THESIS

VALIDATION AND EVALUATION OF COMMERCIALLY AVAILABLE

COMPOUNDS FOR USE AS BEEF AND PORK ANTIMICROBIAL

INTERVENTIONS

Submitted by

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ABSTRACT

VALIDATION AND EVALUATION OF COMMERCIALLY AVAILABLE

COMPOUNDS FOR USE AS BEEF AND PORK ANTIMICROBIAL

INTERVENTIONS

Studies were conducted to validate the use of various antimicrobial chemicals to be used as antimicrobial interventions of chilled subprimals and hot beef carcasses. Chemicals evaluated on surrogate *Escherichia coli* biotype 1 (BAA-1427, BAA-1428, BAA-1429, BAA-1430, and BAA-1431) inoculated at approximately 6.0 log CFU/cm^2 on beef and pork subprimals included lactic acid (2.0, 2.5, and 5.0%), Sodium Bisulfate (2.5%), lactic acid/Sodium Bisulfate mixture (3.0 and 6.0%), Blitz™ (180 and 220 ppm), Inspexx 200™ (100 and 220 ppm), SYNTRx 3300 (pH = 1.2), citrus essential oils (3.0 and 6.0%), and deionized water. Chemicals were applied at in a custom-built spray cabinet with stainless steel slotted conveyor belt (Chad Co., Olathe KS) at two pressures (1.03 and 4.83 bar) and two rate of applications (0.23 and 6.62 lpm). After treatment bacterial counts were lower on samples treated with SYNTRx 3300, compared to other treatments. After treatment counts on samples treated with Blitz, lactic acid, sodium bisulfate, and lactic acid/sodium bisulfate mixtures were similar. Counts after treatment on samples treated with Inspexx and water were similar and higher than other chemicals due to a washing effect and not a chemical effect.

Lactic acid can reduce microbial contamination on beef carcass surfaces when used as a food safety intervention, but effectiveness when applied to the surface of chilled beef subprimals is not well documented. Studies characterizing bacterial reductions from lactic acid on subprimals would be useful in validations of HACCP systems. The objective of this study was to validate initial use of lactic acid as a subprimal intervention during beef fabrication, followed by a secondary application to vacuum-packaged rework product following removal of packaging. Chilled beef subprimal sections (100 cm^2) were either left uninoculated or were inoculated with 6 log CFU/cm^2 of a 5-strain mixture of *Escherichia coli* O157:H7, a 12-strain mixture of non-O157 Shiga toxin-producing *E. coli* (STEC), or a 5-strain mixture of non-pathogenic (biotype I) *E. coli* considered surrogates for *E. coli* O157:H7. Uninoculated and inoculated subprimal sections received an initial or initial and second, "re-work" application of lactic acid in a custom-built spray cabinet at one of 16 different application parameters. Following the initial spray, total inoculum counts were reduced from 6.0 log CFU/cm^2 to 3.6, 4.4, and 4.4 log CFU/cm^2 for the *E. coli* surrogates, *E. coli* O157:H7, and non-O157 STEC inoculation groups, respectively. After the second, re-work application, total inoculum counts remaining were 2.6, 3.2, and 3.6 log CFU/cm^2 for the *E. coli* surrogates, *E. coli* O157:H7, and non-O157 STEC inoculation groups, respectively. Both the initial and secondary lactic acid treatments effectively reduced counts of pathogenic and non-pathogenic strains of *E. coli*, as well as natural microflora, on beef subprimals.

Studies characterizing bacterial reductions when chemicals are applied to beef carcass tissue would be useful in validations of HACCP systems. The objective of this study was to validate use of BoviBrom as a hot carcass intervention during beef slaughter

processes. Beef cutaneous trunci muscle segments (100 cm^2 of exposed surface lean)

were assigned to four groups: 1) inoculated with 4 log of a 5-strain *Escherichia coli*

O157:H7 cocktail, 2) inoculated with 4 log of a 12-strain, non-O157 shiga toxin-

producing *E. coli* (STEC) cocktail, 3) inoculated with 4 log of a 5-strain non-pathogenic

E. coli and *Salmonella* surrogate cocktail, and 4) not inoculated. Application of

BoviBrom occurred in a custom-built spray cabinet at four temperatures (40, 80, 100, and

120°F), three pressures (15, 90, and 120 psi), and three doses (0.5, 1.0, and 1.5 ml/cm^2),

at a single concentration (225 ppm). At sampling time, 100 cm^2 sections were

homogenized for 120 seconds in 100 ml of 0.1% buffered peptone water and serially-

diluted (10-fold) in 0.1% buffered peptone water. Appropriate dilutions were spread

plated (0.1 ml) onto non-selective media [tryptic soy agar (TSA)] and selective media

[TSA plus rifampicin (100μg/ml)] to determine total plate count (TPC) and total

inoculated count (TIC), respectively. Following initial BoviBrom application, varying

application parameters did not affect the after treatment counts, with the exception of

pressure of application as TPC and TIC were far higher when BoviBrom was applied at

15 psi. No difference ($P > 0.05$) was observed between samples treated with different

temperatures or doses. No difference ($P > 0.05$) was observed between samples

enumerated immediately following treatment and samples enumerated at 24 hours post-

treatment. TPC and TIC after treatment counts were lower on samples treated with

BoviBrom when compared to samples treated with only water, showing a portion of the

lower counts can be attributed to the chemical action of BoviBrom. These data would be

beneficial to industry as part of the HACCP validation process.

ACKNOWLEDGMENTS

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CHAPTER I

OBJECTIVES OF THESIS

The objectives of this thesis were:

(1) Validate the use of chemical compounds for use as antimicrobial interventions for subprimal beef and pork.

(2) Evaluate non-pathogenic *E. coli* Biotype I as surrogates for *Escherichia coli* O157:H7 and Non-O157 STEC during subprimal and carcass antimicrobial interventions.

CHAPTER II

LITERATURE REVIEW

INTRODUCTION

Meat products are relied upon by much of the world's population as a source of protein and essential nutrients. Meat and poultry industries contribute approximately $832 billion to the United States' economy, close to 6 percent of the total U.S. gross domestic product (AMI Fact Sheet, 2009). As reliance on meat continues, safety of meat products becomes a concern. The safety of meat products can be compromised by many avenues including contamination with chemical substances (antibiotics, sanitizers, and pesticides), physical hazards (wood, glass, metal) and the introduction of biological contamination such as bacteria, parasites, or viruses. Foods in the U.S. that were contaminated with known microbiological agents cause approximately 9.4 million illnesses, 56,000 hospitalizations, and 1,351 deaths per year (Scallen et al., 2011). Significant advances have been made to food and meat safety through decontamination strategies; however, food safety remains a major concern as contamination continues to occur. Food safety is continually improved by minimizing contamination on all levels.

Escherichia coli O157:H7 was first identified from an outbreak in the 1980s. Riley et al. (1983) described and identified the cause of the outbreak as a rare type of

E. coli, never before identified. *E. coli* O157:H7 was the cause of the largest ground beef

outbreak in the U.S. in 1992 and 1993 which resulted in over 700 illnesses and four

deaths (Bell et al., 1994). Studies implicated ground beef patties from a single

restaurant chain as the source of the illnesses. Holes where identified in processing

and cooking steps which allowed contamination to be present and survive the cooking

process.

     E. coli O157:H7 is estimated to cause 63,153 illnesses, 2,138 hospitalizations,

and 20 deaths a year in the U.S. (Scallen et al., 2011). Fernzen et al. (2005) estimated

the cost of a human infection that did not receive medical attention to be $26 and

could range to $6.2 million from an individual who died of Hemolytic Uremic

Syndrome (HUS), a severe form of *E. coli* O157:H7 infection which leads to kidney

failure. In addition to the cost of treatment and lost wages, the companies whose

product is implicated also suffer. Shareholder wealth is reduced by 1.5 to 3%

(Thomsen and McKenzie, 2001) and company losses can continue for over a month

after the recall. In one instance a child suffered brain damage from *E. coli* O157:H7

contamination from a hamburger and the subsequent lawsuit was settled for $15

million (Buzby et al., 2001).

     E. coli O157:H7 is part of a group of *E. coli* considered Shiga Toxin

producing *E. coli* (STEC) which is a portion of the Enterohemorrhagic *E. coli*

(EHEC) group. Numerous STEC strains, including O157:H7, have the ability to

produce one or both Shiga-toxins (Stx1 and Stx2) (Kaspar and Doyle, 2009). Scallen

et al. (2011) estimates that 112,752 illnesses, 271 hospitalizations, and 0 deaths occur in the U.S. per year that can be attributed to non-O157 STEC. Non-O157 STEC were recognized as a possible cause for cases of HUS in 1975 France (Karmali et al., 1985). *Escherichia coli* O103 was present in some patients; this serotype is part of the non-O157 STEC group referred to as "Top Six". The "Top Six" include serotypes O26, O111, O103, O121, O45, and O145 (Gyles 2007).

Humans become infected with STEC by ingesting contaminated food or water, or with direct contact with bacteria associated with live animals (Karmali 2004) (Figure 2.1). Sources of infection include meat, raw milk, cheeses, unpasteurized apple cider and juice, water, contact with animals, and a variety of produce including lettuce, canaloupes, alfalfa sprouts, radish sprouts (Gyles, 2007). *E. coli* O157 (an STEC) has been shown to have a low effective dose ranging from 50 cells (Tilden et al., 1996) to a few hundred (Bell et al., 1994). Shiga toxin producing *E. coli* infections result in a variety of symptoms including no symptoms, watery diarrhea, bloody diarrhea, or the most severe HUS. Although HUS is characterized by renal failure, other organs are susceptible to STEC including the central nervous system, lungs, pancreas, and heart (Gyles, 2007).

EHEC including STEC colonize the intestinal tract of the victim and do damage through the production of toxins. The colonization process requires STEC to overcome host defense mechanisms, most importantly the range of pH in the tract; very low in the stomach to relatively high in the Small Intestine. The process of infection is seen in Figure 2.2 (Gyles, 2007).

BACTERIAL CONTAMINATION OF BEEF

Many STEC strains including *E. coli* O157:H7 and non-O157 STEC are associated with ruminants and have been found on beef cattle and carcasses. Bacon *et al*. (2000) reported the hides of fed steers/heifers and non-fed cows/bulls carried relatively high levels of aerobic plate count (APC) populations (8.2 to 12.5 log CFU/100 cm^2), total coliform count (TPC) populations (6.0 to 7.9 log CFU/100 cm^2), and *E. coli* (ECC) populations (5.5 to 7.5 log CFU/100 cm^2). Following hide removal, carcasses were sampled and were found to have APC, TCC, and ECC ranging from 6.1 to 9.1, 3.0 to 6.0 and 2.6 to 5.3 log CFU/100 cm^2, respectively. Elder *et al*. (2000) sampled 29 lots of fed cattle before slaughter and reported that 38% had enterohemorrhagic *E. coli* (EHEC) O157 positive hide samples. When the lots were sampled as carcasses, EHEC O157 was isolated, concluding that hide prevalence was significantly correlated to carcass contamination. Barkocy-Gallagher *et al.* (2002) showed that 68.2% of *E. coli* O157:H7 isolated from carcasses genetically matched isolates obtained from the hide. Arthur et al. (2004) further evaluated the relationship between hide and carcass contamination on cattle entering the slaughter floor. *Escherichia coli* O157 was found on 76% of the animal hides. Indicator organisms such as APC and *Enterobacteriaceae* counts on hides averaged 7.8 and 6. 2, respectively. Corresonding chilled carcasses averaged 1.4 and 0.4 log CFU/100 cm^2 APC and *Enterobacteriaceae* counts, respectively. The authors reported carcasses with increased APC and *Enterobacteriaceae* populations were more likely to be contaminated with *E. coli* O157.

Keen and Elder (2002) sampled various areas on finishing cattle including feces, oral cavity and four different hide surface locations to determine microbial contamination. *Escherichia coli* O157 was found to be the most prevalent in the oral cavity (74.8%) followed by the back (73.4%), neck (62.6%), feces (60.4%), flank (54.0%), ventrum (51.1%) and hock (41.0%). Barkocy-Gallagher *et al.* (2003) found that fecal prevalence of *E. coli* O157:H7 peaked at 12.9%, while hide prevalence was never below 29.4% and at times as high as 73.8%. When corresponding, pre-eviscerated carcass were sampled, *E. coli* O157:H7 prevalence ranged from 1.2 to 40.8%. Fecal prevalence of *E. coli* O157:H7 was found to fecal peak in the summer (12.9%), whereas hide prevalence was high from the spring through the fall (67.2 to 73.8%) (Barkocy-Gallagher *et al.* 2003). Similarly, carcass *E. coli* O157:H7 contamination was more comparable to hide prevalence with at least 27.3% of pre-eviscerated carcasses contaminated with *E. coli* O157:H7 during the spring, summer and fall seasons.

Similar to *E. coli* O157:H7, cattle remain the primary reservoir for non-O157 STEC (Kaspar and Doyle, 2009). Barkocy-Gallagher *et al.* (2003) found non-O157 STEC prevalence in feces of fed beef cattle at slaughter to be from 13.9% to 27.1%. Like *E. coli* O157:H7, this was dependent on the season of the year (Van Donkersgoed, Graham, and Gannon, 1999). Barkocy-Gallagher *et al.* (2003) also looked at the prevalence of non-O157 STEC on the hides of cattle. Similar to *E. coli* O157:H7 on hides, non-O157 STEC varies depending on season; a low of 43% in the spring and a high of 78% in the fall.

