, treated wings were placed in a sterile Whirl-Pak bag with 150 ml of designated rinsing solution (Dey Engley neutralizing buffer [D/E; Difco, Becton Dickinson; Franklin Lakes, NJ] or buffered peptone water [BPW; Difco, Becton Dickinson; Franklin Lakes, NJ]). The two rinsing solutions were chosen to determine if there were effects of rinsing solutions on the outcome of plate counts based on solution's ability to neutralize the SSS after treatment. There were a total of four treatment combinations (time + buffer solution): (i) 10 s + D/E, (ii) 10 s + BPW, (iii) 20 s + D/E, and (iv) 20 s + BPW (N = 120; n = 15).

For the second study, inoculated chicken wings were randomly assigned to one of six treatments: (i) SSS (pH 1.1; immersed 20 s; stored 0 h), (ii) SSS (pH 1.1; immersed 20 s; stored 24 h), (iii) cetylpyridinium chloride (CPC, Safe Foods Corporation, Little Rock, AR; 4000 ppm; immersed 10 s; stored 0 h), (iv) CPC (4000 ppm; immersed 10 s; stored 24 h), (v) peroxyacetic acid (PAA, Enviro Tech Chemical Services, Inc., Modesto, CA; 700 ppm; immersed 20 s; stored 0 h), and (vi) PAA (700 ppm; immersed 20 s; stored 24 h). There were a total of 80 chicken wing samples (n = 10 per treatment). Chicken wings were treated for all treatment combinations in two equal day blocks. Solutions were prepared by manufacturer recommendations and parameters approved by USDA-FSIS Directive 7120.1: Safe and Suitable Ingredients used in the

Production of Meat, Poultry, and Egg Products (USDA-FSIS, 2013b) which determined that CPC was limited to a 10 s immersion time and had to be followed by a rinse with water (~ 25 ml). Chicken wings were aseptically immersed into a WhirlPak bag with 350 ml of the designated chemical solution for the specified treatment time. They were then placed on wire racks to allow for a 5 min drip time before placing in a WhirlPak bag.

Microbiological, pH analyses, and color of chicken wings. Both the untreated inoculated and the chemically treated inoculated chicken wing samples were analyzed for inoculated pathogens and total plate counts for each sample. For the second study only, samples were placed into sterile WhirlPak bags with 150 ml of D/E neutralizing broth if the sample was selected for 0 h storage time. The samples selected for 24 hour storage time were placed in individual sterile Whirl-Pak bags and stored at 4°C for 24 hours before 150 ml of D/E neutralizing broth was added to the bag. After the addition of specified rinsing buffer, each sample was vigorously shaken by hand with a strong downward force for 1 min to recover any viable cells from the inoculated chicken wings for microbiological analysis. Samples were serially-diluted (10-fold) in 0.1% buffered peptone water (Difco, Becton Dickinson, Franklin Lakes, NJ). The appropriate dilutions (0.1 ml or 1 ml) were then surface plated onto selective XLDNN and tryptic soy agar with nalidixic acid and novobiocin (TSANN; utilized to compare to the harsh selective media XLDNN in the first study), and tryptic soy agar (Acumedia, Lansing, MI). *Salmonella* inoculated counts were identified on XLDNN as black colonies and also obtained from TSANN after incubation for 24 h at 35°C. In addition, total bacterial population counts were obtained from TSA after incubation for 72 h at 25°C.

In both studies, uninoculated chicken wings that were left untreated to serve as controls and chemically treated chicken wings were analyzed for pH. Control and chemically treated

chicken wing samples were diluted (1:5 dilution) with deionized water, and vigorously shaken by hand with a strong downward force (1 min). The pH was measured with a calibrated pH meter fitted with a glass electrode (Denver Instruments, Arvada, CO).

For the second study, surface color measurements were collected on untreated controls and chemically treated chicken wings to determine effects of treatment on integrity and stability of chicken wing color. Color was measured using a HunterLab MiniScan (45/0-S; Hunter Associates Laboratory Inc., Reston, VA) handheld spectrophotometer to obtain color measurements from the exterior tissue surface of the chicken wings. Measurements were obtained from uninoculated chicken wings that were both untreated control and chemically treated at 0 h and 24 h storage times. Three measurements were obtained from each wing sample to obtain an average color measurement for CIE L* (white vs. black), a* (red vs. green), and b* (yellow vs. blue). Moisture pick-up also was determined and recorded for each treatment compared to the control chicken wings.

Statistical analysis. Both studies were designed as randomized complete block (study 1 was a 2 x 2 factorial, study 2 was a 2 x 3 factorial) using treatment days as blocks. Separate analyses were performed for each treatment to analyze the effect of each treatment against inoculated microbial populations compared to the untreated control samples. Then $\log_{10}$ plate counts obtained from treated chicken wings were analyzed by treatment to determine interactions and main effects of treatment parameters. Data were evaluated using the Mixed Procedure of SAS version 9.3 (Cary, NC) with independent variables including in study 1: immersion time, rinsing solution type, and the respective interactions; and in study 2: chemical treatment, storage time, and the respective interactions. Bacterial populations were expressed as least squares means for log CFU/ml of wing rinsate calculated under an assumption of a log-normal

distribution of plate counts. Color and pH results were compared within treatment to their respective controls to determine treatment effect using the Mixed Procedure of SAS version 9.3 (Cary, NC) and results were expressed as least squares means. All differences were reported using a significance level of $\alpha = 0.05$.

Results and Discussion

Antimicrobial effect of chemical treatments. In the first study, microbial population counts from inoculated chicken wings were obtained from XLDNN, TSANN, and TSA. When comparing SSS treated chicken wing counts between treatments (immersion time: 10 s vs. 20 s; rinsing buffer type: D/E vs. BPW), no interactions ($P > 0.05$) were detected. There were no differences in mean *Salmonella* plate counts ($P > 0.05$) observed between D/E and BPW rinsed chicken wings samples (data not shown), therefore all results were expressed as the main effect ($P < 0.05$) of immersion time (Table 3.1). Compared least squares mean *Salmonella* plate counts for inoculated controls, all of the plate counts obtained from treated chicken wings were reduced (P = 0.0001) after treatment with SSS. Initial inoculation levels of *Salmonella* obtained from chicken wings were approximately 5.5 to 5.7 log CFU/ml of wing rinsate for all media types (Table 3.1). Geornaras et al. (2012a) used SSS in a study to treat inoculated beef trimmings; using a similar inoculation method, they obtained initial inoculation levels of 2.8 to 3.1 log CFU/cm^2 and used different pathogens (*E. coli* O157:H7). A different study conducted by Schmidt et al. (2014) immersed beef cheek meat inoculated with *Salmonella* and shiga toxin-producing *Escherichia coli* in SSS with initial inoculation levels ranging from 3.8 to 4.1 log CFU/cm^2; they may have encountered a lower inoculation level than this study due to species difference, inoculum and inoculation methodology differences.

Salmonella plate counts obtained from chicken wings treated with SSS for 10 s were greater ($P < 0.05$) than *Salmonella* plate counts obtained from chicken wings that were treated for 20 s (Table 3.1). *Salmonella* plate counts obtained from inoculated chicken wings treated with SSS for 20 s were reduced by approximately 1 log CFU/ml; a microbiologically significant improvement to the product. Schmidt et al. (2014) utilized SSS at a pH of 1.8 at three different immersion times (1, 2.5, and 5 min). They found reductions in *Salmonella enterica* on beef cheek meat of 1.0 to 1.5 log CFU/cm^2 based on duration of immersion (Schmidt et al., 2014). Schmidt et al. (2014), even with using a higher pH solution, may have found greater reductions with the treatment of SSS than in this present study due to the longer immersion times when treating the inoculated product. In the present study, chicken wings were immersed for 20 s while Schmidt et al. (2014) immersed beef cheek meat between 1 and 5 min, making this the likely parameter resulting in greater reduction in pathogen populations. Sulfuric acid and sodium sulfate used as an immersion between 10 and 20 s at a pH of 1.1 was an effective microbial intervention for chicken wings. Geornaras et al. (2012a) applied SSS at a pH of 1.2 and immersed inoculated beef trim for 30 s; they found reductions in *Salmonella* Typhimurium and Newport from 0.5 to 0.7 log CFU/cm^2 depending on strain. There were lower reductions in *Salmonella* on inoculated beef after treatment with SSS than found in the present study. Geornaras et al. (2012a) used a very similar immersion time (30 s vs. 20 s), but applied SSS at a higher pH. In the present study, SSS was applied at a pH of 1.1 and immersed 10 to 20 s, which reduced *Salmonella* plate counts obtained from chicken wings from 0.9 to 1.2 log CFU/ml. After 24 h storage, a reduction of 1.6 to 1.8 log CFU/ml was observed. Geornaras et al. (2012a) utilized SSS at a pH of 1.2 and found less than a 1 log reduction, respectively.

In the second study, plate counts were obtained from XLDNN and TSA. There was an interaction ($P < 0.05$) between chemical type and storage time. The initial *Salmonella* inoculation level of chicken wings were 5.7 log CFU/ml of wing rinsate for TSA, and 5.5 log CFU/ml for XLDNN agar (Table 3.2). All least squares mean *Salmonella* plate counts obtained from treated and stored chicken wings were lower ($P < 0.05$) than the control least squares mean plate counts for both TSA and XLDNN agar (Table 3.2).

All chemical treatments reduced (P = 0.0001) *Salmonella* plate counts obtained from the chicken wings when compared to the plate counts of controls on both agar types (Table 3.2). Sulfuric acid and sodium sulfate reduced *Salmonella* populations by approximately 1.1 log CFU/ml on inoculated chicken wings (Table 3.2.) Sulfuric acid and sodium sulfate and PAA additionally reduced ($P < 0.05$) *Salmonella* plate counts obtained from the treated chicken wings from 0 h to 24 h of storage time (Table 3.2). After storage for 24 h, CPC did not differ ($P > 0.05$) compared to 0 h for both agar types (Table 3.2). Sulfuric acid and sodium sulfate treated chicken wings had the greatest reduction (P = 0.0001) after 24 h storage; approximately a 0.5 log CFU/ml reduction was observed from 0 h to 24 h (Table 3.2).

Geornaras et al. (2012b) used PAA as an immersion treatment on inoculated beef trimmings to decontaminate pathogenic *Escherichia coli* O157:H7 and non-O157 shiga toxin producing *E. coli* with an initial inoculation level of approximately 3.2 log CFU/cm^2. In their study, PAA was applied at a concentration of 200 ppm (0.02%) and inoculated beef trimmings were immersed for 30 s; they found reductions of pathogenic *E. coli* from 0.6 to 0.8 log CFU/cm^2 (Geornaras et al., 2012b). They observed lower reductions than found in the present study in which PAA (700 ppm) reduced *Salmonella* populations by 1.5 log CFU/ml with a similar immersion time on inoculated chicken wings (Table 3.2). The Geornaras et al. (2012b)

study may have detected smaller reductions in *E. coli* populations after treatment with PAA due to the difference in initial *Salmonella* populations (3.2 log CFU/cm^2 vs. 5.7 log CFU/ml). Not only were the inoculum initial counts different, but the species were different and PAA concentrations were different (200 ppm vs. 700 ppm) as well, which likely resulted in the difference in PAA effectiveness in these two studies.

Bauermeister et al. (2008) applied PAA at three different levels (25 ppm, 100 ppm, 200 ppm) in a poultry chiller immersion pilot study (1 h immersion). Whole chicken carcasses were inoculated with *Salmonella* Typhimurium and *Campylobacter jejuni*. In the present study, PAA was used at 700 ppm and used as an immersion treatment for a shorter immersion time compared to Bauermeister et al. (2008) and chicken wings were inoculated with *Salmonella* spp. only. In the present study, PAA resulted in a 1.5 log CFU/ml reduction of *Salmonella* and an additional 0.2 log CFU/ml reduction after 24 h of refrigerated storage while Bauermeister et al. (2008) found that PAA reduced *Salmonella* by 1.7 log CFU/carcass. Bauermeister et al. (2008) likely found greater reductions in *Salmonella* populations compared to the present study due to the 1 h immersion versus the 20 s immersion. Although they used a lower concentration of PAA, they still had a longer immersion time and an additional factor of chilled solution which could have contributed to the slightly higher reduction in *Salmonella* they found compared to this chicken wing study.

Li et al. (1997) also used CPC as an antimicrobial pre-chill spray on chicken carcasses inoculated with *Salmonella* Typhimurium. In the present study, CPC was used as an immersion which requires different concentration parameters compared to using it as a spray. They used CPC at 1000 ppm for 90 s at approximately 119 psi which resulted in a 1.6 log reduction of *Salmonella* Typhimurium (Li et al., 1997). In the present study, chicken wings were immersed

in CPC (4000 ppm) for 10 s, followed by a rinse of 25 ml of water because the chicken wings would not be placed in a water immersion chiller. With the differences in treatment parameters, Li et al. (1997) was able to achieve greater reductions (1.6 log reduction) of *Salmonella* compared to this present study (0.7 log CFU/ml). Li et al. (1997) used a lower concentration of CPC but applied it as a spray and did not have to rinse post treatment which may have resulted in greater reductions of *Salmonella* counts. In a different study, Yang et al. (1998) applied CPC using an inside-outside birdwasher on *Salmonella* Typhimurium inoculated chicken carcasses at a concentration of 5000 ppm for 17 s; after a 60 s dwell time, they rinsed the inoculated carcasses off with water to remove chemical residue. This process was very similar to the present study because a similar CPC concentration was used and washed off after treatment. Yang et al. (1998) found that CPC reduced *Salmonella* Typhimurium and total aerobes by approximately 2.0 log CFU/carcass when applied using a bird washer to inoculated whole chicken carcasses. In our study, CPC achieved lower reductions in comparison to Yang et al. (1998), resulting in only 0.7 to 0.8 log CFU/ml at 0 h and 24 h storage times. Yang et al. (1998) applied CPC as a spray, which may have provided additional antimicrobial properties. They also used a higher concentration and allowed the CPC to dwell on the chicken carcasses before rinsing with water, which likely resulted in the difference of effectiveness between these two studies (Yang et al., 1998).

Color, pH, and moisture pick-up analysis of chicken wings. For the second study, both color and pH were measured for all chemical treatments and compared to untreated control chicken wings. Color measurements were obtained at 0 h and 24 h for treated and control chicken wings (the second color measurement was taken at 30 h not 24 h; no differences [$P > 0.05$] were detected). An effect ($P < 0.05$) of storage time was observed for L* and a*

measurements of the chemically treated chicken wings (Table 3.3). A main effect ($P < 0.05$) of chemical was observed for b* color measurements. The only chemical that differed ($P < 0.05$) from the untreated control wings for b* measurement were the SSS treated wings (data not shown). The SSS treated chicken wings were more yellow after treatment and less blue due to the increase in b* values. In a study conducted by Bauermeister et al. (2008), chicken carcasses treated with varying levels of PAA (100 ppm to 200 ppm) resulted in L* values that were the same ($P > 0.05$) as the untreated control after 24 h; however, from days 7 to 14, carcasses treated with 200 ppm were lower ($P < 0.05$) in L* values from the control carcasses. There were differences ($P < 0.05$) in the b* and a* values between 24 h and 15 d storage times for all treatments of PAA (Bauermeister et al., 2008). Results may have differed the present study due to the difference in PAA concentration and storage times compared to the length of storage times observed in Bauermeister et al. (2008).

In the second study, both treated and untreated control chicken wings were measured for pH. All chemically treated chicken wings were lower ($P < 0.05$) in pH from the control chicken wings, excluding the CPC treated chicken wings (Table 3.4). In a study treating beef samples with SSS at a pH of 1.2, the pH of chemically treated beef samples was lower ($P < 0.05$) than the control samples (Geornaras et al., 2012a). Similar results were observed in the present study when the same chemical was treated on different species of product at a different pH (1.1; Geornaras et al., 2012a). Untreated control chicken wings in the present study had a pH measurement of approximately 6.9 for 0 h and 24 h storage time (Table 3.4). Geornaras et al. (2012a) found that the pH of untreated beef samples was approximately 5.7 to 5.9 before chemical treatment, different from chicken wings. In the present study, chicken wings treated with SSS and PAA increased in pH from 0 h to 24 h. The SSS treated chicken wings increased

($P < 0.05$) in pH from 0 h to 24 h, but still differed ($P < 0.05$) from the control chicken wings (Table 3.4). Geornaras et al. (2012a) reported similar results after 24 h of storage when beef was treated with SSS; pH levels were increased compared to the 1 h treated samples which caused the SSS treated beef samples to have the same ($P > 0.05$) pH as the untreated control after 24 h.

Measurements of pH were obtained for the first study from untreated control chicken wings and SSS treated chicken wings following both treatment times (10 s and 20 s). Mean pH of the control wings was 6.3, and both SSS treated chicken wings were different ($P < 0.05$) from the control chicken wings with surface pH of 4.24 to 4.31 (data not shown). The SSS treated chicken wings at both 10 s and 20 s treatment times did not differ ($P > 0.05$) in pH measurements. In addition to the true pH of the untreated and treated chicken wings, buffered chicken wing pH measurements were obtained because two different rinsing types were used to buffer the treated chicken wing samples before microbiological testing. The SSS treated chicken wings did not differ ($P > 0.05$) in pH between both BPW and D/E at both treatment times The pH measurements of the SSS treated chicken wings were not different ($P > 0.05$) between treatment times (10 s and 20 s).

Moisture pick-up for the treated chicken wings did not differ ($P > 0.05$) between 10 s and 20 s immersion times. The approximate moisture pick-up for all treated wings was between 1% and 2 %. In conclusion, the data obtained from both studies demonstrated that SSS used as an immersion at pH 1.1 for 20 s was an effective antimicrobial intervention for chicken wings. Both BPW and D/E are viable rinsing solutions with similar neutralizing capabilities for samples treated with SSS. Overall, chemically treated chicken wings had little color change compared to control chicken wings that would alter the visual quality of the product. SSS, PAA, and CPC were effective antimicrobial interventions for chicken wings stored at 0 h or 24 h. When when

effects of SSS treatment were compared to currently used industry chemicals, SSS performed at least equally and could be used in the poultry industry to treat parts as effectively against *Salmonella*.

Table 3.1. Least squares mean (SE) plate counts (log CFU/ml) obtained from xylose lysine deoxycholate agar with novobiocin and nalidixic acid (XLDNN), tryptic soy agar with NN (TSANN), and tryptic soy agar (TSA) obtained from inoculated chicken wing samples after each treatment compared to the untreated controls.

	XLDNN			TSANN			TSA		
Treatment	Untreated	Treated*	*P*-value	Untreated	Treated*	*P*-value	Untreated	Treated*	*P*-value
10 s	5.5 (0.1)	4.6 (0.1)	<0.0001	5.7 (0.0)	4.9 (0.0)	<0.0001	5.6 (0.1)	4.8 (0.1)	<0.0001
20 s	5.5 (0.0)	4.3 (0.0)	<0.0001	5.7 (0.1)	4.6 (0.1)	<0.0001	5.6 (0.1)	4.5 (0.1)	<0.0001

* Inoculated chicken wings immersed with sulfuric acid and sodium sulfate (SSS; pH 1.1); P-values < 0.05 are significant.