Before evisceration, 53.9% of beef carcasses in large U.S. processing plants were positive for at least one strain of non-O157 STEC and this prevalence was reduced to 8.3% on the carcass after various interventions were applied (Arthur et al., 2002). It is important to note that the samples from the Arthur et al. (2002) study were collected during summer months, which have been associated with high prevalence for STEC. A similar study found non-O157 STEC on 64.9% of carcasses before evisceration and on 4% of carcasses following various interventions (Barkocy-Gallagher *et al.* 2003). This same study found the *stx* gene on 98.7% of pre-evisceration carcasses and on 10.6% of the carcasses following interventions.

PREHARVEST PATHOGEN INTERVENTIONS

Live animal contamination, in addition to environmental plant contamination, is considered to be the most likely source of carcass and meat contamination (Chapman et al., 1993). A potential solution to controlling the amount of contamination on the surfaces of cattle is to wash them with chemical solutions to either loosen debris from the hide or to reduce/eliminate the bacterial populations on the hide. Byrne et al. (2000) looked at washing cattle with chemical solutions during lairage, which resulted in higher levels of stress on the animals, and minimal reduction in populations. Due to the excess water remaining on washed cattle, bacterial populations did not differ from dirty (but dry) cattle.

Bosilevac et al. (2003) showed that washing cattle with 1% cetylpyridinium chloride (CPC) resulted in a lower prevalence of *E. coli* O157:H7 on cattle washed

with CPC (56%) versus cattle washed with water (34%). In addition, samples from

subsequent carcasses of cattle that were washed either with CPC or water, resulted in

3% and 23% *E. coli* O157:H7-positive samples, respectively. Tovich (2003)

described a system that washes stunned and exsanguinated cattle in a three chamber

washing system with a water/sodium hydroxide mixture, high pressure water, and

then lactic acid; washed cattle are then vacuumed along the pattern-mark before the

hide is first opened

Several chemical interventions have been evaluated for their ability to reduce

microbial populations on pathogen inoculated hides. Mies *et al.* (2004) assessed the

antimicrobial efficacy of chlorine (100, 200, and 400 ppm), lactic acid (2, 4, and 6%),

ethanol (70, 80, and 90%), acetic acid (2, 4, and 6%) and Oxy-Sept (0.5, 2, and 4%)

when applied to hides inoculated with *Salmonella* Typhimurium. All ethanol

concentrations as well as the 4 and 6% acetic and lactic acid treatments resulted in

significantly greater reductions of *Salmonella* Typhimurium when compared to the

remaining treatments and a distilled water spray. Furthermore, Bosilevac *et al.*

(2005a) evaluated solutions of 1.6% sodium hydroxide, 4% trisodium phosphate, 4%

chlorofoam or 4% phosphoric acid with treatment followed by a rinse of either water

or acidified chlorine (pH 7.0) at 200 or 500 ppm for their ability to reduce microbial

contamination from hides. Total coliform counts were reduced by 3.7 to 4.1 log

CFU/100 cm^2 when the chlorofoam, phosphoric acid and sodium hydroxide

treatments were applied to hides and followed by a 500 ppm acidified chlorine rinse.

Innovative water washing technologies such as ozonated and electrolyzed oxidative

water also have been evaluated for their efficacy at lowering aerobic bacteria and *E.*

coli O157:H7 on hides (Bosilevac *et al.,* 2005b). Electrolyzed water treatments reduced APC and *Enterobacteriaceae* by at least 3.5 and 4.3 log CFU/100 cm^2, respectively. Comparable results were reported for reductions of APC and *Enterobacteriaceae* when hides were treated with ozonated water. Microbial reductions could be a function of temperature and application pressure as the ozonated water treatments were applied at 48.26 bar and the electrolyzed water treatments were applied at 17.23 bar and at 60°C. Both treatments effectively reduced *E. coli* O157:H7 prevalence on the hide.

Mies *et al.* (2004) found similar results when they evaluated a commercial cattle wash system that incorporated treatments of a water wash, double water wash, 0.5% lactic acid or a wash with 50 ppm chlorine. All four treatments were found to actually increase bacterial populations from the range of 0.1 to 0.8 log CFU/cm^2. Researchers have assessed cetylpyridinum chloride (CPC), an antimicrobial found in mouthwash, to decrease the microbial populations on the hides of live cattle. Ransom *et al.* (2003) found that the application of a 1% CPC solution to the hides of cattle immediately before incapacitation reduced *E. coli* O157:H7 prevalence on hides from 14.5% (controls) to 5.5%. Carcasses corresponding to CPC treatment were found to have greater prevalence of *E. coli* O157:H7 than carcasses from control animals. Further research (Bosilevac *et al.,* 2004) of CPC found that treating cattle with a 1% CPC solution decreased *E. coli* O157:H7 prevalence on the hides from 56% on control animals to 34% on treated. Prevalence on pre-eviscerated carcasses fell from 23% on controls to 3% on treated.

POST-HARVEST PATHOGEN INTERVENTIONS

Beef carcasses are initially sterile, but become contaminated with bacteria during slaughter and dressing procedures (Sofos et al., 1999). Various carcass decontamination processes exist for the elimination or reduction of spoilage or pathogenic bacteria including use of spot cleaning techniques or the use of water or chemicals in spraying or washing applications (Belk, 2001). Gorman et al. (1995) concluded that knife trimming alone significantly reduced microbiological contamination compared to a control; however, significant contamination remained following trimming. Steam vacuuming, an alternative method to knife trimming (FSIS – USDA, 1996), also has been shown to reduce microbial contamination on beef carcasses (Gorman et al., 1995). Knife trimming and steam vacuuming can only reduce bacterial populations in localized areas and cannot be used efficiently for the entire carcass (Dorsa et al., 1997).

There is a concern with spraying/washing methods as the application of treatments "may cause penetration of bacteria into the meat or spreading and redistribution on the carcass" (Sofos et al., 1999). To combat this concern, methods have been developed to reduce/eliminate bacteria populations instead of solely relying on removal. Spraying/washing decontamination methods range from hot water and steam to chemical solutions including organic acids and other novel solutions.

Hot water (Gorman et al., 1995; Castillo et al., 1998; Sofos et al., 1999) and steam (Gorman et al., 1995; Sofos et al., 1999) have been shown to be effective

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against bacterial populations while increasing the temperature of the carcass surfaces. Prevalence of *E. coli* O157:H7 was reduced by 81% when hot water was used as a pre-evisceration wash (Koohmaraie et al., 2005). It is important to note, hot water only works if temperatures are maintained and the carcass surface reaches 74°C for greater than 5 seconds (Koohmaraie et al., 2005).

Lactic and acetic acid have been shown to be effective when applied at varying concentrations, temperatures, and doses (Smulders and Greer, 1998; Sofos et al., 1999). Treatments of lactic, acetic, or citric acids when applied from 1 to 5% have reduced bacterial populations by 1 to 3 logs (Castillo et al., 1998; Gorman et al., 1995; Cutter and Siragusa, 1994). The antimicrobial effect of organic acids has been attributed to undissociated acid molecules that interfere with both cellular metabolism and biological activity due to the decreased pH of the environment (Cutter and Siragusa, 1994). Cutter and Siragusa (1994) found a linear relationship (r = 0.86) between sustained low pH and greater bacterial population reductions. Corresponding to this, the pH of adipose tissue following acid treatment remained lower when compared to the pH of lean tissue and greater reductions were observed on adipose tissue.

Similar to water, temperature of applied acid treatments has been shown to impact the reductions observed following treatment. Anderson and Marshall (1990) and Greer and Dilts (1992) both reported an increase in bacterial reductions on beef when the temperature of the acid treatment was increased. Contrary to these studies, Cutter, Dorsa, and Siragusa (1997) found increased reductions when temperature was

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increased with the water treatments, but change in reduction was observed with increased temperatures of acid treatments.

MULTIPLE SEQUENTIAL INTERVENTION STRATEGIES

Although single interventions reduce bacterial population numbers, residual presence of bacteria remains a concern. The use of multiple sequential interventions has been shown to be more effective than individual interventions (Sofos and Smith, 1998). Because of this, many intervention systems employ multiple combinations of knife trimming, steam vacuuming, hot water washes and chemical sprays to take advantage of the additive effects.

Steam vacuuming or knife trimming has been shown to be effective on localized areas, most commonly associated with knives or machines during the hide removal process. Following knife trimming or steam vacuuming, a pre-evisceration wash with hot water or organic acid further reduces bacterial loads. Following evisceration and splitting of the carcass, an additional hot water or steam washing step is utilized. Before entering the hotbox or sales cooler, a final organic acid or chemical wash is applied (Koohmarie et al., 2005).

Hardin et al. (1997) and Cutter et al. (1997), showed increased water temperature enhanced the effect of acid solutions. Lactic acid function is enhanced following treatments with hot water (Castillo et al., 1998). Koutsoumanis et al. (2004) found support for this as lactic acid application following hot water application as found to be the most effective combination.

Bacon et al. (2000) found that bacterial populations decreased after each processing step including hide removal, final wash, and chilling. Starting hide TPC, TCC, and ECC levels ranged from 8.2 to 12.5, 6.0 to 7.9, and 5.5 to 7.5 log CFU/100 cm^2, respectively. After hide removal, prior to evisceration, TPC, TCC, and ECC counts ranged from 6.1 to 9.1, 3.0 to 6.0, and 2.6 to 5.3 log CFU/100 cm^2, respectively. The authors did note a difference between plant locations sampled in the study and some plant locations had similar counts before and after hide removal showing large amount of contamination. This high level of post-hide removal contamination was the target for multiple-sequential interventions. After final wash, TPC, TCC, and ECC counts ranged from 3.8 to 7.1, 1.5 to 3.7, and 1.0 to 3.0 log CFU/100 cm^2, respectively. Chilling is the final carcass intervention and TPC, TCC, and ECC ranged from 2.3 to 5.3, 0.9 (0.9 log CFU/ cm^2 was the detection limit) to 1.3, and 0.9 log CFU/100 cm^2, respectively. The entire intervention system was responsible for a 4.3, 3.6, and 3.2 log CFU/100 cm^2 reduction in mean log values for TPC, TCC, and ECC, respectively, showing the multiple system approach is more effective than any single intervention.

SUBPRIMAL INTERVENTIONS

Antimicrobial interventions have traditionally focused on animal hides or carcasses. Traditionally, sustained refrigeration temperatures following the dressing process through fabrication have been considered sufficient to control bacterial growth on meat (Palumbo, 1986). However, surviving bacterial populations may

continue to grow during temperature abuse periods. Additionally, cross-contamination via employees, improperly sanitized contact surfaces, such as belts, tables, saws, cutting boards, knives, hooks, or other carcasses could reintroduce pathogenic bacteria to meat surfaces (Upmann, Jakob, and Reuter, 2000). Gill, Badoni, and McGinnis (1999) found ECC counts increased from immediately prior to entry into the fabrication process and its subsequent exit. The authors found organic material on improperly cleaned equipment to be the source of the contamination, which, while running, was transferring contamination onto cutting surfaces without product being present. The contamination of both the cutting surfaces and the equipment ensured a good chance of cross-contamination occurring.

In a similar study, Gill, McGinnis, and Bryant (2000) looked at bacterial populations on the surface of beef carcasses and primal cuts before and following fabrication, respectively. TCC and ECC counts increased from 4.0 and 3.5 log CFU/500 carcasses, respectively, to >6.0 and 5.5 log CFU/500 cuts. The increased populations on primal cuts was attributed to contact surfaces of tables. Because of this, interventions to control bacterial growth before, during, and after fabrication would be of similar importance as whole carcass interventions.

Bacon et al. (2002) found that lactic acid, which is relied upon as a carcass intervention, resulted in minimal reductions of TPC, TCC, and ECC (< 0.5 log CFU/100 cm^2) when applied to top sirloin butts from initial mean values of 5.7, 3.8, and 3.3 log CFU/100 cm^2. The authors postulated that application of multiple decontamination treatments prior to fabrication could have decreased the impact of subprimal lactic acid application.

Ransom et al. (2003) found, when cetylpyridinium chloride was applied to beef short plate and lean tissue, that counts of *E. coli* O157:H7 went from 5.8 to 3.7 log CFU/g for the and from 4.2 to 2.3 log CFU/g, depending on the starting amount inoculated. In the same study, lactic acid, applied at 2% and 55°C, reduced the high inoculum group by 1.1 log CFU/g and 1.5 log CFU/g for the low inoculum group. Similarly, 2% acetic acid reduced *E. coli* O157:H7 by 1.1 and 1.4 log CFU/g for the high and low inoculation groups, respectively.

Heller et al. (2007) inoculated beef outside rounds with a three-strain cocktail of *E. coli* O157:H7 and applied one of five interventions or no intervention; surface trimming, hot water (82°C), warm 2.5% lactic acid (55°C), warm (55°C) 5.0% lactic acid, or 2% activated lactoferrin followed by a warm (55°C) lactic acid. Interventions were applied with a handheld sprayer for 20s at 3.1 bar. *Escherichia coli* O157:H7 mean populations were reduced from 3.6 log CFU/100 cm^2 by 0.9 to 1.1 log CFU/100 cm^2. The authors did not find any differences between applied treatments.

Although many methods exist to combat bacterial contamination of beef carcasses, there is a continued need for novel methods, as concerns about selecting acid tolerant organisms and continued equipment corrosion exist (Sofos et al., 1999; Smulders and Greer, 1998).

ANTIMICROBIAL EFFECT OF ESSENTIAL OILS

Essential oils (EO), have long been used for flavoring of food, fragrances, and pharmaceuticals (Burt, 2004). Essential oils' antibacterial properties have been relied

upon for various products including dental root canal sealers, antiseptics, feed

supplements for animals, and food preservatives (Burt, 2004).