Table 3.2. Least squares mean plate counts (SE) obtained from tryptic soy agar (TSA) and xylose lysine deoxycholate agar supplemented with nalidixic acid and novobiocin (XLDNN) of inoculated chicken wings treated with different antimicrobial chemicals and stored at different times compared to untreated control chicken wings.

Storage Time	TSA				XLDNN			
	Untreated	SSS*	PAA*	CPC**	Untreated	SSS*	PAA*	CPC**
0 h	5.7 (0.1)^{a}	4.6 (0.1)^{c}	4.2 (0.1)^{d}	5.0 (0.1)^{b}	5.5 (0.1)^{a}	4.3 (0.1)^{c}	4.0 (0.1)^{d}	4.7 (0.1)^{b}
24 h	5.7 (0.1)^{a}	4.1 (0.1)^{de}	4.0 (0.1)^{e}	4.9 (0.1)^{b}	5.5 (0.1)^{a}	3.7 (0.1)^{e}	3.8 (0.1)^{de}	4.8 (0.1)^{b}

$^{a\text{-}e}$ Under each type of media (TSA and XLDNN), LSMeans with different superscript letters are different ($P < 0.05$).

* Inoculated chicken wings were immersed into both sulfuric acid and sodium sulfate (SSS; pH 1.1) and peroxyacetic acid (PAA; 700 ppm) solutions for 20 s.

** Inoculated chicken wings were immersed into cetylpyridinium chloride (CPC; 4000 ppm) solution for 10 s, followed by 25 ml water spray.

Table 3.3. Least squares means for L* (white/black; SE), a* (red/green; SE) and b* (yellow/blue; SE) measurements of chicken wings at different storage times.

Storage Time	L*	a*	b*
0 h	68.92 (1.78)^{b}	-1.8 (0.17)^{a}	4.48 (0.39)
24 h	74.79 (1.78)^{a}	-2.4 (0.17)^{b}	5.05 (0.39)

^{a,b} LSMeans, within same column, bearing different superscript letters, differ ($P < 0.05$)

Table 3.4. Least squares mean (SE) pH measurements of chicken wings treated with different antimicrobial chemicals and stored at different times.

Storage Time	Untreated	SSS*	PAA*	CPC**
0 h	6.9 (0.1)^{a}	4.4 (0.1)^{d}	6.3 (0.1)^{b}	7.0 (0.1)^{a}
24 h	6.9 (0.1)^{a}	5.6 (0.1)^{c}	6.7 (0.1)^{ab}	6.7 (0.1)^{ab}

$^{a\text{-}d}$ LSMeans bearing different superscript letters are different ($P < 0.05$).
* Inoculated chicken wings were immersed into both sulfuric acid and sodium sulfate (SSS; pH 1.1) and peroxyacetic acid (PAA; 700 ppm) solutions for 20 s.
** Inoculated chicken wings were immersed into cetylpyridinium chloride (CPC; 4000 ppm) solution for 10 s, followed by 25 ml water spray.

CHAPTER 4

Experiment B

Summary

A study was conducted to: (i) validate that inoculants of non-pathogenic *Escherichia coli* effectively serve as surrogates for pathogenic *Escherichia coli* O157:H7, non-O157 shiga toxin-producing *Escherichia coli* (STEC), and *Salmonella* spp. when warm beef carcass tissue is treated with SSS, and (ii) validate that SSS applied between low and high pH and pressure levels is an effective intervention when applied to warm beef carcass surface tissue. The study was repeated over three days with two samples analyzed per treatment on each day (n = 6). This study utilized four inoculum, including: a 5-strain mixture of *E. coli* O157:H7, a 12-strain mixture of non-O157 STEC (two strains per serogroup), a 6-strain mixture of *Salmonella*, and a 5-strain mixture of non-pathogenic *E. coli*. Treatment application parameters included: (i) two SSS pH levels (pH 1.5 [high] and pH 1.0 [low]), and (ii) two application pressures (13 psi [low] and 22 psi [high]) for a total of four treatments. Samples were assigned randomly to one of the four possible SSS treatments which were administered in a custom-built spray cabinet (Chad Co., Olathe, KS) designed in every way to simulate commercial beef slaughter floor operations. The surrogate and pathogen inocula responded similarly ($P > 0.05$), to application of SSS at high and low parameters on warm beef carcass surface tissue, making the surrogate inoculum a viable option to use in a plant for validation purposes. Treatments at all parameter combinations reduced ($P < 0.05$) bacterial contamination from 6.3 log CFU/cm^2 to 4.7 to 5.7 log CFU/ cm^2. Based on the findings of this study, SSS was an effective ($P < 0.05$) antimicrobial intervention for the surrogate and pathogen inocula when applied between the high and low pH and pressure parameters on warm beef carcass surface tissue.

Introduction

The most prevalent pathogens causing concern in the meat industry are: pathogenic *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella spp* (Sofos, 2008). Between 2009 and 2010, the pathogen contributing to the most foodborne illness was *Salmonella* responsible for 49% of the foodborne related outbreaks. *Escherichia coli*, another prevalent pathogen related to beef products, contributed to 16% of the illnesses sourced from food related outbreaks (CDC, 2013). From the total cases of food that caused the outbreaks, beef was responsible for 13% of the illnesses (CDC, 2013).

The three pathogen groups that cause the most foodborne illnesses in fresh beef products are *Escherichia coli* O157:H7, non-O157 shiga toxin-producing *Escherichia coli* (STEC), and *Salmonella spp.* (Koohmaraie et al., 2005; Sofos, 2008). Usually, these bacteria are primarily sourced from cattle hides (Koohmaraie et al., 2005). The most effective approach to address these pathogens in beef products is by using the multiple hurdles technology that provides sequential applications of antimicrobial interventions to reduce risk of their presence (Graves Delmore et al., 1998; Keeton et al., 2006; Koohmaraie et al., 2005; Pohlman et al., 2002; Sofos, 2008). Specifically, *E. coli* O157:H7 and non-O157 STECs are adulterants in ground beef and must be eliminated through the multiple hurdles intervention systems -- beginning with control on the harvest floor of beef processing facilities (Graves Delmore et al., 1998; Keeton et al., 2006; Koohmaraie et al., 2005; Pohlman et al., 2002; Sofos, 2008; USDA-FSIS, 2004; USDA-FSIS, 2013).

Multiple hurdles include several food safety interventions in a system to prevent, eliminate, and reduce pathogens on meat (Graves Delmore et al., 1998; Keeton et al., 2006;

Koohmaraie et al., 2005; Pohlman et al., 2002; Sofos, 2008). Food safety interventions on the harvest floor consist of both physical and chemical interventions (e.g. steam pasteurization, hot water pasteurization, and chemical treatments; Koohmaraie et al., 2005; Sofos, 2008). There are many options for chemical interventions and the beef industry continually explores options for the best chemical to fit their operations (Koohmaraie et al., 2005; Sofos, 2008). A new commercially available antimicrobial, sulfuric acid and sodium sulfate, has not been adequately investigated to determine its efficacy in beef processing facilities.

Beef processing facilities are required to verify process control by validating that their interventions are effective in controlling prevalent pathogens. Validation of food safety systems may be difficult due to the small number of pathogenic cells contaminated on the carcasses, making the measurement of efficacy challenging to achieve (Arthur et al., 2004). Therefore, use of surrogate bacteria can be a helpful tool to validate efficacy of an intervention on a harvest floor to show that the system is working properly by having the ability to inoculate carcasses with higher populations of bacteria (Arthur et al., 2004). Objectives of this study were to (i) validate that inoculants of non-pathogenic *Escherichia coli* effectively serve as surrogates for pathogenic *Escherichia coli* O157:H7, non-O157 shiga toxin-producing *Escherichia coli* (STECs), and *Salmonella* spp. when warm beef carcass tissue is treated with sulfuric acid and sodium sulfate (SSS) between designated parameters, and (ii) validate that SSS applied between high and low pH and pressure levels is an effective intervention when applied to warm beef carcass surface tissue in a spray cabinet for potential use as supporting evidence for beef processing plants.

Materials and Methods

Preparation of Inocula. This study utilized four inoculum mixtures, including: (i) a 5-strain mixture of *E. coli* O157:H7 (ATCC 43895, C1-072, C1-109, C1-154, C1-158), (ii) 12-strain mixture of non-O157 STEC (two strains of each of the serogroups O26, O45, O103, O111, O121, and O145), (iii) a 6-strain mixture of *Salmonella* (serotypes Agona, Anatum, Saint-Paul, Reading, Newport and Typhimurium DT104 var. Copenhagen), and (iv) a 5-strain mixture of non-pathogenic *E. coli* (ATCC BAA-1427, ATCC BAA-1428, ATCC BAA-1429, ATCC BAA-1430, ATCC BAA-1431) that could serve as surrogates for *E. coli* O157:H7, STEC, and *Salmonella* spp. Rifampicin-resistant cultures of the *E. coli* O157:H7, non-pathogenic *E. coli* and non-O157 STEC strains were used to allow selection and differentiation of the inoculum from natural flora associated with beef used in the study. Xylose lysine deoxycholate (XLD; Acumedia, Lansing, MI) agar was used for selective enumeration of the *Salmonella* inoculum. Strains were activated and subcultured (35°C, 24 ± 2 h) in 10 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD; for *Salmonella* strains) or TSB supplemented with 100 μg/ml rifampicin (Sigma-Aldrich Inc., St. Louis, MO; for all rifampicin-resistant *E.coli* strains). Broth cultures of the strains belonging to the same inoculum type were combined and cells harvested by centrifugation (3220 x *g*, 20 min, 4°C, Eppendorf model 5810 R, Brinkman Instruments Inc., Hamburg, Germany), washed with 10 ml phosphate buffered saline (PBS, pH 7.4; Sigma, St. Louis, MO), re-centrifuged, and resuspended in PBS to the original inoculum volume (*E. coli* O157:H7 [50 ml], non-O157 STEC [120 ml], *Salmonella* [60 ml] to obtain a concentration of approximately 8 to 9 log CFU/ml.

Sample collection and inoculation of warm beef carcass surface tissue. The study was conducted on three equal day blocks. A total of 240 sections of warm beef carcass surface

brisket tissue were obtained from hot beef carcasses after being subjected to hot-water pasteurization during harvest at a commercial beef processing facility, and transferred to the Center for Meat Safety & Quality at Colorado State University. Each piece of beef tissue was divided into two 10 x 10 cm^2 portions for microbial tests and randomly assigned to either (i) untreated control or (ii) chemical treatment groups. Beef tissue portions were either left uninoculated (to evaluate the antimicrobial effects of SSS against naturally-contaminated beef tissue), or were spot-inoculated (droplets randomly dispersed on the surface of the beef) on one side (external fat side) with 200 µl of one of the four inoculum types to obtain a target inoculation level of approximately 6 log CFU/cm^2 before chemical treatment application. Inoculated samples were held for 15 min at room temperature to allow for bacterial cell attachment (reflecting the maximum amount of time that would be expected for bacteria to attach to the outer carcass surface on a commercial harvest floor under normal operating conditions). After cell attachment, half of the warm beef samples were randomly assigned to a treatment and the other half were left untreated to serve as untreated control samples to obtain initial plate counts.

Chemical treatment of the warm beef carcass surface tissue. Half of the uninoculated and inoculated samples were randomly assigned to one of four possible SSS (Zoetis Florham Park, NJ) treatments applied in a custom-built spray cabinet (Chad Co., Olathe, KS) designed to simulate commercial beef harvest floor operations. Each sample was aseptically placed on a meat hook attached to the spray cabinet to simulate a chain moving a carcass on the harvest floor. The chain speed was constant for all treatments. The remaining uninoculated and inoculated samples served as untreated controls. Treatment application parameters included: (i) two SSS pH levels (pH 1.5 [high] and pH 1.0 [low]), and (ii) two application pressures (13 psi

[low] and 22 psi [high]). The four treatments included: (i) High pH + Low Pressure, (ii) High pH + High Pressure, (iii) Low pH + Low Pressure, and (iv) Low pH + High Pressure. Additional pieces of uninoculated beef (extra portion from each piece of beef brisket) were treated at each SSS application level and parameter for pH analysis.

Microbiological and pH analyses. All samples for microbial analysis were mechanically pummeled (Masticator, IUL Industries, Barcelona, Spain) for 2 min in 175 ml of Dey/Engley neutralizing broth (D/E; Difco, Becton Dickinson, Franklin Lakes, NJ) and serially-diluted (10-fold) in 0.1% buffered peptone water (BPW; Difco, Becton Dickinson, Franklin Lakes, NJ). Appropriate dilutions were surface-plated (0.1 ml or 1 ml) onto tryptic soy agar (TSA; Acumedia, Lansing, MI) to determine total bacterial populations, TSA supplemented with rifampicin (100 µg/ml; TSA + rif) to enumerate rifampicin-resistant *E. coli* O157:H7, non-O157 STEC, and non-pathogenic *E. coli* populations, and on XLD agar (Acumedia, Lansing, MI) for *Salmonella* populations. Colonies were counted after incubation of plates at 35°C for 24 h (TSA + rif and XLD agar) or 25°C for 72 h (TSA).

Untreated and SSS-treated samples were diluted (1:5 dilution) with deionized water and mechanically pummeled (2 min) to obtain pH measurements. The pH was measured with a calibrated pH meter fitted with a glass electrode (Denver Instruments, Arvada, CO).

Statistical analysis. This study was designed as a randomized complete block with a 2 x 2 factorial. The study was repeated on three separate days with two samples analyzed per treatment on each day (i.e., a total of six samples per treatment level; n = 6 replicates; day as random block effect). Bacterial populations are expressed as least squares means for log CFU/cm^2 (SE) calculated under an assumption of a log-normal distribution of plate counts. Data

were evaluated using the Mixed Procedure of SAS version 9.3 (Cary, NC) with independent variables including inoculum type, pH level, pressure and the respective interactions. To compare surviving populations of the surrogates to those of each pathogen group (non-O157 STEC, *E. coli* O157:H7, *Salmonella*) after application of SSS, data were analyzed using a Mixed Procedure such that the model included the plate counts before chemical treatment (after inoculation) as a covariate to adjust least squares means to a common pre-treatment inoculated plate count. All differences were reported using a significance level of $\alpha = 0.05$.

Results and Discussion

Surrogate behavior. Adjusted (to a common inoculation level) non-pathogenic *E. coli* surrogate mean plate counts obtained from inoculated beef samples treated with SSS, plated on selective and non-selective media were the same ($P > 0.05$) as for those counts for the pathogens. Studies conducted by Cabrera-Diaz et al. (2009) and Ingham et al. (2010) determined suitability for different non-pathogenic *E. coli* strains as suitability for surrogate organisms for *E. coli* O157:H7 and *Salmonella,* similarly to this study. Cabrera-Diaz et al. (2009) determined surrogate behavior using a different approach than this study. Instead of conducting a study to create parameter "book-ends" for a certain intervention, as was conducted in this study and Ingham et al. (2010), they subjected surrogate bacteria and the pathogen inocula to various categorical treatments. For example, they evaluated resistance and growth curves on TSA and found no difference ($P > 0.05$) in behavior (Cabrera-Diaz et al., 2009). This was similar to the present study in that surrogate and inocula populations were evaluated on TSA agar before and after beef samples were treated to compare cell attachment and growth behaviors when inoculated onto beef tissue. Additionally Cabrera-Diaz et al. (2009) evaluated D-values in heated PBS of the bacteria and found no difference ($P > 0.05$) in thermal resistance between the

target pathogens and the surrogates; this is potential evidence for use in thermal treatment of beef products. Lastly they compared acid resistant properties using lactic acid acidified PBS and determined that log reductions were the same ($P > 0.05$) for the surrogates, *E. coli* O157:H7, and most of the *Salmonella* strains. Similar conclusions were drawn when evaluating use of SSS at high and low pH levels (low = 1.0; high = 1.5) and at differing application pressures (low = 13 psi; high = 22 psi) in the present study; no differences ($P > 0.05$) occurred in acid resistant behavior (Tables 4.1 and 4.2).

No main effects of treatment were detected ($P > 0.05$) for any of the differing types of inocula based on counts obtained on selective media (TSA + rif), other than for *Salmonella* which differed by pH level (P = 0.0001) in this current study. A main effect of pH was obtained for counts recovered with TSA agar for STEC-inoculated samples (P = 0.0291), while the others only showed a trend of pH ($P < 0.10$). Due to the non-selective TSA, the main effect of pH for STEC-inoculated samples was likely detected because of the background bacteria causing a difference on this media only. Cabrera-Diaz et al. (2009) conducted an evaluation of their surrogate bacteria with a general approach to any antimicrobial situation, while the present study, as well as the study Ingham et al. (2010), was aimed at validating use of the surrogates for specific intervention applications, all concluding that the non-pathogenic *E. coli* were appropriate surrogates for *E. coli* O157:H7 and *Salmonella.* Application of SSS between high and low pH and pressure on warm beef carcass surface tissue resulted in surrogate and pathogen inocula responding similarly, making the surrogate inoculum a viable option to use in a plant for validation purposes.

Antimicrobial effect of SSS treatments. All treatment combinations effectively reduced ($P < 0.05$) bacterial contamination on inoculated warm beef carcass surface tissue

(Tables 4.3 to 4.5). Initial inoculation levels on beef samples was approximately 6.1 to 6.3 log CFU/cm^2. Geornaras et al. (2012b) conducted a study using SSS to treat inoculated beef trimmings against *E. coli* O157:H7 and non-O157 STECs. Inoculation level was similar to this study, except that they applied a different initial inoculation of 3.2 log CFU/cm^2. The average natural contamination level of warm carcass surface tissue was ≤ 1.4 log CFU/cm^2 and bacterial counts of all treated uninoculated samples were less than the initial contamination level for all treatment combinations (data not shown). Approximately 12.5% of the treated, naturally-contaminated samples were below the detection limit of the test (< 0.2 log CFU/cm^2).

Geornaras et al. (2012b) treated inoculated beef trimmings, differently from this study; instead of using a spray treatment, beef trimmings were immersed for 30 s and were also treated with a pH of 1.2 SSS versus SSS at 1.0 or 1.5 pH. In the present study, SSS at pH 1.0 applied to inoculated warm beef carcass tissue resulted in reductions in *E. coli* O157:H7, non-O157 STEC, and *Salmonella* populations of 0.9 to 1.5 log CFU/cm^2 depending on the pathogen. Geornaras et al. (2012b) found reductions that were lower in magnitude in *E. coli* populations than found in the present study; after treatment of SSS, we observed reductions of 0.1 to 0.3 log CFU/cm^2. Geornaras et al. (2012b) found SSS to be less effective than in the present study, and procedural differences that may have resulted in such discrepancies were multifaceted. In the present study, SSS was used at a pH 1.0 and applied as a spray treatment, while Geornaras et al. (2012b) applied SSS as an immersion for 30 s at a pH 1.2, thereby limiting the physical properties of removing bacteria when applied as a spray and using a less concentrated solution. Results of the present study showed that, when SSS was applied at 1.5 pH, reductions observed in *E. coli* populations were 0.4 to 0.6 log CFU/cm^2 which were still greater reductions than observed by

Geornaras et al. (2012b) on beef trim. This indicated that the spray application may be a factor that inhibited greater efficacy in their study.