Many studies in recent years have looked at the efficacy of EOs for use on

food and food products. Smid and Gorris (1999) found that a greater concentration of

EO is needed to achieve the same effect as in vitro studies. Pandit and Shelef (1994)

showed a 50-fold increase in EO was necessary for use in pork liver sausage. Both

the intrinsic properties of the food products (fat, protein, and water content, pH,

presence of antioxidants or preservatives) and extrinsic factors (temperature,

availability of oxygen, light) could be attributed to the decreased effect on bacteria in

practice (Burt, 2004). Essential oil activity has been shown to increase antimicrobial

effect with decreases in the pH of food, the storage temperature, and the amount of

oxygen available (Tassou et al., 1995; Skandamis and Nychas, 2000; Tsigarida et al.,

2000).

High levels of fat in products have resulted in a decreased activity of EOs.

Essential oils incorporate into the lipids of cellular membrane, leading to leakage of

cell components and subsequent lysis (Juven et al., 1994; Skandamis et al., 2001).

Because of this, EOs incorporate into the lipid component of foods and less is

available to target the bacterial cell (Mejlholm and Dalgaard, 2002).

Because of the increased activity of EOs with decreasing pH, temperature, and

oxygen availability, it is a logical choice for use a subprimal intervention.

Subprimals are generally vacuum packaged, and stored under refrigeration, which

would increase the activity of EOs.

HACCP VALIDATION

On July 25, 1996, United States Department of Agriculture – Food Safety Inspection Service (USDA-FSIS) published the Pathogen Reduction: Hazard Analysis and Critical Control Point (HACCP) Systems Final Rule. This regulation states that each establishment is required to evaluate and validate the effectiveness of its HACCP system's ability to control food safety hazards (USDA-FSIS, 1996). More recently (May 2010), USDA-FSIS issued the Draft Guidance: HACCP Systems Validation document which clarified the expectation of validation (USDA-FSIS, 2010). This document addressed the importance of validating the entire HACCP system including prerequisite programs. It also stipulated what was necessary for validation and defined validation as "the process of demonstrating that the HACCP system as designed can adequately control identified hazards to produce a safe, unadulterated product" (USDA-FSIS, 2010). Validation had two elements: (1) scientific or technical support for the HACCP system, and (2) practical in-plant demonstration showing that the HACCP system is effective. If the scientific documentation used defines a particular parameter, that parameter must be used for the process. In addition to proving the HACCP system is theoretically sound, based on scientific support, the establishment must show its system is able to reach the desired effect, through in-plant demonstration. The first step of in-plant validation is defining critical operational parameters including time, temperature, pressure, concentration or microbial log reduction. In-plant validation must provide data sufficient to show the process can operate effectively on a daily basis (USDA-FSIS 2010).

To effectively demonstrate a HACCP system is working properly to control pathogens, surrogate indicator organisms are essential. Surrogate organisms can include *Enterobacteriaceae*, coliforms, or generic *E. coli*. The effective indicator organisms would show the ability of the HACCP system to reduce pathogen populations without artificial introduction of pathogens into the plant. Recently, non-pathogenic *E. coli* biotype I isolates were described as surrogates for *E. coli* O157:H7 (Marshall et al., 2005). Specifically, Marshall et al. (2005) evaluated bacteria isolated from beef hides and identified five non-pathogenic *E. coli* isolates as surrogates for *E. coli* O157:H7 growth and reduction following antimicrobial intervention. These isolates were further evaluated during cooking, fermentation, freezing, and refrigerated storage of meat (2009). Keeling et al. (2009) found no difference between two of the isolates (BAA-1428 and BAA-1430) and *E. coli* O157:H7 during frozen storage, which even survived at a slightly higher rate than *E. coli* O157:H7. Under refrigeration, all five isolates were found to be similar to *E. coli* O157:H7. No difference was found between the isolates and *E. coli* O157:H7 during fermentation and the isolates survived at higher levels than *E. coli* O157:H7, adding an additional layer of security with over prediction (Keeling et al. 2009).

On September 20, 2011, USDA-FSIS published a proposed rule for Shiga Toxin-Producing *Escherichia coli* in Certain Raw Beef Products, declaring six serotypes (O26, O45, O103, O111, O121, and O145) of non-O157 Shiga toxin-producing *E. coli* (STEC) adulterants of non-intact raw beef products. Because of this, validation of an antimicrobial intervention's ability to control these bacteria would be very important (USDA-FSIS, 2011).

CONCLUSION

  E. coli O157:H7 and other shiga-toxin producing *E. coli,* including the "Top Six" non-O157 STEC, are a continued concern for the meat industry. To combat this issue, many antimicrobial interventions have been developed and implemented at the processing level. In order to be in compliance with the USAD-FSIS, all HACCP systems must be validated to show the ability of the system to function with science and actual pathogens, and in plant with the surrogates.
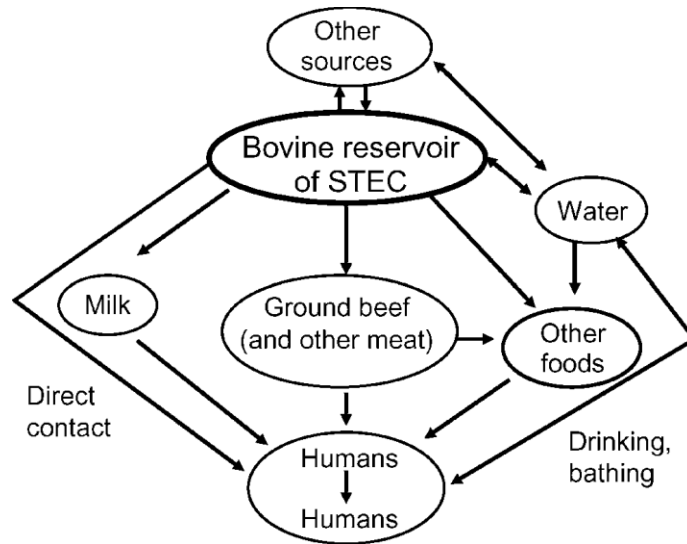
Figure 2.1. The central role of cattle in transmission of Shiga toxin-producing *Escherichia coli* (STEC) to humans. Cattle constitute a large reservoir of STEC, which may be transmitted to humans through consumption of meat and milk, direct contact with cattle, consumption of water or foods contaminated with cattle manure, or bathing in contaminated water. Ground beef is a frequent source of human disease due to O157:H7 STEC. The STEC population in cattle may be passed on to other sources such as birds and may also contain STEC that originated with other animal species. Infected humans may transfer the organisms to other humans. (Gyles, 2007)
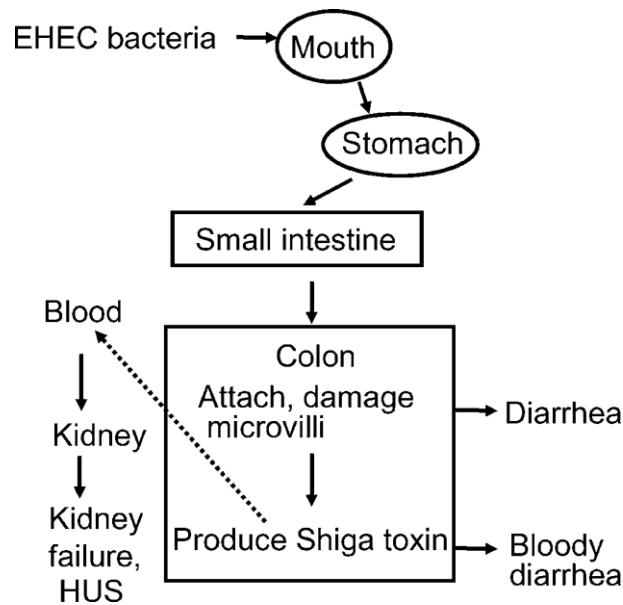
Figure 2.2. Overview of disease in humans due to Enterohemorrhagic *Escherichia coli* (EHEC). Infection begins with entry of the bacteria through food or water taken in the mouth. Acid resistance of EHEC facilitates their survival through the low pH of the stomach. The bacteria pass through the small intestine, and virulence genes are turned on by environmental signals in the colon. The EHEC adhere to the enterocytes of the colon in a characteristic intimate adherence and cause effacement of the microvilli and diarrhea. If sufficient Shiga toxin (Stx) is produced, local damage to blood vessels in the colon result in bloody diarrhea. If sufficient Stx is absorbed into the circulation, vascular endothelial sites rich in the toxin receptor are damaged, leading to impaired function. The
kidneys and central nervous system are sites that are frequently affected, and hemolytic uremic syndrome (HUS) may develop. (Gyles, 2007)

VALIDATION OF COMMERCIALLY AVAILABLE COMPOUNDS FOR

ANTIMICROBIAL INTERVENTION FOR SUBPRIMAL BEEF AND PORK

SUMMARY

Studies were conducted to validate the use of various antimicrobial chemicals to be used as antimicrobial interventions of chilled subprimals and hot beef carcasses. Chemicals evaluated on surrogate *Escherichia coli* biotype 1 (BAA-1427, BAA-1428, BAA-1429, BAA-1430, and BAA-1431) inoculated at approximately 6.0 log CFU/cm^2 on beef and pork subprimals included lactic acid (2.0, 2.5, and 5.0%), Sodium Bisulfate (2.5%), lactic acid/Sodium Bisulfate mixture (3.0 and 6.0%), Blitz™ (180 and 220 ppm), Inspexx 200™ (100 and 220 ppm), SYNTRx 3300 (pH = 1.2), citrus essential oils (3.0 and 6.0%), and deionized water. Chemicals were applied at in a custom-built spray cabinet with stainless steel slotted conveyor belt (Chad Co., Olathe KS) at two pressures (1.03 and 4.83 bar) and two rate of applications (0.23 and 6.62 lpm). After treatment bacterial counts were lower on samples treated with SYNTRx 3300, compared to other treatments. After treatment counts on samples treated with Blitz, lactic acid, sodium bisulfate, and lactic acid/sodium bisulfate mixtures were similar. Counts after treatment on samples treated with Inspexx and water were similar and higher than other chemicals due to a washing effect and not a chemical effect.

INTRODUCTION

Carcasses and meat from healthy animals is initially sterile, but becomes contaminated with bacteria when exposed to the environment through fabrication and processing (Sofos et al., 1999). Extensive research has been conducted on beef carcass decontamination looking at various methodologies including spot, thermal, and chemical decontamination, in addition to other novel techniques (13). Although single interventions reduce bacterial population numbers, residual presence of bacteria remains a concern. The use of multiple sequential interventions has been shown to be more effective than individual interventions (Bacon et al., 2000). Because of this, many beef decontamination systems in the United States employ multiple combinations of knife trimming, steam vacuuming, hot water washes and chemical sprays to take advantage of the additive effects.

Antimicrobial interventions have traditionally focused on animal hides or carcasses. Sustained refrigeration temperatures following the dressing process through fabrication have been considered sufficient to control bacterial growth on meat (Palumbo 1986). However, surviving bacterial populations may continue to grow during temperature abuse periods. Additionally, cross-contamination from employees, improperly sanitized contact surfaces, such as belts, tables, saws, cutting boards, knives, hooks, or other carcasses could reintroduce pathogenic bacteria to meat surfaces (Upmann, Jakob, and Reuter 2000). Gill et al. (1999) reported that *Escherichia coli* counts on beef carcasses increased from immediately prior to entry into the fabrication process and its subsequent exit. In a similar study, Gill et al. (2000) looked at bacterial

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populations on the surface of beef carcasses and primal cuts prior to and following

fabrication. Total coliform and *E. coli* counts increased from 4.0 and 3.5 log CFU/500

carcasses to >6.0 and 5.5 log CFU/500 cuts, respectively. The increased populations on

primal cuts were attributed to contact with cutting surfaces such as tables (2000).

Because of this, interventions to control bacterial growth before, during, and after

fabrication would be of similar importance as whole carcass interventions.

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and Critical Control Point (HACCP) Systems Final Rule. This regulation states that each

establishment is required to evaluate and validate the effectiveness of its HACCP

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which clarified the expectation of validation (USDA-FSIS, 2010). This document

addressed the importance of validating the entire HACCP system including prerequisite

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Validation had two elements: (1) scientific or technical support for the HACCP system,

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the scientific documentation used defines a particular parameter, that parameter must be

used for the process. In addition to proving the HACCP system is theoretically sound,

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defining critical operational parameters including time, temperature, pressure, concentration or microbial log reduction. In-plant validation must provide data sufficient to show the process can operate effectively on a daily basis (USDA-FSIS 2010).

To effectively demonstrate a HACCP system is working properly to control pathogens, surrogate indicator organisms are essential. Surrogate organisms can include *Enterobacteriaceae*, coliforms, or generic *E. coli*. The effective indicator organisms would show the ability of the HACCP system to reduce pathogen populations without artificial introduction of pathogens into the plant. Recently, non-pathogenic *E. coli* biotype I isolates were described as surrogates for *E. coli* O157:H7 (Marshall et al., 2005). Specifically, Marshall et al. (2005) evaluated bacteria isolated from beef hides and identified five non-pathogenic *E. coli* isolates as surrogates for *E. coli* O157:H7 growth and reduction following antimicrobial intervention. These isolates were further evaluated during cooking, fermentation, freezing, and refrigerated storage of meat (2009). Keeling et al. (2009) found no difference between two of the isolates (BAA-1428 and BAA-1430) and *E. coli* O157:H7 during frozen storage, which even survived at a slightly higher rate than *E. coli* O157:H7. Under refrigeration, all five isolates were found to be similar to *E. coli* O157:H7. No difference was found between the isolates and *E. coli* O157:H7 during fermentation and the isolates survived at higher levels than *E. coli* O157:H7, adding an additional layer of security with over prediction (Keeling et al. 2009).