Schmidt et al. (2014) used SSS (pH 1.8) as an immersion to treat beef cheek meat inoculated with *Salmonella* and shiga toxin-producing *Escherichia coli* with initial inoculation levels ranging from 3.8 to 4.1 log CFU/cm^2; lower inoculation levels than achieved in this study. They found reductions of *Salmonella enterica* obtained from the lean cheek meat of 1.0 to 1.5 log CFU/cm^2 varying based on duration of immersion; similar results were observed in the present study (Table 4.3 to 4.5; Schmidt et al., 2014). In the present study, the greatest reductions found in *Salmonella* inocula were obtained when beef samples were spray treated with SSS at pH 1.0; however, Schmidt et al. (2014) achieved similar results when using higher pH (1.8) and as an immersion. This may have occurred due to the extended length of immersion time (1, 2.5, and 5 min) compared to a shorter chain speed spray application in this study. Both studies provided two effective ways to reduce pathogen contamination at different SSS concentrations and application methods. Based on the findings of this study, SSS is an effective antimicrobial intervention for the surrogate and pathogen inocula when applied within the tested high and low parameters of pH and pressure onto warm beef carcass surface tissue.

pH analysis. The pH of both untreated control and SSS treated warm beef carcass surface tissue samples was determined and compared to assess any residual effect of the acid treatment on the surface of the beef tissue. Samples treated with high pH levels (1.5) were not different ($P > 0.05$) from the control samples, while the low pH (1.0) treatment samples had lower ($P < 0.05$) surface pH values compared to the controls (Table 4.6). In a study in which chilled beef trim samples treated with SSS, the chemically treated beef samples had significantly lower surface pH values compared to the untreated control when the samples were immersed in

SSS at a pH of 1.2 (Geornaras et al., 2012a). In another study, beef trim treated with SSS, resulted in surface pH values that were lower ($P < 0.05$) compared to the SSS treated beef samples (Geornaras et al., 2012b). In the present study, untreated beef samples had pH values of 5.7 to 5.8 (Table 4.6). Geornaras et al. (2012a) found that the pH of untreated beef samples was 5.7 to 5.9 before chemical treatment, which were the same as control pH measurements found in the present study. Likewise, in another study, control beef samples had pH values of 5.4 to 6.0 (Geornaras et al., 2012b). In the present study, warm beef carcass tissue samples treated with the low pH SSS generated surface pH values that were lower ($P < 0.05$) than controls (pH 4.7 to 4.8; Table 6). Geornaras et al. (2012a) found that pH of sample surfaces was similar for beef trim (pH of 4.8) after treatment with a spray application of SSS at a pH of 1.2, and concluded that residual pH of SSS applied at either 1.0 or 1.2 as a spray or immersion have the same impact on surface pH. Application of SSS at a low pH (1.0) on warm beef carcass surface tissue samples caused reduced surface pH value for beef samples compared to treatments with SSS at pH above 1.5.

Table 4.1. For each treatment combination, P-values express the direct comparison of each pathogen to the surrogates for adjusted treated plate count results (initial plate counts used as a covariate) obtained with selective media (tryptic soy agar with rifampicin [TSA + rif] and xylose lysine deoxycholate [XLD] agar) from treated beef samples.

	Non-O157 STEC vs. Surrogate (TSA + rif)	***E. coli* O157:H7 vs. Surrogate (TSA + rif)**	***Salmonella* vs. Surrogate (XLD agar)**
*Treatment (pH, Pressure)	**P-value	**P-value	**P-value
High, Low	0.5293	0.6972	0.7812
High, High	0.2918	0.3325	0.0723
Low, Low	0.1475	0.8773	0.0647

* The pH and pressure of the sulfuric acid and sodium sulfate solution are expressed in all combinations for the high (pH 1.5 and 22 psi) and low (pH 1.0 and 13 psi) parameters tested.

** P-values > 0.05 indicate no difference in behavior of the pathogenic group to the surrogate inoculum.

Table 4.2. For each treatment combination, P-values express the direct comparison of each pathogen to the surrogates for adjusted treated plate count results (initial plate counts used as a covariate) obtained with tryptic soy agar from treated beef samples.

	Non-O157 STEC vs. Surrogate	***E. coli* O157:H7 vs. Surrogate**	***Salmonella* vs. Surrogate**
*Treatment (pH, Pressure)	**P-value	**P-value	**P-value
High, Low	0.5697	0.6940	0.9477
High, High	0.3988	0.5941	0.1509
Low, Low	0.3712	0.2893	0.2370
Low, High	0.6506	0.4950	0.4019

* The pH and pressure of the sulfuric acid and sodium sulfate solution are expressed in all combinations for the high (pH 1.5 and 22 psi) and low (pH 1.0 and 13 psi) parameters tested.
** P-values > 0.05 indicate no difference in behavior of the pathogenic group to the surrogate inoculum.

Table 4.3. Adjusted least squares mean (SE) plate counts (log CFU/cm^2) obtained with tryptic soy agar with rifampicin [TSA + rif] and xylose lysine deoxycholate [XLD] agar from warm carcass surface tissue after treatment of sulfuric acid and sodium sulfate applied at high and low pH compared to the untreated plate counts for each inoculum type.

	Non-O157 STEC TSA + rif		***E. coli* O157:H7 TSA + rif**		***Salmonella* XLD agar**	
Treatment	Untreated	Treated	Untreated	Treated	Untreated	Treated
High pH (1.5)	6.3^a (0.05)	5.8^b (0.10)	6.2^a (0.07)	5.5^b (0.09)	6.1^a (0.04)	5.3^b (0.07)
Low pH (1.0)	6.3^a (0.05)	5.6^b (0.10)	6.2^a (0.07)	5.2^b (0.08)	6.1^a (0.04)	4.6^c (0.08)

^{a,b,c} Least squares means with the same bacteria with no common superscript letter are different ($P < 0.05$).

Table 4.4. Adjusted least squares mean (SE) plate counts (log CFU/cm^2) obtained with tryptic soy agar from warm beef carcass surface tissue with an application of sulfuric acid and sodium sulfate at high and low pH compared to the untreated before plate counts for each inoculum type.

	Non-O157 STEC		***E. coli* O157:H7**		***Salmonella***	
Treatment	Untreated	Treated	Untreated	Treated	Untreated	Treated
High pH (1.5)	6.3^a (0.03)	5.8^b (0.07)	6.2^a (0.06)	5.5^b (0.08)	6.2^a (0.05)	5.4^b (0.07)
Low pH (1.0)	6.3^a (0.03)	5.5^c (0.08)	6.2^a (0.06)	5.2^b (0.10)	6.2^a (0.05)	4.9^c (0.07)

^{a,b,c} Least squares means with the same bacteria with no common superscript letter are different ($P < 0.05$).

Table 4.5. Adjusted least squares mean (SE) treated plate counts (log CFU/cm^2) obtained with tryptic soy agar (TSA) and tryptic soy agar with rifampicin (TSA + rif) from warm beef carcass surface tissue with an application of sulfuric acid and sodium sulfate at high and low pH, compared to the untreated before plate counts for the surrogate inoculum; P-values < 0.05 are significant.

	Surrogates TSA			**Surrogates TSA + rif**		
Treatment (pH, Pressure)	Untreated	Treated	P-value	Untreated	Treated	P-value
High pH (pH 1.5)	6.4 (0.04)	5.7 (0.08)	<0.0001	6.4 (0.04)	5.6 (0.08)	<0.0001
Low pH (pH 1.0)	6.4 (0.04)	5.4 (0.07)	<0.0001	6.4 (0.04)	5.4 (0.06)	<0.0001

Table 4.6. Least squares means for pH (SE) of warm beef carcass tissue treated with sulfuric acid and sodium sulfate at different treatment parameters, high (pH 1.5 and 22 psi) and low (pH 1.0 and 13 psi), compared to the untreated control pH samples; P-values < 0.05 are significant.

Treatment (pH, Pressure)	**pH Samples**		
	Untreated (Control)	Treated	P-value
High, Low	5.82 (0.12)	5.60 (0.12)	0.2302
High, High	5.73 (0.11)	5.47 (0.11)	0.0968
Low, Low	5.87 (0.08)	4.84 (0.08)	0.0032
Low, High	5.77 (0.06)	4.76 (0.06)	0.0003

CHAPTER 5

Experiment C

Summary

Two studies were conducted with the objectives of (i) determining that inoculants of non-pathogenic *Escherichia coli* effectively serve as surrogates for pathogenic *Escherichia coli* O157:H7, non-O157 shiga toxin-producing *Escherichia coli* (STEC), and *Salmonella* spp. when warm beef carcass tissue was treated with a commercially available blend of lactic acid and citric acid (LCA) between high and low parameters, and (ii) validate that LCA between designated low and high application parameters was an effective antimicrobial intervention when applied to warm beef carcass surface tissue, and (iii) utilize the surrogate organisms to validate the use of hot water pasteurization in combination with LCA as a carcass antimicrobial spray intervention in a commercial beef harvest facility. Beef tissue (10 x 10 cm^2) was randomly assigned to either a (i) before treatment or (ii) after treatment group for each inoculum mixture. Four inoculum mixtures were used: a 5-strain mixture of *E. coli* O157:H7, 12-strain mixture of non-O157 STEC, a 6-strain mixture of *Salmonella*, and a 5-strain mixture of non-pathogenic *E. coli* (6.0 log CFU/cm^2). A lactic acid and citric acid blend was applied to inoculated beef tissue in a spray cabinet at low and high application parameters of temperature (43, 60 ^{o}C), concentration (1.9, 2.5%), and application pressure (15, 30 psi). The surrogate organisms were used to inoculate beef carcasses to validate the use of hot water pasteurization (92.2 to 92.8^{o}C at 13 to 15 psi) combined with a LCA spray intervention (1.9%; 13 to 15 psi; 50 to 51.7°C) at a commercial beef harvest facility. After treatment of LCA, surrogate counts were compared to each of the pathogen counts by using the pretreatment plate counts as a covariate in the model; they responded the same ($P > 0.05$) to the application of LCA. All treatment combinations effectively

reduced ($P < 0.05$) bacterial contamination. A hot water pasteurization cabinet and a LCA spray cabinet at indicated parameters significantly ($P < 0.05$) reduced bacterial contamination on inoculated beef carcasses, thus validating the intervention as a system.

Introduction

Escherichia coli O157:H7, non-O157 shiga toxin-producing *E. coli*, and *Salmonella* spp. are the most prevalent pathogens in fresh beef products (Huffman, 2002; Koohmaraie et al., 2005; Sofos, 2008; USDA, 2012). Considerable effort has been expended since the 1990s to control pathogens in beef products (Wheeler et al., 2014). The greatest progress for control of pathogens in beef began after *E. coli* O157:H7 became an adulterant in ground beef and, additionally, in 2011 when six serotypes of shiga toxin-producing *E. coli* were declared an adulterant (USDA-FSIS, 2012; Wheeler et al., 2014). Industry and government agencies have invested in research efforts to reduce pathogens on beef and have shown success. United States Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) microbiological testing results for raw ground beef and raw ground beef components for *E. coli* O157:H7 or non-O157 STEC have declined in the number of positive samples between 2001 and 2012 from 0.77% to 0.24% (Wheeler et al., 2014).

Pathogens that contaminate beef products primarily originate from cattle hides (Koohmaraie et al., 2005). Multiple hurdles technology is the most common and most effective method for controlling risk of pathogens on meat (Graves-Delmore et al., 1998; Keeton et al., 2006; Koohmaraie et al., 2005; Pohlman et al., 2002; Sofos, 2008). These systems include several sequential food safety interventions in a process to continually provide control through elimination and reduction of pathogens (Graves-Delmore et al., 1998; Keeton et al., 2006;

Koohmaraie et al., 2005; Pohlman et al., 2002; Sofos, 2008). Physical and chemical interventions such as steam pasteurization, hot water pasteurization, and chemical treatments are the most effective interventions used on a beef harvest floor today (Koohmaraie et al., 2005; Sofos, 2008). There are a variety of chemical interventions that are considered for use in the meat industry, varying in efficacy and cost (Koohmaraie et al., 2005; Sofos, 2008).

The USDA-FSIS requires verification of process control by validating that CCP food safety interventions are effective in controlling prevalent pathogens (9 CFR 413, 417, 430). Using pathogen testing or natural microflora to verify process control may be difficult due to the low number of bacteria cells to use as data to support effectiveness (Arthur et al., 2004). Use of high levels of surrogate organisms may be a helpful mechanism to validate efficacy of a food safety intervention and to provide evidence that a system is working properly (Arthur et al., 2004). One antimicrobial chemical that is a combination of lactic acid and citric acid (LCA) has not been adequately researched to provide validating evidence to beef processing facilities in regards to its effectiveness against pathogens.

Thus, the objectives of this laboratory study were to (i) determine if inoculants of non-pathogenic *Escherichia coli* effectively serve as surrogates for pathogenic *Escherichia coli* O157:H7, non-O157 shiga toxin-producing *Escherichia coli* (STECs), and *Salmonella* spp. when warm beef carcass surface tissue was treated with a commercially available blend of lactic acid and citric acid (LCA) between designated parameters, and (ii) determine if LCA between designated low and high application temperatures, pressures and concentrations was an effective antimicrobial intervention when applied to warm beef carcass surface tissue inoculated with surrogate and pathogenic organisms. The objectives of a second in-plant study were to utilize the

surrogate organisms to validate that hot water pasteurization, in combination with LCA, provides adequate carcass antimicrobial effectiveness in a commercial beef harvest facility.

Materials and Methods

Preparation of Inocula. This study utilized four inoculum mixtures from bovine origin, including: (i) a 5-strain mixture of *E. coli* O157:H7 (ATCC 43895, C1-072, C1-109, C1-154, C1-158), (ii) 12-strain mixture of non-O157 STEC (two strains of each serogroups: O26, O45, O103, O111, O121, and O145), (iii) a 6-strain mixture of *Salmonella* (serotypes Agona, Anatum, Saint-Paul, Reading, Newport and Typhimurium DT104 var. Copenhagen), and (iv) a 5-strain mixture of non-pathogenic *E. coli* (ATCC BAA-1427, ATCC BAA-1428, ATCC BAA-1429, ATCC BAA-1430, ATCC BAA-1431) that could serve as surrogates for *E. coli* O157:H7, STEC, and *Salmonella* spp. Cultures of rifampicin resistant *E. coli* O157:H7, non-pathogenic *E. coli* and non-O157 STEC strains were used for selection and differentiation of the inoculum from natural contamination. Xylose lysine deoxycholate (XLD; Acumedia, Lansing, MI) agar was used for selective enumeration of the *Salmonella* inoculum. Strains were activated and subcultured (35°C, 24 ± 2 h) in 10 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD; for *Salmonella* strains) or TSB supplemented with 100 µg/ml rifampicin (Sigma-Aldrich Inc., St. Louis, MO; for all rifampicin-resistant *E.coli* strains). Each inocula were individually combined and cells harvested by centrifugation (3220 x *g*, 20 min, 4°C, Eppendorf model 5810 R, Brinkman Instruments Inc., Hamburg, Germany), washed with 10 ml phosphate buffered saline (PBS, pH 7.4; Sigma, St. Louis, MO), re-centrifuged, and resuspended in PBS to the original volume (*E. coli* O157:H7 [50 ml], non-O157 STEC [120 ml], *Salmonella* [60 ml]) to obtain a concentration of 8 to 9 log CFU/ml.

The inoculum used in the commercial beef plant consisted of a five-strain mixture of non-pathogenic *E. coli* (ATCC BAA-1427, ATCC BAA-1428, ATCC BAA-1429, ATCC BAA-1430, and ATCC BAA-1431) that were considered surrogates for *E. coli* O157:H7, non-O157 *E. coli*, and *Salmonella* from the laboratory study. The strains were individually cultured and subcultured (35°C, 24 ± 2 h) in 10 ml of TSB. Broth cultures (10 ml) of all five strains were then combined and cells harvested by centrifugation. Cell pellets were washed with 10 ml of PBS re-centrifuged, and resuspended to the original volume in PBS to obtain a concentration of approximately 8 log CFU/ml.

Sample collection/inoculation of warm beef carcass surface tissue. The laboratory study was conducted over five days. A total of 200 sections of warm beef carcass surface brisket tissue were collected from hot beef carcasses, after being subjected to hot-water pasteurization during harvest at a commercial beef processing facility, and transferred to the Center for Meat Safety & Quality at Colorado State University. Beef tissue samples were divided into two 10 x 10 cm^2 portions for microbial tests (n = 5) and randomly assigned to either (i) untreated control or (ii) chemical treatment groups. Beef tissue portions were either left uninoculated, for evaluation of the antimicrobial effects of LCA against natural microflora, or were spot-inoculated (drops randomly dispersed on the surface of the beef) onto the external adipose side with 200 µl of one of the four inoculum types for a target inoculation level of approximately 6 log CFU/cm^2. Inoculated samples sat for 15 min at room temperature to allow for bacterial cell attachment; this time reflected the maximum amount of time that would be expected for bacteria to attach to the carcass surface under normal operating conditions at a commercial beef facility. After cell attachment, half of the beef samples were randomly assigned a treatment and the other half were left untreated to serve as untreated control samples to obtain initial plate counts.

The surrogate bacteria were used to validate the use of a hot water pasteurization cabinet and a LCA spray cabinet, as a combined system, to control pathogenic *E. coli* and *Salmonella* at a commercial beef facility. Carcass sides (n = 40) were inoculated on the out rail to allow ease of inoculation and to prevent carcasses from touching. The external carcass surface was inoculated within three 10×10 cm^2 areas that were designated aseptically with carcass ink on the chuck. Inoculation was performed using separate sterile sampling sponges hydrated with 10 ml of the inoculum and then sponging the carcass surface within the marked zones (i.e., separate inoculum-hydrated sponges were used for each zone). The target inoculation level was 6 log CFU/cm^2. After inoculation, carcasses were left stationary for approximately 10 min before treatment to allow for bacterial cell attachment. On each sampling day, 10 carcasses were inoculated and subjected to treatment.

LCA treatment/ sampling of warm beef carcass surface tissue. For the laboratory study, uninoculated and inoculated samples were randomly assigned to one of eight possible LCA (Birko Corp.; Henderson, CO) treatments applied in a custom-built spray cabinet (Chad Co., Olathe, KS) designed to simulate a commercial beef carcass spray cabinet. Samples were aseptically placed on the spray cabinet hook to simulate a chain moving a carcass on the harvest floor; the chain speed was constant for all treatments. Remaining uninoculated and inoculated samples served as untreated controls. The LCA solution was applied to inoculated beef tissue at low and high application parameters of temperature (43, 60 ºC), concentration (1.9, 2.5%), and application pressure (15, 30 psi) for a total of eight treatments. Samples had a 10 min dwell time before buffer was added. Additional pieces of uninoculated beef (extra portion from each piece of beef brisket) were treated at each LCA application level and parameter for pH evaluation.