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(STEC) adulterants of non-intact raw beef products. Because of this, validation of an antimicrobial intervention's ability to control these bacteria would be very important (USDA-FSIS, 2011).

A subprimal decontamination step is often implemented before packaging in an effort to control any recontamination that could have occurred during fabrication. There is limited literature available on the validation of chemical antimicrobial treatments applied using a subprimal spray cabinet on chilled subprimals.

MATERIALS AND METHODS

Bacterial strains and preparation of inocula. This experiment was composed of a single inoculum composed of five strains of non-pathogenic *E. coli* (ATCC BAA-1427, ATCC BAA-1428, ATCC BAA-1429, ATCC BAA-1430, ATCC BAA-1431). The strains were activated and subcultured (35°C, 24±2 h) in 10 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD). Each strain (10 ml) was harvested individually by centrifugation (Eppendorf model 5810 R, Brinkman Instruments Inc., Westbury, NY; 4,629×g, 15 min, 4°C), washed with 10 ml phosphate buffered saline (PBS; pH 7.4; 0.2 g KH_2PO_4, 1.5 g $NaHPO_4 \cdot H_2O$, 8.0 g of NaCl, and 0.2 g of KCL in 1 L sterile distilled water), re-centrifuged, and resuspended in PBS to obtain a concentration of 8 log CFU/ml. Strains were combined (200µl of each strain) and vortexed vigorously for 120s.

Sample inoculation and treatment. Beef brisket flats (IMPS 120A) and pork Boston butts (IMPS 406) were obtained from a commercial processing facility and transported to the Center for Meat Safety and Quality at Colorado State University.

Briskets and Boston butts were randomly assigned to two groups of 12 each consisting of (1) no inoculation and (2) inoculation with non-pathogenic strains. The brisket flats and Boston butts were portioned into sections with 100 cm^2 of exposed surface lean, approximately 5 cm x 10 cm x 0.5 cm, and were spot inoculated (50 μl of inoculant cocktail onto both sides of sections), to a target level of approximately 6 log CFU/cm^2. Sections were inoculated with approximately 6 log/cm^2 of the non-pathogenic *E. coli* cocktail. Sterile PBS (50 μl of PBS both sides of sections) was placed on sections assigned as the non-inoculated controls to mimic the inoculated sections. Samples were placed at 4°C for 30 min to allow for bacterial cell attachment.

The chemical and concentrations used were:

1) Lactic Acid

    a. 2.5%

    b. 5.0%

2) Sodium Bisulfate / Lactic Acid

    a. 3.0% (1.5% Sodium Bisulfate, 1.5% Lactic Acid)

    b. 5.0% (2.5% Sodium Bisulfate, 2.5% Lactic Acid)

3) Peroxyacetic Acid – Blitz™ (Birko Corporation, Denver, CO

    a. 180 and 220 ppm

4) Peroxyacetic Acid – Inspexx™ (Ecolab, St. Paul, MN)

    a. 100 and 220 ppm

5) Lactic Acid applied at 22±2°C (Purac Inc., Arlington Heights, Ill.)

    a. 2.5 and 5.0%

6) Lactic Acid applied at 48±2°C (Purac Inc., Arlington Heights, Ill.)

a. 2.5 and 5.0%

7) Buffered Citric Acid - SYNTRx 3300 (Synergy Technologies, Inc., Shreveport, LA)

a. pH = 1.2 (≈3.0%)

8) Deionized Water

9) No Treatment

Deionized water was used to make final concentrations. Final concentrations of peroxyacetic acid compounds (Blitz ™ and Inspexx™) were measured using standard Quality Assurance procedures (Ecolab, St. Paul, MN). Lactic acid solutions were mixed with deionized water and 88% L-lactic acid (Purac Inc., Arlington Heights, IL.); concentrations were confirmed with a Lactic Acid Test Kit [Code: TK1325-Z (Birko Corporation, Denver, CO). SYNTRx 3300 solution was titrated to pH = 1.2, approximately 3.0% solution. The pH of the solution was measured throughout titration using a digital pH meter fitted with a glass electrode (Denver Instruments, Arvada, CO). Sodium Bisulfate / Lactic Acid solutions were mixed from a stock solution of 50% to a final concentration of 3.0 and 5.0%. This was done using the formula: Concentration_1 x $\text{Volume}_1 = \text{Concentration}_2$ x Volume_2. The pH of all applied solutions (Table 1) was measured using a digital pH meter fitted with a glass electrode (Denver Instruments, Arvada, CO).

Beef brisket and pork Boston butt sections were treated with chemical compounds in a custom-built spray cabinet with stainless steel slotted conveyor belt (Chad Co., Olathe KS) at two pressures (1.03 and 4.83 bar) and two rate of applications (0.23 and 6.62 lpm). Solutions were applied as a spray from a pressurized canister (3 liter capacity)

and exposed to the meat samples from 12 nozzles [WashJet HSS 1/8 MEG 2510 (Spraying Systems, Carol Stream, IL), four for the top of the meat samples and eight for the bottom. Sections were allowed to drip dry for 5 s and placed into vacuum bags (20.32 x 35.56 cm; Cryovac, Duncan, SC). Treated sections were vacuum packaged (Hollymatic, Countryside, IL) and stored at 4°C until sampled.

Microbiological analyses. Sections (100 cm^2) from each subprimal (beef brisket or pork Boston butt) were sampled before inoculation to determine initial total bacterial populations. Sections were placed in 625 ml filter bags (19 x 30cm; Nasco Whirl-Pak, Modesto CA) to which 100 ml of 0.1% buffered peptone water (Difco, Becton Dickinson) was added, followed by pummeling (Masticator, IUL Industries, Barcelona, Spain) for 2 min. Sample homogenates were serially diluted (10-fold) in 0.1% BPW and appropriate dilutions were surface plated (0.1 ml) and spread on tryptic soy agar (TSA: Difco, Becton Dickinson) to determine initial total plate counts (TPC). Following treatment, samples were plated in like manner on TSA and Violet Red Bile Agar with 4-methylumbelliferyl-β-D-glucuronide (VRBA-MUG) to enumerate TPC and bacterial populations of *E. coli* including the inoculum [*E. coli* Counts (ECC)], respectively. Non-inoculated sections were sampled likewise and surface plated (0.1ml) on TSA and VRBA –MUG to enumerate TPC and ECC, respectively. Plates were incubated at 37°C for 18-24 hours prior to enumeration.

Statistical analysis. Samples were run in duplicate, with the entire experiment repeated three times for a total of six samples per treatment. Main effects of specie, temperature, pressure, rate, concentration, time of sampling and the respective

interactions were analyzed using the General Linear Model of SAS v. 9.2. *P* values less than 0.05 (*P* < 0.05) were considered statistically significant.

Results and Discussion. TPC mirrored ECC for all beef and pork samples. Additionally, no difference (*P* > 0.05) was observed in TPC enumerated from either beef or pork samples at any time period (Table 3.1). TPC and ECC immediately after treatment (Hour 0) varied based on the parameter of application, with chemical used having the greatest effect on after treatment counts. The greatest difference between before and after treatment counts was observed on samples treated with SYNTRx 3300. Lactic acid and Blitz caused similar after treatment counts, but the difference between the before and after treatment counts was smaller than those observed with SYNTRx 3300. Samples treated with water and Inspexx did not differ in after treatment counts, showing minimal change between before and after treatment counts were from a washing effect.

After treatment counts did not vary when Blitz was applied at different concentrations. However, after treatment counts on samples treated with lactic acid and Inspexx were lower on samples where the higher concentration was applied. Slightly higher after treatment counts were observed on samples treated with lactic acid at 48°C compared to application at 22°C.

Pressure of application did not affect after treatment counts. Rate of application did effect the after treatment counts observed with water and Inspexx. This is most likely due to a washing effect. Interestingly, rate of application had minimal effect on after treatment count differences with Blitz, lactic acid, and SYNTRx treated samples.

Although subprimal beef and pork after treatment counts differed significantly (*P* < 0.05) at Hour 0 and Hour 24 post-treatment, the differences are not practical. TPC and ECC immediately after treatment (Hour 0) varied based on the parameter of application, with chemical used having the greatest effect on after treatment counts. The greatest

difference between before and after treatment counts was observed on samples treated with SYNTRx 3300. Lactic acid and Blitz caused similar after treatment counts, but the difference between the before and after treatment counts was smaller than those observed with SYNTRx 3300. Samples treated with water and Inspexx did not differ in after treatment counts, showing minimal change between before and after treatment counts were from a washing effect.

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Pressure of application did not affect after treatment counts ($P < 0.05$). Rate of application did effect the after treatment counts observed with water and Inspexx. This is most likely due to a washing effect. Interestingly, rate of application had minimal effect on after treatment count differences with Blitz, lactic acid, and SYNTRx treated samples.

Table 3.1 Differences in total plate counts (log CFU/cm^2 ± standard deviation) enumerated from beef brisket or pork Boston butt at different times, averaged over all application parameters.

	Before Treatment	0 hr After Treatment	24 hr After Treatment	48 hr After Treatment
Beef	6.0 ± 0.2	4.5 ± 1.0	4.5 ± 1.2	4.5 ± 1.1
Pork	6.0 ± 0.2	4.5 ± 1.0	4.4 ± 1.3	4.5 ± 1.1

Table 3.2 Effect of Chemical*Pressure*Rate*Concentration Interaction on Total Plate Counts (log CFU/cm^2) averaged over beef and pork samples

Chemical	Press ure	Rate	Conc	TPC from Non-Selective Media		
				0 hr After Treatment	24 hr After Treatment	48 hr After Treatment
Lactic Acid	15	0.23	2.5%	4.58 (0.47)	4.48 (0.45)	4.40 (0.26)
Lactic Acid	15	0.23	5.0%	4.35 (0.10)	4.26 (0.12)	4.17 (0.23)
Lactic Acid	15	6.62	2.5%	4.57 (0.24)	4.44 (0.09)	3.73 (0.39)
Lactic Acid	15	6.62	5.0%	4.37 (0.24)	4.23 (0.09)	3.52 (0.38)
Lactic Acid	70	0.23	2.5%	4.72 (0.32)	4.61 (0.28)	4.34 (0.27)
Lactic Acid	70	0.23	5.0%	4.51 (0.33)	4.39 (0.28)	4.55 (0.27)
Lactic Acid	70	6.62	2.5%	4.15 (0.46)	4.00 (0.13)	4.11 (0.09)
Lactic Acid	70	6.62	5.0%	4.39 (0.19)	4.35 (0.07)	3.67 (0.37)
Sodium Bisulfate	15	0.23	2.5%	4.66 (0.09)	4.70 (0.12)	4.28 (0.07)
Sodium Bisulfate	15	0.23	2.5%	4.66 (0.09)	4.70 (0.12)	4.28 (0.07)
Sodium Bisulfate	70	6.62	2.5%	4.23 (0.07)	4.24 (0.05)	4.27 (0.07)
Sodium Bisulfate	70	6.62	2.5%	4.23 (0.08)	4.24 (0.04)	4.24 (0.04)
Sodium Bisulfate / Lactic Acid	15	0.23	3.0%	3.91 (0.05)	3.89 (0.11)	3.92 (0.05)
Sodium Bisulfate / Lactic Acid	15	0.23	5.0%	4.07 (0.09)	3.95 (0.07)	3.71 (0.09)
Sodium Bisulfate / Lactic Acid	15	6.62	3.0%	4.11 (0.07)	4.16 (0.08)	3.77 (0.20)
Sodium Bisulfate / Lactic Acid	15	6.62	5.0%	4.00 (0.09)	4.06 (0.13)	3.66 (0.20)
Sodium Bisulfate / Lactic Acid	70	0.23	3.0%	4.04 (0.21)	3.99 (0.17)	3.86 (0.06)
Sodium Bisulfate / Lactic Acid	70	0.23	5.0%	3.80 (0.24)	3.81 (0.15)	4.18 (0.16)
Sodium Bisulfate / Lactic Acid	70	6.62	3.0%	3.49 (0.21)	3.70 (0.08)	3.84 (0.07)
Sodium Bisulfate / Lactic Acid	70	6.62	5.0%	4.06 (0.18)	4.11 (0.05)	3.96 (0.02)
Water	15	0.23		5.79 (0.27)	5.67 (0.10)	5.63 (0.10)
Water	15	6.62		5.65 (0.15)	5.64 (0.28)	5.33 (0.09)
Water	70	0.23		5.77 (0.10)	5.73 (0.13)	5.83 (0.07)
Water	70	6.62		5.63 (0.11)	5.59 (0.11)	5.69 (0.06)
No Treatment				6.34 (0.21)	6.55 (0.13)	6.47 (0.31)

Table 3.3 Effect of Chemical*Pressure*Rate*Concentration Interaction on Total Plate Counts (log CFU/cm^2) averaged over beef and pork samples