The in-plant study treatment parameters included: a hot water pasteurization cabinet (92.2 to 92.7ºC at 13 to 15 psi) combined with a LCA carcass spray intervention (1.9%; 13 to 15 psi; 50 to 51.7°C). Over two days of treatment, 10 carcasses (20 sides) were inoculated and treated with the hot water pasteurization cabinet and the LCA spray cabinet Before entering the hot water pasteurization cabinet, both an inoculated and uninoculated (naturally-contaminated) zone were sampled to serve as the "Before" sample for the whole system. The carcasses continued through the hot water pasteurization cabinet followed by sampling of a second zone (in the middle of the system). The carcasses then continued through the LCA cabinet and then the chain was stopped to obtain samples from different inoculated and uninoculated-treated zones to serve as the "After" samples for the whole system. This study was repeated on two separate days. Prior to intervention treatments, the lower inoculated zones, once inoculated, were sampled with a 3M sampling sponge hydrated with 10 ml D/E neutralizing broth (3M; St. Paul, MN). Zones were sampled by making 10 vertical and 10 horizontal passes within the 10×10 cm^2 area. After treatment with the hot water pasteurization and the LCA, the remaining uninoculated and inoculated zones were sponge sampled likewise for the whole system.

Microbiological and pH analyses. Samples in the in-lab study for microbial analysis were mechanically pummeled (Masticator, IUL Industries, Barcelona, Spain) for 2 min in 175 ml of Dey/Engley neutralizing broth (D/E; Difco, Becton Dickinson, Franklin Lakes, NJ) and serially-diluted (10-fold) in 0.1% buffered peptone water (BPW; Difco, Becton Dickinson, Franklin Lakes, NJ). Appropriate dilutions were surface-plated (0.1 ml or 1 ml) onto tryptic soy agar (TSA; Acumedia, Lansing, MI) to determine total bacterial populations, TSA supplemented with rifampicin (100 µg/ml; TSA + rif) to enumerate rifampicin-resistant *E. coli* O157:H7, non-O157 STEC, and non-pathogenic *E. coli* populations, and on XLD agar (Acumedia, Lansing,

MI) for *Salmonella* populations. Colonies were counted after incubation of plates at 35°C for 24 h (TSA + rif and XLD agar) or 25°C for 72 h (TSA). Untreated and LCA-treated samples were diluted (1:5 dilution) with deionized water, and mechanically pummeled (2 min) to obtain pH measurements. The pH was measured with a calibrated pH meter fitted with a glass electrode (Denver Instruments, Arvada, CO).

For the in-plant study, sponge samples were sent to a commercial lab (Food Safety Net Services) for microbial analysis. Upon arrival, 15 ml of butterfield's buffer (Hardy Diagnostics; Santa Maria, CA) were added to all samples for a total of 25 ml of diluent (10 ml D/E + 15 ml butterfield's buffer). Samples were mechanically pummeled for 2 min and serially diluted (10-fold) in buffered peptone water (BPW; Difco, Becton Dickinson, Franklin Lakes, NJ). Appropriate dilutions were surface-plated in duplicate to enumerate aerobic plate counts (3M Petrifilm Aerobic Count Plates; St. Paul, MN) and *Enterobacteriaceae* counts (3M Petrifilm Enterobacteriaceae Count Plates; St. Paul, MN). Colonies on Petrifilm plates were enumerated following 48 hours incubation at 37°C.

Statistical analysis. The laboratory study was designed as a randomized complete block with a 2 x 2 x 2 factorial. Bacterial populations are expressed as least squares means for log CFU/cm^2 (standard error; SE) calculated under an assumption of a log-normal distribution of plate counts. Data were evaluated using the Mixed Procedure of SAS version 9.3 (Cary, NC) with independent variables including inoculum type, concentration, temperature, pressure and the respective interactions. To compare surviving populations of the surrogates to those of each pathogen group (non-O157 STEC, *E. coli* O157:H7, *Salmonella*) after application of LCA, data were analyzed using a Mixed Procedure such that the model included the plate counts before chemical treatment (after inoculation) as a covariate to adjust least squares means to a common

pre-treatment inoculated plate count. All differences were reported using a significance level of $\alpha = 0.05$. The study conducted in-plant was designed as a paired comparison and data were presented as least squares mans with differences reported using a significance level of $\alpha = 0.05$.

Results and Discussion

Surrogate behavior. Treatment parameters for application of LCA onto warm beef carcass surface tissue of temperature (low = 43°C; high = 60°C), pressure (low = 15 psi; high = 30 psi), and concentration (low = 1.90%; high = 2.30%) did not cause the surrogates to behave differently than the pathogenic inoculums when plated onto TSA (Table 5.1). A difference in response to treatment between the surrogate and *Salmonella* spp. inoculums ($P < 0.05$) was detected at one treatment combination (Low, High, High), but that difference was not microbiologically meaningful (less than 1.0 log CFU/cm^2); this difference may be due to background bacteria recovered from the non-selective TSA. When surrogate and pathogen inocula were compared on selective media, no differences ($P > 0.05$) were detected (data not shown).

Cabrera-Diaz et al. (2009) compared fluorescent protein marked non-pathogenic *E. coli* to pathogenic *Salmonella* strains and *E. coli* O157:H7 to be used as surrogates for validation purposes in commercial beef facilities. They evaluated both non-pathogenic and pathogenic bacteria attachment, resistance, and growth curves on TSA and found no difference ($P > 0.05$) in behavior between the groups; this was done similarly when comparing inocula and surrogate organism populations for attachment and growth differences in the current study. Additionally, they evaluated D-values in heated PBS of the bacteria and found no difference ($P > 0.05$) in thermal resistance between the target pathogens and the surrogates (Cabrera-Diaz et al., 2009).

Lastly, they compared acid resistant properties using lactic acid acidified PBS and determined that log reductions were the same ($P > 0.05$) for the surrogates, *E. coli* O157:H7, and most of the *Salmonella* strains; however, some *Salmonella* serotypes were less resistant than the surrogate bacteria (Cabrera-Diaz et al., 2009). Although the methods were different between Cabrera-Diaz et al. (2009) and the current study, the conclusions were the same between studies. This study utilized a specific intervention to determine if surrogate organisms would appropriately represent the pathogens in that environment; Cabrera-Diaz et al. (2009) took a more general approach to ensure that the surrogate organisms behaved as the pathogens in any antimicrobial situation for various validation purposes in beef production.

Ingham et al. (2010) used a more direct approach to compare potential of non-pathogenic *E. coli* as indicator organisms to serve as surrogate bacteria for *E. coli* O157:H7 for use in commercial beef plants. A surrogate was considered a suitable indicator organism for *E. coli* O157:H7 if the intervention produced a reduction in surrogate levels that was not greater ($P > 0.05$) than that observed for *E. coli* O157:H7, which were the same characteristics used to draw conclusions in the present study (Ingham et al., 2010). They treated samples with a 6 d dry aging, three different acids (acetic acid, lactic acid and Fresh Bloom [a mix of erythorbic, ascorbic, and citric acids]), followed by 1 d dry aging, and hot water (Ingham et al., 2010). All three surrogate inocula were suitable as *E. coli* O157:H7 surrogates for use in dry aging and acid spray plus dry-aging treatments of beef; the surrogates in the present study agreed with Ingham et al. (2010). In the present study, application of LCA on warm beef carcass surface tissue caused the surrogates and pathogen inoculums to respond similarly, making the surrogate inoculum a viable option to use in a plant for validation purposes.

In-lab antimicrobial effect of LCA treatments. All treatment combinations effectively reduced ($P < 0.05$) bacterial contamination on inoculated warm carcass surface tissue (Tables 5.2 to 5.4). The average natural contamination on warm beef carcass surface tissue was ≤ 1.30 log CFU/cm^2 and all warm beef carcass samples had lower counts following treatment; 32.50% of the natural bacterial contamination of the treated samples were below the detection limit (0.2 log CFU/cm^2). No main effects ($P > 0.05$) of treatment parameters (temperature, pressure or concentration) were detected, all data are presented as treated counts versus the controls (Tables 5.2 to 5.4). Laury et al. (2009) determined that the effects of 2.5% LCA as spray and immersion treatments on the reduction of *Salmonella* on whole broiler carcasses during processing and the reduction of *Salmonella* and *E. coli* O157:H7 on beef trim (beef tips). They inoculated the broiler carcasses to a 5.0 log CFU/ml level of *Salmonella* and the beef tips with 4.0 log CFU/100 cm^2 of either *Salmonella* or *E. coli* O157:H7; inoculation levels were very similar to those of the present study (Laury et al., 2009). After application of LCA, contamination was reduced ($P < 0.05$) for *Salmonella* populations by 1.3 log CFU/ml with a spray treatment and 2.3 log CFU/ml for all immersion treatments of poultry products (Laury et al., 2009). In the present study, similar results were achieved for reduction in *Salmonella* populations (1.5 to 1.8 log CFU/cm^2) when treated at the same concentration as a spray treatment. Laury et al. (2009) may have achieved higher reduction in *Salmonella* populations when applying LCA as an immersion due to the increased treatment time compared to the spray treatments. They found that LCA reduced ($P < 0.05$) *E. coli* O157:H7 populations by 1.4 log CFU/100 cm^2 and *Salmonella* populations by 1.1 log CFU/100 cm^2 compared with the control samples for the inoculated beef trim (Laury et al., 2009). Laury et al. (2009) achieved similar reductions of *E. coli* O157:H7 and *Salmonella* on inoculated beef trim compared to our results when inoculated warm beef carcass tissue was

treated with LCA. Results indicated that LCA was an effective antimicrobial intervention for the surrogate and pathogen inoculums when applied within the tested high and low parameters of temperature, pressure, and concentration on warm carcass surface tissue.

pH analysis. There were no main effects ($P > 0.05$) of treatment on the outcome of the surface pH of warm beef carcass surface tissue. Surface pH of the samples from all the treatment combinations were different ($P < 0.05$) than the surface pH of the control warm beef carcasses surface tissue samples. The surface pH of the control samples were 5.99 while the pH of the treated samples ranged from 4.62 to 5.01 (data not shown). No studies previously have evaluated the surface pH following treatment with LCA; however, a study conducted by Cutter and Siragusa (1994) evaluated surface pH on beef carcass adipose tissue for varying lactic and citric acid treatments separately. Treatment with LCA on warm beef carcasses surface tissue reduced ($P < 0.05$) the final surface pH compared to untreated samples. Agreeable to this study, Cutter and Siragusa (1996) found all surface pH to be reduced ($P < 0.05$) from those of control samples. They observed surface pH on adipose beef carcass tissue to be approximately 3.55 for a 3.0% citric acid treatment and a pH of 3.72 for a 3.0% lactic acid treatment, which was lower than that following an LCA treatment ranging from 4.62 to 5.01. Differences in results between those studies and the present study were likely due to the difference in chemicals and chemical concentration, as the LCA treated beef samples in the present study were at a lower concentration.