Chemical	Pressure	Rate	Conc	TPC from Non-Selective Media		
				0 hr After Treatment	24 hr After Treatment	48 hr After Treatment
Blitz™	15	0.23	180 ppm	4.30 ±0.15	4.28 ±0.08	4.15 ±0.10
Blitz™	15	0.23	220 ppm	4.12 ±0.09	3.68 ±0.11	2.99 ±0.38
Blitz™	15	6.62	180 ppm	4.04 ±0.13	4.44 ±0.12	4.64 ±0.19
Blitz™	15	6.62	220 ppm	3.83 ±0.13	4.23 ±0.12	4.43 ±0.18
Blitz™	70	0.23	180 ppm	4.22 ±0.09	4.18 ±0.10	3.28 ±0.05
Blitz™	70	0.23	220 ppm	4.00 ±0.10	3.97 ±0.10	3.49 ±0.07
Blitz™	70	6.62	180 ppm	4.11 ±0.11	4.07 ±0.08	4.03 ±0.12
Blitz™	70	6.62	220 ppm	4.13 ±0.11	4.49 ±0.17	4.72 ±0.26
Inspexx™	15	0.23	100 ppm	5.52 ±0.08	5.55 ±0.20	5.98 ±0.09
Inspexx™	15	0.23	220 ppm	5.84 ±0.15	5.84 ±0.10	5.53 ±0.22
Inspexx™	15	6.62	100 ppm	5.61 ±0.18	5.67 ±0.07	5.68 ±0.04
Inspexx™	15	6.62	220 ppm	5.40 ±0.19	5.46 ±0.07	5.48 ±0.04
Inspexx™	70	0.23	100 ppm	5.84 ±0.15	5.87 ±0.11	5.89 ±0.17
Inspexx™	70	0.23	220 ppm	5.62 ±0.15	5.65 ±0.11	6.10 ±0.17
Inspexx™	70	6.62	100 ppm	5.22 ±0.23	5.94 ±0.20	5.95 ±0.18
Inspexx™	70	6.62	220 ppm	5.69 ±0.24	5.85 ±0.08	5.89 ±0.07
Lactic Acid – 22°C	15	0.23	2.5%	4.57 ±0.15	4.77 ±0.07	4.57 ±0.15
Lactic Acid – 22°C	15	0.23	5.0%	4.93 ±0.07	4.46 ±0.07	4.17 ±0.07
Lactic Acid – 22°C	15	6.62	2.5%	4.50 ±0.24	4.29 ±0.08	4.11 ±0.18
Lactic Acid – 22°C	15	6.62	5.0%	4.47 ±0.07	4.52 ±0.07	4.46 ±0.07
Lactic Acid – 22°C	70	0.23	2.5%	4.92 ±0.07	4.60 ±0.09	4.79 ±0.08
Lactic Acid – 22°C	70	0.23	5.0%	4.21 ±0.18	4.11 ±0.17	4.26 ±0.08
Lactic Acid – 22°C	70	6.62	2.5%	4.79 ±0.08	4.60 ±0.11	4.92 ±0.07
Lactic Acid – 22°C	70	6.62	5.0%	4.17 ±0.07	4.42 ±0.07	4.52 ±0.07
Lactic Acid – 48°C	15	0.23	2.5%	4.46 ±0.07	4.17 ±0.07	4.21 ±0.07
Lactic Acid – 48°C	15	0.23	5.0%	4.28 ±0.07	4.23 ±0.07	4.26 ±0.07
Lactic Acid – 48°C	15	6.62	2.5%	4.41 ±0.10	4.35 ±0.10	4.27 ±0.20
Lactic Acid – 48°C	15	6.62	5.0%	4.46 ±0.07	4.40 ±0.07	4.21 ±0.07
Lactic Acid – 48°C	70	0.23	2.5%	4.28 ±0.07	4.23 ±0.07	4.26 ±0.07
Lactic Acid – 48°C	70	0.23	5.0%	4.21 ±0.07	4.17 ±0.07	4.27 ±0.17
Lactic Acid – 48°C	70	6.62	2.5%	4.46 ±0.07	4.40 ±0.07	4.21 ±0.07
Lactic Acid – 48°C	70	6.62	5.0%	4.28 ±0.07	4.23 ±0.07	4.26 ±0.07
SYNTRx 3300	15	0.23	pH = 1.2	2.32 ±0.07	1.10 ±0.15	2.17 ±0.08
SYNTRx 3300	15	6.62	pH = 1.2	2.26 ±0.12	1.78 ±0.72	1.78 ±0.36
SYNTRx 3300	70	0.23	pH = 1.2	1.60 ±0.55	1.63 ±0.59	2.35 ±0.10
SYNTRx 3300	70	6.62	pH = 1.2	2.26 ±0.11	1.06 ±0.06	2.23 ±0.10
Water	15	0.23		5.79 ±0.27	5.67 ±0.10	5.63 ±0.10
Water	15	6.62		5.65 ±0.15	5.64 ±0.28	5.33 ±0.09
Water	70	0.23		5.77 ±0.10	5.73 ±0.13	5.83 ±0.07
Water	70	6.62		5.63 ±0.11	5.59 ±0.11	5.69 ±0.06
No Treatment				6.34 ±0.21	6.55 ±0.13	6.47 ±0.31

VALIDATION OF CITRUS ESSENTIAL OILS AS AN ANTIMICROBIAL INTERVEN FOR SURROGATE *ESCHERICHIA COLI* ON BEEF SUBPRIMALS

SUMMARY

Escherichia coli O157:H7 and *Salmonella* spp. are bacterial pathogens often associated with beef, and cause many cases of foodborne illness each year in the United States. During beef slaughter and processing these bacteria may spread from the hide or intestines to the carcass. The objective of this research was to investigate the use of naturally occurring compounds citrus essential oils (CEOs) extracted from orange peel to reduce or eliminate these pathogens at the chilling stage of processing, or during fabrication. Brisket flats (used to simulate beef subprimals) were spot inoculated with approximately 6 log of surrogate generic *E. coli* cocktail (previously shown to be identical in growth and survival parameters to *E. coli* O157:H7 and *Salmonella* spp.). Following drying, CEOs were applied by spraying at concentrations of 3% and 6% to the surface of different pieces of meat. Treatments were applied using a custom built spray cabinet at 2.07 bar and applied at a rate of 3.79 liters per minute to replicate commercial practices. The CEOs significantly reduced (p<0.05) the concentration of *E. coli* on the brisket flats in comparison to inoculated- no spray or water-sprayed controls over aperiod of 90 days, while causing an initial reduction of approximately 1.4 log units. Total

aerobic bacteria and psychrotrophic counts were also reduced on uninoculated briskets following treatment. These results indicate that 3% cold pressed terpeneless Valencia orange oil could be used as an additional intervention against *E. coli* O157:H7 and *Salmonella* spp. at the refrigerated storage stage of processing.

Key words: *Escherichia coli* O157:H7, citrus essential oils (CEOs), intervention, beef subprimals.

INTRODUCTION

Various interventions for the reduction of *E. coli* O157:H7 contamination on beef carcasses, cuts and trimmings have been studied. The use of chemical agents, including acetic, lactic and peroxyacetic acid, ammonium hydroxide, cetylpyridinium chloride, hydrogen peroxide, activated lactoferrin, ozone, acidified sodium chlorite, sodium hydroxide, sodium hypochlorite, sodium metasilicate and trisodium phosphate have been studied (Heller et al., 2005). However, most intervention steps are implemented prior to chilling, therefore any bacterial contamination present at the chilling step has potential to be passed on to the consumer. As such, there has been increased interest in the application of antimicrobial treatments to beef trimmings prior to grinding for the reduction of microbial contamination in ground beef.

Essential oils (EOs) have been in use for centuries as pharmaceuticals, and are currently mainly used for fragrances and flavors (Van de Braak and Leijten 1999). In the late 19th century, the antimicrobial properties of EOs first began to be studied (Burt 2004). Though EOs are mainly used as flavor agents and in fragrances, there has been an emphasis on the possible role of essential oils as antimicrobials (Cowan 1999, Holley and Patel 2005). EOs from thyme, cinnamon, sage, rosemary, sassafras, clove, gardenia, jasmine and more have been shown to exert antimicrobial effect against microorganisms such as *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* Typhimurium*,* and *Campylobacter jejuni,* (Cosentino et al., 1999; Friedman et al., 2002; Valero and Salmerón 2003; Moreira et al., 2005).

Citrus EOs have shown good antimicrobial activity *in vitro* against both Gram positive and Gram negative foodborne pathogens (Fisher and Phillips 2006; 2009; Nannapaneni et al., 2008, 2009; O'Bryan et al., 2008; Friedly et al., 2009). Nevertheless, more studies are needed to prove the usefulness of application of citrus EOs as antimicrobials for foodstuffs at chilling temperature.

MATERIALS AND METHODS

Bacterial strains and preparation of inocula. Five *E. coli* isolates were used as surrogates for *E. coli* O157:H7 and *Salmonella* spp. during meat inoculation studies. These surrogate microorganisms are nonpathogenic *E. coli* isolates designated as P1, P3, P8, P14 and P68 (ATCC BAA-1427, ATCC BAA-1428, ATCC BAA-1429, ATCC BAA-1430, ATCC BAA-1431, respectively), which have been shown to behave similarly to *E. coli* O157:H7 and *Salmonella* spp. isolates in meat, including during processing (Niebuhr and others 2008; Keeling and others 2009). All isolates came from the American Type Culture Collection (ATCC). The surrogate strains were combined to form a cocktail, prior to artificial inoculation experiments. Strains were reactivated from -70˚C stocks by initial streaking onto Tryptic Soy Agar (TSA, Becton Dickinson, Sparks, MD) and incubating at 37°C for 24 hours. For each isolate, a single colony was transferred to 10 ml of Tryptic Soy Broth (TSB, Becton Dickinson) and incubated at 37°C for 18 hours. Ten microliters of bacterial inoculum was transferred into fresh TSB (10 ml) and incubated at 37°C for 18 hours. Each strain (10 ml) was harvested individually by centrifugation (Eppendorf model 5810 R, Brinkman Instruments Inc., Westbury, NY, 4,629 x g, 15 min, 4˚C), then washed twice with 10 ml phosphate buffered saline (PBS; pH 7.4; 0.2 g KH_2PO_4, 1.5 g $NaHPO_4^.H_2O$, 8.0g NaCl, and 0.2g KCL in 1L sterile

distilled water), then resuspended in PBS to obtain a concentration of 8 $\log_{10}$ CFU/ml.

Two milliliters of each bacterial suspension in PBS was combined in a 10 ml centrifuge

tube.

Sample inoculation and treatment. Beef brisket flats (IMPS 120A) were

obtained from a local commercial processing facility and used to investigate the ability of

the CEOs to reduce or eliminate the *E. coli* surrogates from the chilled subprimal surface.

Brisket flats were collected from a commercial processing facility prior to any subprimal

antimicrobial intervention and transported in commercial packaging to Colorado State

University's Meat Science Laboratory. Brisket flats were cut into 100 cm^2 sections (10 x

10 x 0.2 cm) and spot inoculated with approximately 6 log of the surrogate *E. coli*

cocktail. Inoculated meat sections were placed in a single layer on a fiberglass tray,

covered with aluminum foil, and stored at 4°C for 30 min to allow bacteria to attach to

the meat surface. After drying, citrus EOs were applied in two concentrations to the

surface of different pieces of meat. Treatments included citrus essential oils applied at

concentrations of 3.0% and 6.0%, deionized water (water spray), and no spray. Deionized

water was used to mix the citrus EO sprays and Tween 80 was added at 0.25% to act as

an emulsifier to the oils. The water, oil, and Tween 80 mixture was sonicated (MiSonix

Incorporated, Farmingdale, NY) for 120 seconds until oils went into a stable emulsion.

The mixtures were kept at 4°C for 180 minutes prior to application to the samples.

Treatments were applied using a custom built spray cabinet (Chad Co, Olathe, KS).

Product was exposed to treatments from eight nozzles (WashJet HSS 1/8 MEG 2510 –

Spraying Systems Co, Wheaton, IL) four above and four below. Nozzles faced the

product at 45° angles facing opposite directions ensuring the surface area of the product

was completely covered with the desired treatment. Spray was applied at 2.07 bar and at a rate of 3.79 liters per minute to replicate commercial practices.

Following treatment, samples were placed in vacuum packaging bags (20.32 by 35.56 cm, Cryovac , Duncan, SC) and stored anaerobically at 4°C until microbial sampling was performed. Microbial samples were analyzed at day 0 (20 minutes after spraying and packaging), at days 1, 2, 3, 4, and 5, then every five days from day 5 to 90. Temperature fluctuations during refrigerated storage during all 90 days never exceeded ±0.3˚C.

Microbiological analyses. 100 cm^2 section samples were processed by adding 100 ml of Buffered Peptone Water (BPW, Becton Dickinson) to the original packaging followed by maceration for 120 seconds (RUL Instruments Masticator, Barcelona, Spain). One ml of the homogenized sample was placed in a sterile 2 ml microcentrifuge tube (Eppendorf, Hauppauge, NY), centrifuged at 5,000 rpm, washed with one ml of 0.1% BPW, and vortexed for 120 seconds to resuspend the pellet. The process was repeated to remove any residual CEO that would interfere with bacterial growth during plating and enumeration. Serial dilutions were performed in BPW and appropriate dilutions were surface plated onto Violet Red Bile Agar (VRB) with 4-methylumbelliferyl-β-D-glucuronide (Becton Dickinson) and Tryptic Soy Agar (Becton Dickinson) to enumerate total *E. coli* and total aerobic or psychrotrophic bacteria, respectively. VRBA plates were incubated for up to 24 hours at 37°C followed by colony enumeration based on typical colony morphology. In addition, the activity of the citrus EO on normal microbial flora was investigated by spraying non-inoculated pieces of brisket as described above, followed by analysis for total aerobic counts and

psychrotrophic counts, by plating masticated samples on TSA followed by incubation at 37˚C for up to 24 hours and 10^oC for 7 days, respectively.

Statistical Analysis. Three independent replications were performed using duplicate samples for each replication. Bacterial populations were expressed as mean $\log_{10}$ CF/cm^2 with standard deviations calculated using the assumption of log-normal distribution of microorganisms. The experiment was repeated three times, with a total of six samples per treatment. Data was evaluated using the GLM procedure of SAS version 9.2 (SAS Institute Inc., Cary, NC). Means were separated with the least significant difference procedure at the significance level of alpha = 0.05.

Results and Discussion. *E. coli* counts were reduced by approximately 1.5 log unit from 6.0 log CFU/cm^2 (3% or 6% CEO) immediately after treatment (day 0) (Figure 1).There was no difference in initial reduction of *E. coli* (p>0.05) between using the higher or lower treatment of citrus EO. Counts on the samples treated with 3% or 6% citrus EO remained static until day 35, after which the *E. coli* counts slowly began to increase. By day 90, the counts had increased to approximately 5 $\log_{10}$ CFU/cm^2 while in the untreated control the counts had already reached approximately 6.5 log. At the end of the study the counts remained approximately one log unit lower than the initial inoculum level, even for the 3% citrus EO treatment. At the end of the experiment, the differences between the treated and non-treated samples were aproximately1.2 log units (3% citrus EO) and 1.4 log units (6% citrus EO). Spraying uninoculated meat with 3% citrus EO and 6% citrus EO significantly (p<0.05) lowered initial counts, however the total counts grew to the same levels as the controls within 5 and 15 days of storage for 3% and 6% citrus EO treatment, respectively (Figure 2). For the remainder of the storage there were no

differences in the total aerobic counts between treated and untreated samples (p>0.05). In both treated and untreated samples total aerobic counts remained fairly static for up to 65 days, and then grew by approximately 4 logs compared to initial levels. 3% and 6% citrus EO treatments reduced initial psychrotrophic counts by approximately 0.5 and 0.7 log units, respectively as compared to the untreated controls (Figure 3). The treatments continued to exert bacteriostatic activity for up to 55 days, after which 3% citrus EO treatment was not significantly different (p>0.05) from the controls. However, 6% citrus EO treated samples exhibited lower psychrotrophic counts until the end of storage period at 90 days. In all samples, (treated, water, and no spray), counts started to increase after 35 days, and after 90 days the concentrations of bacteria in all samples increased by approximately 4 log units (Figure 3).

In addition to the fact that the results of this study provide complimentary support and further validation of the use of citrus EOs to control the growth of foodborne pathogenic bacteria, the study is noteworthy because it demonstrates the ability of the oils to control the growth of the bacteria, both in pure culture and on meat, at low temperatures. When concentrations of 3% and 6% citrus essential oils were sprayed onto pieces of brisket used to simulate beef subprimals , the oils significantly reduced the concentration of *E. coli* that was artificially inoculated onto the beef, and total aerobic bacterial counts, in comparison to inoculated, no spray or water sprayed controls, over a period of 90 days (Figures 1 and 2). These results show that citrus EOs could be used as a cold active antimicrobial, in an additional step to inhibit foodborne bacterial pathogens on beef subprimals during fabrication. Other studies have also indicated that citrus EOs are active at lower temperatures. Citrus oils and their fractionated components were

found to be effective at refrigeration temperatures (4°C) for reducing spoilage organisms in beef over 12 days (Fernandez-Lopez et al., 2005). As such, cold pressed terpeneless Valencia orange oil is able to maintain activity at low temperatures, and thus has potential as an intervention for use during refrigerated storage of the subprimal .

In the beef industry, carcasses are chilled to temperatures close to or at 4°C within 24 hours (Beef Industry Food Safety Council 2009). A solution of 3% cold pressed terpeneless Valencia orange oil could be used as an additional intervention against *E. coli* O157:H7 and *Salmonella* spp. at the chilling stage of processing. The oils could also be used as an intervention on cuts of beef, as the results of this study show the ability of the citrus essential oils to control growth of bacteria on vacuum packaged meats for various periods of time.
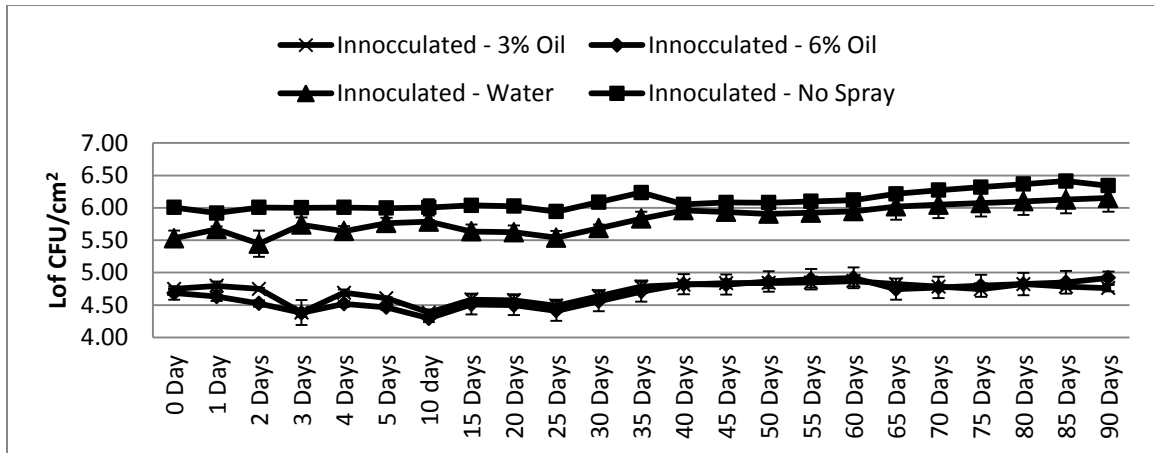
Figure 4.1 – Effect of terpeneless Valencia citrus essential oils on a five-strain cocktail of biotype I *Escherichia coli* spiked onto 100 cm² portions of beef brisket-pectoralis major during storage at 4˚C for up to 90 days. The beef subprimal cuts were inoculated with 6 $\log_{10}$ CFU/cm² of the surrogate *E. coli*. Means and standard deviations for duplicate samples from three different independent replications are shown.
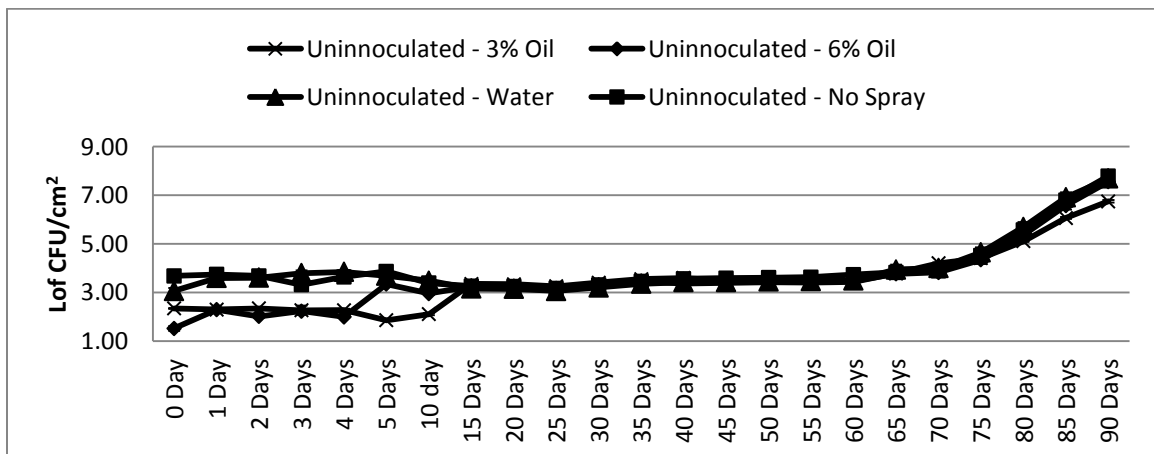

Figure 4.2 – Effect of terpeneless citrus essential oils on total aerobic counts for uninoculated 100 cm² portions of beef brisket-pectoralis major during storage at 4˚C for up to 90 days. Means and standard deviations for duplicate samples from three different independent replications are shown.

EVALUATION OF LACTIC ACID AS AN INITIAL AND SECONDARY SUBPRIMAL INTERVENTION FOR *ESCHERICHIA COLI* O157:H7, NON-O157 SHIGA TOXIN-PRODUCING *E. COLI*, AND A NON-PATHOGENIC *E. COLI* SURROGATE FOR *E. COLI* O157:H7

SUMMARY

Lactic acid can reduce microbial contamination on beef carcass surfaces when used as a food safety intervention, but effectiveness when applied to the surface of chilled beef subprimals is not well documented. Studies characterizing bacterial reductions from lactic acid on subprimals would be useful in validations of HACCP systems. The objective of this study was to validate initial use of lactic acid as a subprimal intervention during beef fabrication, followed by a secondary application to vacuum-packaged rework product following removal of packaging. Chilled beef subprimal sections (100 cm^2) were either left uninoculated or were inoculated with 6 log CFU/cm^2 of a 5-strain mixture of *Escherichia coli* O157:H7, a 12-strain mixture of non-O157 Shiga toxin-producing *E. coli* (STEC), or a 5-strain mixture of non-pathogenic (biotype I) *E. coli* considered surrogates for *E. coli* O157:H7. Uninoculated and inoculated subprimal sections received an initial or initial and second, "re-work" application of lactic acid in a custom-built spray cabinet at one of 16 different application parameters. Following the initial spray, total inoculum counts were reduced from 6.0 log CFU/cm^2 to 3.6, 4.4, and 4.4 log CFU/cm^2 for the *E. coli* surrogates, *E. coli* O157:H7, and non-O157 STEC inoculation

groups, respectively. After the second, re-work application, total inoculum counts remaining were 2.6, 3.2, and 3.6 log CFU/cm^2 for the *E. coli* surrogates, *E. coli* O157:H7, and non-O157 STEC inoculation groups, respectively. Both the initial and secondary lactic acid treatments effectively reduced counts of pathogenic and non-pathogenic strains of *E. coli*, as well as natural microflora, on beef subprimals. These data would be beneficial to industry as part of the HACCP validation process.

INTRODUCTION

Carcasses and meat from healthy animals is initially sterile, but becomes contaminated with bacteria when exposed to the environment through fabrication and processing (Sofos et al., 1999). Extensive research has been conducted on beef carcass decontamination looking at various methodologies including spot, thermal, and chemical decontamination, in addition to other novel techniques (Sofos et al., 1999). Although single interventions reduce bacterial population numbers, residual presence of bacteria remains a concern. The use of multiple sequential interventions has been shown to be more effective than individual interventions (Bacon et al., 2000). Because of this, many beef decontamination systems in the United States employ multiple combinations of knife trimming, steam vacuuming, hot water washes and chemical sprays to take advantage of the additive effects.

Antimicrobial interventions have traditionally focused on animal hides or carcasses. Sustained refrigeration temperatures following the dressing process through fabrication have been considered sufficient to control bacterial growth on meat (Palumbo 1986). However, surviving bacterial populations may continue to grow during temperature abuse periods. Additionally, cross-contamination from employees, improperly sanitized contact surfaces, such as belts, tables, saws, cutting boards, knives, hooks, or other carcasses could reintroduce pathogenic bacteria to meat surfaces (Upmann, Jakob, Reuter, 2000). Gill et al. (1999) reported that *Escherichia coli* counts on beef carcasses increased from immediately prior to entry into the fabrication process and its subsequent exit. In a similar study, Gill et al. (2000) looked at bacterial populations on the surface of beef carcasses and primal cuts prior to and following

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fabrication. Total coliform and *E. coli* counts increased from 4.0 and 3.5 log CFU/500 carcasses to >6.0 and 5.5 log CFU/500 cuts, respectively. The increased populations on primal cuts were attributed to contact with cutting surfaces such as tables (Gill et al., 2000). Because of this, interventions to control bacterial growth before, during, and after fabrication would be of similar importance as whole carcass interventions.

On July 25, 1996, United States Department of Agriculture – Food Safety Inspection Service (USDA-FSIS) published the Pathogen Reduction: Hazard Analysis and Critical Control Point (HACCP) Systems Final Rule. This regulation states that each establishment is required to evaluate and validate the effectiveness of its HACCP system's ability to control food safety hazards (USDA-FSIS 1996). More recently (May 2010), USDA-FSIS issued the Draft Guidance: HACCP Systems Validation document which clarified the expectation of validation (USDA-FSIS 2010). This document addressed the importance of validating the entire HACCP system including prerequisite programs. It also stipulated what was necessary for validation and defined validation as "the process of demonstrating that the HACCP system as designed can adequately control identified hazards to produce a safe, unadulterated product" (USDA-FSIS 2010). Validation had two elements: (1) scientific or technical support for the HACCP system, and (2) practical in-plant demonstration showing that the HACCP system is effective. If the scientific documentation used defines a particular parameter, that parameter must be used for the process. In addition to proving the HACCP system is theoretically sound, based on scientific support, the establishment must show its system is able to reach the desired effect, through in-plant demonstration. The first step of in-plant validation is defining critical operational parameters including time, temperature, pressure,

concentration or microbial log reduction. In-plant validation must provide data sufficient to show the process can operate effectively on a daily basis (USDA-FSIS 2010).