In-plant validation. The hot water pasteurization cabinet reduced the level of inoculated APC bacterial contamination from 6.2 log CFU/cm^2 to 2.7 log CFU/cm^2 (Table 5.6). Bolsilevac et al. (2006) conducted a study at a commercial beef harvest facility treating hot beef carcasses as a pre-evisceration intervention with 2.0% lactic acid spray, hot water wash (165ºC, 5.5 s), or a

combination of both treatments. They found similar results when subjecting carcasses to hot water pasteurization treatment and observed a reduction in APC contamination from 6.2 log CFU/100 cm^2 to 3.5 log CFU/100 cm^2. It is likely that, in the present study, an additional 0.8 log CFU/cm^2 reduction in APC populations was achieved with hot water due to the higher water temperature used (74ºC). An additional 0.4 log CFU/cm^2 reduction of APC bacterial contamination level were detected on carcasses subsequently treated with LCA, increasing the system effectiveness against APC (Table 5.6). On the contrary, Bolsilevac et al. (2006) found that the whole system (hot water + lactic acid) reduced APC contamination from 6.4 to 4.2 log CFU/100 cm^2, which was no more effective than the hot water pasteurization alone. Intuitively, the combination of hot water and lactic acid should have resulted in a greater reduction of APC contamination; however, the authors believed this finding to be chance and the possibility that the lactic acid may have had a cooling effect, reducing the effectiveness of the hot water (Bolsilevac et al., 2006). Carcasses were not inoculated in their study, so it is possible that the carcass variations may have caused the unexpected results of the whole intervention system.

Natural microflora recovered on APC petrifilm resulted in 70% of the samples below detection limit (-0.9 log CFU/cm^2) after hot water pasteurization in the current study (data not shown). Hot water pasteurization reduced the level of EB bacteria from 5.8 log CFU/cm^2 to 2.6 log CFU/cm^2 (Table 5.5). Bolsilevac et al. (2006) recovered EB populations before and after hot water pasteurization; initial counts were lower for EB (4.4 log CFU/100 cm^2) due to natural contamination and were not initially inoculated as were carcasses in the present study. They were able to achieve a 2.7 log reduction in EB populations compared to a 3.2 log reduction in the present study; the difference in effectiveness was most likely due to temperature differences (Table 5.5; Bolsilevac et al., 2006). With the application of LCA, an additional 0.5 log CFU/cm^2

reduction of EB bacteria contamination was achieved (Table 5.5). Natural microflora recovered on EB petrifilm resulted in 30% of the samples below detection limit after hot water pasteurization and 40% after treatment of LCA. Both inoculated and uninoculated warm beef carcass surface tissue, treated with a hot water pasteurization cabinet and a LCA spray cabinet at indicated parameters, reduced ($P < 0.05$) bacterial contamination. A hot water pasteurization cabinet with an LCA spray cabinet is an effective post-harvest carcass intervention to serve as a biological critical control point.

Table 5.1. For each treatment combination, P-values express the direct comparison of each pathogen to the surrogates for plate count results obtained with Tryptic Soy Agar (TSA) from lactic acid and citric acid (LCA) treated beef samples.

*Treatment (Temp., Press., Conc.)	**Non-O157 STEC vs. Surrogate**	***E. coli* O157:H7 vs. Surrogate**	***Salmonella* vs. Surrogate**
	**P-value	**P-value	**P-value
Low, Low, Low	0.3428	0.8316	0.2396
Low, High, Low	0.0751	0.1552	0.2447
High, Low, Low	0.3136	0.9770	0.7929
High, High, Low	0.3300	0.3887	0.6287
Low, Low, High	0.2724	0.3412	0.2499
Low, High, High	0.4652	0.4166	0.0056
High, Low, High	0.1103	0.0830	0.0591
High, High, High	0.7481	0.9257	0.3771

* The temperature (Temp.), pressure (Press.), and concentration (Conc.) of (LCA) are expressed in all combinations for the high (60 ^{o}C, 30 psi, 2.5%) and low (43 ^{o}C, 15 psi, 1.9%) parameters tested

** P-values > 0.05 indicate no difference in behavior of the pathogenic group to the surrogate inoculum

Table 5.2. Adjusted least squares mean (SE) plate counts (log CFU/cm^2) on tryptic soy agar from warm beef carcass surface tissue with an application of a blend of lactic acid and citric acid (LCA) spray treatment, inoculated with different pathogen groups; P-values < 0.05 are significant.

	Non-O157 STEC			***E. coli* O157:H7**			***Salmonella***		
Treatment	Untreated	Treated	P-value	Untreated	Treated	P-value	Untreated	Treated	P-value
LCA spray	6.2 (0.02)	5.3 (0.06)	<0.0001	6.2 (0.03)	5.2 (0.04)	<0.0001	6.2 (0.03)	4.9 (0.05)	<0.0001

Table 5.3. Adjusted least squares mean (SE) plate counts (log CFU/cm^2) on selective media (tryptic soy agar with rifampicin [TSA] and xylose lysine deoxycholate [XLD]) from warm beef carcass surface tissue with an application of a blend of lactic acid and citric acid (LCA) spray treatment, inoculated with different pathogen groups; P-values < 0.05 are significant.

	Non-O157 STEC TSA + Rifampcin			***E. coli* O157:H7 TSA + Rifampcin**			***Salmonella* XLD agar**		
Treatment	Untreated	Treated	P-value	Untreated	Treated	P-value	Untreated	Treated	P-value
LCA spray	6.2 (0.02)	5.2 (0.06)	<0.0001	6.2 (0.02)	5.3 (0.04)	<0.0001	6.0 (0.02)	4.5 (0.04)	<0.0001

Table 5.4. Adjusted least squares mean (SE) plate counts (log CFU/cm^2) on tryptic soy agar (TSA) and TSA with rifampicin from warm beef carcass surface tissue with an application of a blend of lactic acid and citric acid (LCA) spray for the surrogate inoculum; P-values < 0.05 are significant.

	Surrogates TSA			**Surrogates TSA + Rifampcin**		
Treatment	Untreated	Treated	P-value	Untreated	Treated	P-value
LCA spray	6.3 (0.02)	5.2 (0.06)	<0.0001	6.3 (0.02)	5.2 (0.06)	<0.0001

Table 5.5. Adjusted least squares mean (SE) *Enterobacteriaceae* counts (log CFU/cm^2) from warm beef carcass surface tissue before and after treatments of hot water pasteurization (H.P.; ≥ 70°C [carcass surface water], cabinet water temperature 92.2 – 92.7ºC, 13 - 15 psi) and lactic acid and citric acid (LCA) spray cabinet (1.9% LCA, 13 - 15 psi, 50 – 51.7°C); P-values < 0.05 are significant.

Treatment	**Inoculated Surrogates**			**Uninoculated/Natural Microflora**		
	Before	After	P-value	Before	After	P-value
After hot water pasteurization	5.8 (0.12)	2.6 (0.11)	<0.0001	< 0.6 (0.12)	< -0.4 (0.12)	<0.0001
After LCA spray cabinet (whole CCP system)	5.8 (0.12)	2.1 (0.10)	<0.0001	< 0.6 (0.12)	< -0.7 (0.10)	<0.0001

Least squares means with a less than symbol (<) indicate one or more of the samples within the treatment had plate counts below the analysis detection limit (-0.9 log. CFU/cm^2).

Table 5.6. Adjusted least squares mean (SE) aerobic plate counts (log CFU/cm^2) from warm beef carcass surface tissue before and after treatments of hot water pasteurization (H.P.; ≥ 70°C [carcass surface water], cabinet water temperature 92.2 - 92.7ºC, 13 - 15 psi) and lactic acid and citric acid (LCA) spray cabinet (1.9% LCA, 13 - 15 psi, 50 – 51.7°C); P-values < 0.05 are significant.

Treatment	Inoculated Surrogates			Uninoculated/Natural Microflora		
	Before	After	P-value	Before	After	P-value
After hot water pasteurization	6.2 (0.11)	2.7 (0.10)	<0.0001	< 1.2 (0.14)	< -0.2 (0.13)	<0.0001
After LCA spray cabinet (whole CCP system)	6.2 (0.11)	2.3 (0.10)	<0.0001	< 1.2 (0.14)	< -0.4 (0.12)	<0.0001

Least squares means with a less than symbol (<) indicate one or more of the samples within the treatment had plate counts below the analysis detection limit (-0.9 log. CFU/cm^2).

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