To effectively demonstrate a HACCP system is working properly to control pathogens, surrogate indicator organisms are essential. Surrogate organisms can include *Enterobacteriaceae*, coliforms, or generic *E. coli*. The effective indicator organisms would show the ability of the HACCP system to reduce pathogen populations without artificial introduction of pathogens into the plant. Recently, non-pathogenic *E. coli* biotype I isolates were described as surrogates for *E. coli* O157:H7 (Marshall et al., 2005). Specifically, Marshall et al. (2005) evaluated bacteria isolated from beef hides and identified five non-pathogenic *E. coli* isolates as surrogates for *E. coli* O157:H7 growth and reduction following antimicrobial intervention. These isolates were further evaluated during cooking, fermentation, freezing, and refrigerated storage of meat (Keeling et al., 2009). Keeling et al. (2009) found no difference between two of the isolates (BAA-1428 and BAA-1430) and *E. coli* O157:H7 during frozen storage, which even survived at a slightly higher rate than *E. coli* O157:H7. Under refrigeration, all five isolates were found to be similar to *E. coli* O157:H7. No difference was found between the isolates and *E. coli* O157:H7 during fermentation and the isolates survived at higher levels than *E. coli* O157:H7, adding an additional layer of security with over prediction (Keeling et al., 2009).

On September 20, 2011,   USDA-FSIS published a proposed rule for Shiga Toxin-Producing *Escherichia coli* in Certain Raw Beef Products, declaring six serotypes (O26, O45, O103, O111, O121, and O145) of non-O157 Shiga toxin-producing *E. coli* (STEC) adulterants of non-intact raw beef products. Because of this, validation of an

antimicrobial intervention's ability to control these bacteria would be very important (USDA-FSIS 2011).

A subprimal decontamination step is often implemented before packaging in an effort to control any recontamination that could have occurred during fabrication. There is limited literature available on the validation of chemical antimicrobial treatments applied using a subprimal spray cabinet on chilled subprimals. Lactic acid is a common antimicrobial agent used to reduce bacterial populations on meat. However, its ability to reduce bacterial populations on re-worked subprimals needs to be validated. The objective of this study was to validate the use of lactic acid as an initial subprimal intervention and as a secondary intervention on reworked product when applied at industry operating parameters. Additionally, limited literature exists on non-O157 STEC. Specifically, data are needed on the effectiveness of antimicrobial interventions, currently used by the meat industry, against non-O157 STEC, and whether the antimicrobial effects obtained are similar to those obtained for *E. coli* O157:H7 or the biotype I *E. coli* surrogates. Therefore, a study comparing the ability of chemical interventions to reduce *E. coli* O157:H7, non-O157 STEC, and the biotype I *E. coli* surrogates when applied to chilled subprimals using industry parameters would be extremely useful as a validation tool for industry systems.

Material and Methods

Bacterial strains and preparation of inocula. Three different inocula were used in this study; one comprised of five *E. coli* O157:H7 strains [ATCC 43888, ATCC 43895, C1-057, C1-072, C1-109 (isolated from bovine feces (Carlson et al., 2009))], the

second comprised of five strains of non-pathogenic *E. coli* that are surrogates for *E. coli* O157:H7, as described by Marshall et al. (8) (ATCC BAA-1427, ATCC BAA-1428, ATCC BAA-1429, ATCC BAA-1430, ATCC BAA-1431), and a third comprised of 12 strains of non-O157 STEC (i.e., two strains each of serotypes O26, O45, O103, O111, O121, and O145) described in Table 1. Rifampicin resistant cultures of each of the strains were developed, based on the method described by Kaspar and Tamplin (1993), to allow selection and differentiation of inoculum populations from natural flora associated with meat. The rifampicin-resistant strains were individually cultured and subcultured (35°C, 24±2 h) in 10 ml of tryptic soy broth (Difco, Becton Dickinson, Sparks, MD) supplemented with 100 µg/ml rifampicin (Sigma-Aldrich Inc., St. Louis, MO). Cell cultures (10 ml) of each strain were harvested individually by centrifugation (Eppendorf model 5810 R, Brinkmann Instruments Inc., Westbury, NY; 4,629×g, 15 min, 4°C), washed with 10 ml phosphate-buffered saline (PBS, pH 7.4; 0.2 g/liter KH_2PO_4, 1.5 g/liter $Na_2HPO4 \cdot H_2O$, 8.0 g/liter of NaCl, and 0.2 g/liter KCl), re-centrifuged, and suspended in PBS to obtain a concentration of 8 log CFU/ml. Inocula were then prepared by adding 10 ml of the respective strains within each inoculum type together, and vortexing vigorously for 2 min.

Sample inoculation and treatment. Two different subprimals were used in the study; beef round peeled knuckle (IMPS 167A) and beef brisket flats (IMPS 120A). Chilled subprimals were obtained prior to any post-chill antimicrobial intervention at three different times but post-harvest aging was held constant (48 to 72 hours post-mortem). The subprimals were portioned into five sections with 100 cm^2 of exposed lean surface and were spot inoculated (50 µl of inoculant cocktail onto both sides of sections),

to a target level of approximately 6 log CFU/cm^2, with either the pathogenic *E. coli* O157:H7 mixture, the pathogenic non-O157 STEC mixture, or the surrogate non-pathogenic *E. coli* mixture. Sterile PBS (50 µl of PBS both sides of sections) was placed on a fourth set of sections assigned as the non-inoculated controls to mimic the inoculated sections. Samples were placed at 4°C for 30 min to allow for bacterial cell attachment.

Uninoculated and inoculated beef knuckle or brisket sections were treated with lactic acid in a custom-built spray cabinet (Chad Co., Olathe KS) at one of 16 combinations of two lactic acid concentrations (2.0 and 5.0%), two lactic acid temperatures (22 and 48°C), two pressures (1.03 and 4.83 bar), and two flow rates (0.22 and 6.22 lpm). Concentrated lactic acid (88% L-lactic acid; Purac, Linconshire, IL) was mixed with tap water to the desired concentrations. The pH of the lactic acid treatments was measured at the time of application. Lactic acid was applied to all sections initially and allowed to drip for 10 seconds and were then vacuum packaged in 20.3 x 35.6 cm vacuum bags (Cryovac, Duncan, SC), sealed in a sing;e chamber vacuum packager (Hollymatic, Countryside, IL) and stored at 4°C until enumerated or second application of lactic acid, 24 hours after first application.

Microbiological analyses. Sections (100 cm^2) from each subprimal (beef knuckle or brisket) were sampled before inoculation to determine initial total bacterial populations. Sections were placed in 625 ml filter bags (19 x 30cm; Nasco Whirl-Pak, Modesto CA) to which 100 ml of D/E neutralizing broth (Difco, Becton Dickinson) was added, followed by pummeling (Masticator, IUL Industries, Barcelona, Spain) for 2 min. Sample homogenates were serially diluted (10-fold) in 0.1% buffered peptone water (Difco, Becton Dickinson) and appropriate dilutions were surface-plated on tryptic soy

agar (TSA; Difco, Becton Dickinson). After inoculation and prior to initial treatment

with lactic acid, subprimal sections were sampled likewise and surface-plated on TSA

plus rifampicin (100 μg/ml) for enumeration of rifampicin-resistant *E. coli* inoculum

populations; thus, determining inoculation level. Samples analyzed immediately

following initial treatment, prior to second treatment (i.e., after vacuum-packaged storage

at 4°C), and following second treatment were plated on TSA and TSA plus rifampicin

(100μg/ml) for enumeration of total bacterial populations and rifampicin-resistant *E. coli*

inoculum populations, respectively. Uninoculated treated sections were sampled likewise

and surface-plated on TSA to enumerate total plate counts. Samples were incubated at

37°C for 18 to 24 hours prior to enumeration.

Statistical analysis. Samples were run in triplicate, with the entire experiment

repeated twice for a total of six samples per treatment.   Main effects of inoculum,

subprimal, sampling time, and temperature, pressure, rate, and concentration of lactic

acid application were analyzed. After initial analysis, temperature, pressure, rate, and

concentration of lactic acid application were analyzed together as the application

parameter. Additionally, all interactions between inoculum, subprimal, time and

application parameter were analyzed.   Statistical analysis was conducted using the

General Linear Model of SAS v. 9.2. *P* values less than 0.05 ($P < 0.05$) were considered

statistically significant.

Results and Discussion. Natural microflora, enumerated on the uninoculated

samples had an average Total Plate Count (TPC) of 3.5 ($\pm$ 0.2) log CFU/cm^2 across all

samples. Following the first lactic acid treatment, average TPC from the uninoculated

samples were reduced to 2.4 $\pm$ 0.3 log CFU/cm^2 (Figure 1). Similarly, average TPC prior

to the second treatment were 3.4 ± 0.2 log CFU/cm^2 and were reduced to 2.3 ± 0.3 log

CFU/cm^2 averaged across all samples (Figure 1). Some differences ($P < 0.05$) were

found between application parameters. After the first application of lactic acid at varying

parameters, total plate counts ranged from $0.9 \pm 0.3 - 1.5 \pm 0.2$ log CFU/cm^2 lower than

the starting counts for samples (Table 2). Similarly, after the second application of lactic

acid, total plate counts ranged from $1.0 \pm 0.1 - 1.2 \pm 0.5$ log CFU/cm^2 lower than the

starting counts depending on the application parameter (Table 3). After-treatment, TPC

mirrored Total Inoculum Counts (TIC), showing that the majority of the bacteria

enumerated as TPC were the artificially inoculated bacteria. Following the initial spray

application of lactic acid, mean TIC were reduced from 6.0 log CFU/cm^2 to 3.6 ± 0.1, 4.4

± 0.1, and 4.4 ± 0.1 log CFU/cm^2 for the *E. coli* surrogates, *E. coli* O157:H7, and non-

O157 STEC inoculation groups, respectively. Following the first lactic acid treatment,

there was an observed difference ($P < 0.05$) of 0.1 ± 0.0 and 0.1 ± 0.0 log CFU/cm^2 on

different subprimals for both TPC and TIC, respectively. Although the difference was

significant, it was not meaningful on a microbiological scale (NACMCF, 2009).

Similarly, differences ($P < 0.05$) were observed in TIC when lactic acid was

applied at different parameters. There was no difference in TPC for the uninoculated

samples after the first application when lactic acid was applied at different

concentrations, or pressures. However, a difference of 0.1 ± 0.0 log CFU/cm^2 was

observed when lactic acid was applied at different rates, and the same difference in TPC

was observed when lactic acid was applied at varying temperature. The small differences

$(0.0-0.1 \pm 0.0$ log CFU/cm^2) between counts at varying application parameters were

significant, but not large enough to affect differences in microbial growth (NACMCF, 2009).

For the *E. coli* surrogates and *E. coli* O157:H7, TIC were 0.1 ± 0.0 and 0.1 ± 0.0 log CFU/cm^2 lower when lactic acid was applied at 5.0% compared to 2.5%, respectively. Conversely, non-O157 STEC TIC were 0.1 ± 0.1 log CFU/cm^2 lower when lactic acid was applied at 2.5% compared to 5.0%. After the first application at varying pressures, the *E. coli* surrogates and non-O157 STEC 0.2 ± 0.1 log CFU/cm^2 higher TIC when lactic acid was applied at 4.83 vs. 1.03 bar. When the first lactic acid treatment was applied at 1.03 bar, *Escherichia coli* O157:H7 TIC were 0.1 ± 0.0 log CFU/cm^2 higher when compared to application at 4.83 bar. Similar differences were with varying rates. TIC were 0.2 ± 0.1, 0.1 ± 0.0, and 0.0 ± 0.0 higher when lactic acid was applied at 6.22 lpm compared to application at 0.22 lpm. Likewise, application of lactic acid at varying temperatures resulted in TIC 0.1 ± 0.0, 0.0 ± 0.0, and 0.0 ± 0.0 log CFU/cm^2 higher following the first application at 48°C vs. 22°C for the *E. coli* surrogates, *E. coli* O157:H7, and non-O157 STEC inoculation groups, respectively.

During storage at 4°C, TIC decreased an average 0.1 ± 0.1, 0.2 ± 0.1, and 0.0 ± 0.1 log CFU/ cm^2 for the *E. coli* surrogates, *E. coli* O157:H7, and non-O157 STEC inoculation groups, respectively.

After the second lactic acid application, remaining TIC averaged 2.6 ± 0.1, 3.2 ± 0.1, and 3.6 ± 0.1 log CFU/cm^2 for the *E. coli* surrogates, *E. coli* O157:H7, and non-O157 STEC inoculation groups, respectively. However, following the second lactic acid

application, lower after-treatment counts were observed when lactic acid was applied at 5.0% versus 2.0%.

Following the first and second lactic acid applications a significant difference was observed when lactic acid was applied at varying parameters. However, as the range of after treatment TIC was 0.3 and 0.2 log CFU/cm^2 for the first and second application, respectively, the difference in parameter of application was not deemed microbiologically meaningful (NACMCF, 2009). Similarly, the differences between inoculants were significant but not meaningful on a as they consisted of less than 1.0 log CFU/cm^2 difference (NACMCF, 2009). Similarly, the difference between subprimals was significant ($P < 0.05$), but not meaningful on a microbiological scale. Following the first lactic acid application, TIC on beef brisket samples were slightly higher than TIC on beef knuckles (3.7 and 3.6 log CFU/cm^2, respectively). Following the second application of lactic acid, TIC enumerated from the beef brisket samples were 0.04 CFU/cm^2 higher than TIC enumerated from the beef knuckle sections, a statistical ($P < 0.05$), but microbiologically meaningless difference.

Bacon et al. (2002) found lactic acid, which is relied upon as a whole carcass intervention, resulted in minimal reductions (< 0.5 log CFU/100 cm^2) of total plate counts, total coliform counts, and *E. coli* counts when applied to top sirloin butts from initial mean values of 5.7, 3.8, and 3.3 log CFU/100 cm^2. Contrary to this, at the applied parameters in the present study, lactic acid was shown to reduce counts of all three inocula and the uninoculated background flora. Bacon et al. (2002) found that counts of *E. coli* O157:H7 were reduced from 5.8 to 4.7 log CFU/g and from 4.2 to 2.7 log CFU/g, for a high and low inoculation group, respectively, when lactic acid was applied at 2%

and 55°C. Similar results were observed in the present study when lactic acid was applied at one of 16 combinations of two concentrations (2.0 and 5.0%), two temperatures (22 and 48°C), two pressures (1.03 and 4.83 bar), and two flow rates (0.22 and 6.22 gpm), on either the beef brisket or knuckle sections. Heller et al. (2007) inoculated beef outside rounds with a three-strain cocktail of *E. coli* O157:H7 and applied lactic acid at 55°C at two concentrations, 2.5 and 5.0%. *E. coli* O157:H7 mean populations were reduced from 3.6 log CFU/100 cm^2 by 0.9 to 1.1 log CFU/100 cm^2. The authors did not find any differences between applied treatments.

Following the initial lactic acid treatment in our study, the surrogate inoculum comprised of non-pathogenic *E. coli,* had the lowest after-treatment TIC compared to both pathogenic inoculants. This was most likely due to the fact that the surrogates were developed for use on beef hot carcass tissue and this study evaluated them on beef chilled subprimals, in addition to using rifampicin-resistant derivatives of the strains. However, the *E. coli* surrogates were still closely associated with the behavior of the other inocula due to lactic acid action on chilled beef subprimals. Because the counts were lower on the sections inoculated with the surrogates after the initial lactic acid application, the counts after the second application were also lower.

Both the initial and secondary lactic acid treatments successfully resulted in lower after-treatment TPC and TIC. A second lactic acid spray, applied as a re-work procedure to product treated formerly with lactic acid, can be applied at the same operating parameters regardless of subprimal. Although the lactic acid treatments did not reduce microbial populations to below detectable limits, the double lactic acid spray was shown to be effective as a primary and rework intervention against *E. coli* O157:H7 and non-

O157 STEC. These data would be beneficial to industry as part of the HACCP validation

process.

Table 5.1. Strains included in the non-O157 STEC inoculum

Non-O157 STEC Strain	Serotype	Source
E1-092[a]	O26:H11	Human
E1-098[a]	O45:NM	Human
E1-102 [a]	O103:HN	Human
E1-106 [a]	O111:NM	Human
E1-109 [a]	O121	Human
E1-110 [a]	O121:H19	Human
E1-112 [a]	O145:H28	Human
E1-115 [a]	O26:H11	Cattle
E1-118 [a]	O145:NM	Cattle
E1-121 [a]	O45:NM	Cattle
E1-123 [a]	O103:N	Cattle
A1-002 [b]	O111	Cattle

[a] Elder et al. 2011. Unpublished
[b] Ahlstrom et al. 2011. Unpublished

Table 5.2. Remaining Total Plate Counts (log CFU/cm^2) enumerated on tryptic soy agar from uninoculated samples treated with lactic acid at varying parameters (means ± standard deviation shown)

Lactic Acid Application Parameters	First Application of Lactic Acid		Second Application of Lactic Acid	
	Before Treatment	After Treatment	Before Treatment	After Treatment
2.0%, 22°C, 1.03 bar, 0.22 lpm	3.6 ± 0.2	2.4 ± 0.3	3.6 ± 0.1	2.6 ± 0.1
2.0%, 22°C, 4.83 bar, 0.22 lpm	3.4 ± 0.2	2.3 ± 0.3	3.3 ± 0.1	2.2 ± 0.2
2.0%, 22°C, 1.03 bar, 6.22 lpm	3.3 ± 0.1	2.4 ± 0.2	3.3 ± 0.1	2.2 ± 0.2
2.0%, 22°C, 4.83 bar, 6.22 lpm	3.6 ± 0.1	2.4 ± 0.3	3.5 ± 0.2	2.5 ± 0.2
2.0%, 48°C, 1.03 bar, 0.22 lpm	3.8 ± 0.2	2.3 ± 0.2	3.8 ± 0.2	2.8 ± 0.2
2.0%, 48°C, 4.83 bar, 0.22 lpm	3.2 ± 0.2	2.3 ± 0.3	3.0 ± 0.3	1.9 ± 0.5
2.0%, 48°C, 1.03 bar, 6.22 lpm	3.6 ± 0.1	2.4 ± 0.3	3.5 ± 0.2	2.5 ± 0.2
2.0%, 48°C, 4.83 bar, 6.22 lpm	3.5 ± 0.1	2.3 ± 0.3	2.9 ± 0.4	1.7 ± 0.5
5.0%, 22°C, 1.03 bar, 0.22 lpm	3.6 ± 0.1	2.4 ± 0.3	3.4 ± 0.2	2.4 ± 0.3
5.0%, 22°C, 4.83 bar, 0.22 lpm	3.5 ± 0.1	2.3 ± 0.3	3.2 ± 0.1	2.2 ± 0.2
5.0%, 22°C, 1.03 bar, 6.22 lpm	3.6 ± 0.3	2.4 ± 0.2	3.6 ± 0.3	2.6 ± 0.3
5.0%, 22°C, 4.83 bar, 6.22 lpm	3.6 ± 0.1	2.4 ± 0.3	3.2 ± 0.3	2.1 ± 0.5
5.0%, 48°C, 1.03 bar, 0.22 lpm	3.7 ± 0.2	2.3 ± 0.2	3.4 ± 0.3	2.3 ± 0.3
5.0%, 48°C, 4.83 bar, 0.22 lpm	3.7 ± 0.2	2.3 ± 0.3	3.4 ± 0.4	2.3 ± 0.5
5.0%, 48°C, 1.03 bar, 6.22 lpm	3.3 ± 0.1	2.4 ± 0.3	3.2 ± 0.1	2.2 ± 0.2
5.0%, 48°C, 4.83 bar, 6.22 lpm	3.4 ± 0.1	2.3 ± 0.3	3.3 ± 0.2	2.3 ± 0.2

lpm: liters per minute

Table 5.3. Remaining Total Plate Counts (log CFU/cm^2) enumerated on tryptic soy agar from inoculated samples treated with lactic acid at varying parameters (means ± standard deviation shown)

Lactic Acid Application Parameters	E. coli Surrogates			E. coli O157:H7			Non-O157 STEC		
	After first application of LA	Before second application of LA	After second application of LA	After first application of LA	Before second application of LA	After second application of LA	After first application of LA	Before second application of LA	After second application of LA
2.0%, 22°C, 1.03 bar, 0.22 lpm	4.1 ± 0.1	3.4 ± 0.1	2.6 ± 0.0	4.4 ± 0.1	3.9 ± 0.0	2.9 ± 0.1	4.3 ± 0.0	4.4 ± 0.0	3.6 ± 0.0
2.0%, 22°C, 4.83 bar, 0.22 lpm	3.6 ± 0.2	3.8 ± 0.2	2.9 ± 0.1	4.4 ± 0.1	4.4 ± 0.3	3.2 ± 0.2	4.2 ± 0.1	4.3 ± 0.1	3.6 ± 0.0
2.0%, 22°C, 1.03 bar, 6.22 lpm	3.5 ± 0.0	3.6 ± 0.0	2.8 ± 0.0	4.4 ± 0.1	4.6 ± 0.1	3.3 ± 0.1	4.4 ± 0.2	4.4 ± 0.2	3.7 ± 0.1
2.0%, 22°C, 4.83 bar, 6.22 lpm	3.6 ± 0.1	3.7 ± 0.1	2.8 ± 0.1	4.6 ± 0.1	4.7 ± 0.1	3.5 ± 0.2	4.2 ± 0.1	4.3 ± 0.1	3.6 ± 0.0
2.0%, 48°C, 1.03 bar, 0.22 lpm	3.2 ± 0.2	3.5 ± 0.1	2.7 ± 0.1	4.3 ± 0.1	4.5 ± 0.0	3.3 ± 0.1	4.3 ± 0.1	4.3 ± 0.1	3.6 ± 0.0
2.0%, 48°C, 4.83 bar, 0.22 lpm	3.7 ± 0.1	3.8 ± 0.0	2.9 ± 0.0	4.3 ± 0.0	4.5 ± 0.0	3.3 ± 0.0	4.3 ± 0.2	4.4 ± 0.2	3.6 ± 0.0
2.0%, 48°C, 1.03 bar, 6.22 lpm	3.3 ± 0.1	3.5 ± 0.1	2.7 ± 0.0	3.9 ± 0.2	4.3 ± 0.1	2.7 ± 0.3	3.9 ± 0.2	3.9 ± 0.1	3.1 ± 0.1
2.0%, 48°C, 4.83 bar, 6.22 lpm	3.8 ± 0.1	3.9 ± 0.1	3.0 ± 0.1	4.4 ± 0.1	4.6 ± 0.1	3.4 ± 0.2	4.0 ± 0.1	4.0 ± 0.1	3.1 ± 0.0
5.0%, 22°C, 1.03 bar, 0.22 lpm	3.8 ± 0.2	3.9 ± 0.1	3.0 ± 0.1	4.4 ± 0.1	4.6 ± 0.1	3.4 ± 0.1	4.6 ± 0.0	4.6 ± 0.0	3.8 ± 0.0
5.0%, 22°C, 4.83 bar, 0.22 lpm	3.7 ± 0.2	3.8 ± 0.1	2.9 ± 0.1	4.4 ± 0.1	4.6 ± 0.1	3.4 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	3.8 ± 0.0
5.0%, 22°C, 1.03 bar, 6.22 lpm	3.6 ± 0.0	3.7 ± 0.0	2.9 ± 0.0	4.2 ± 0.2	4.6 ± 0.3	3.9 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	3.8 ± 0.0
5.0%, 22°C, 4.83 bar, 6.22 lpm	3.7 ± 0.1	3.8 ± 0.1	2.9 ± 0.0	4.3 ± 0.1	5.0 ± 0.1	2.9 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	3.8 ± 0.0
5.0%, 48°C, 1.03 bar, 0.22 lpm	3.4 ± 0.1	3.6 ± 0.1	2.8 ± 0.0	4.5 ± 0.1	4.7 ± 0.1	3.5 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	3.8 ± 0.0
5.0%, 48°C, 4.83 bar, 0.22 lpm	3.7 ± 0.1	3.8 ± 0.0	2.9 ± 0.0	4.6 ± 0.1	4.7 ± 0.1	3.6 ± 0.1	4.6 ± 0.1	4.7 ± 0.1	3.8 ± 0.0
5.0%, 48°C, 1.03 bar, 6.22 lpm	3.5 ± 0.1	3.7 ± 0.1	2.8 ± 0.0	4.6 ± 0.1	4.7 ± 0.1	3.6 ± 0.1	4.1 ± 0.2	4.2 ± 0.2	3.6 ± 0.0
5.0%, 48°C, 4.83 bar, 6.22 lpm	3.9 ± 0.1	3.9 ± 0.1	3.0 ± 0.1	4.5 ± 0.0	4.7 ± 0.0	3.5 ± 0.0	4.1 ± 0.1	4.1 ± 0.1	3.2 ± 0.0

LA: lactic acid; lpm: liters per minute

Table 5.4. Remaining Total Inoculum Counts (log CFU/cm^2) enumerated on tryptic soy agar plus rifampicin (100µg/ml) from inoculated samples treated with lactic acid at varying parameters (means ± standard deviation shown)

| Lactic Acid Application Parameters	*E. coli* Surrogates			*E. coli* O157:H7			Non-O157 STEC		
	After first application of LA	Before second application of LA	After second application of LA	After first application of LA	Before second application of LA	After second application of LA	After first application of LA	Before second application of LA	After second application of LA
2.0%, 22°C, 1.03 bar, 0.22 lpm	3.3 ± 0.2	4.0 ± 0.1	2.9 ± 0.1	4.3 ± 0.1	4.4 ± 0.1	3.2 ± 0.1	4.4 ± 0.0	4.4 ± 0.0	3.5 ± 0.0
2.0%, 22°C, 4.83 bar, 0.22 lpm	3.6 ± 0.2	3.5 ± 0.1	2.5 ± 0.1	4.3 ± 0.1	4.4 ± 0.1	3.2 ± 0.2	4.4 ± 0.1	4.4 ± 0.1	3.5 ± 0.1
2.0%, 22°C, 1.03 bar, 6.22 lpm	3.6 ± 0.2	3.6 ± 0.2	2.5 ± 0.2	4.6 ± 0.0	4.3 ± 0.2	3.6 ± 0.0	4.4 ± 0.3	4.4 ± 0.2	3.5 ± 0.2
2.0%, 22°C, 4.83 bar, 6.22 lpm	3.8 ± 0.1	3.4 ± 0.1	2.4 ± 0.1	4.3 ± 0.1	4.5 ± 0.1	3.2 ± 0.1	4.4 ± 0.2	4.4 ± 0.2	3.5 ± 0.1
2.0%, 48°C, 1.03 bar, 0.22 lpm	3.6 ± 0.1	3.6 ± 0.1	2.6 ± 0.1	4.4 ± 0.0	4.3 ± 0.1	3.2 ± 0.0	4.3 ± 0.1	4.4 ± 0.1	3.5 ± 0.1
2.0%, 48°C, 4.83 bar, 0.22 lpm	3.7 ± 0.1	3.6 ± 0.1	2.6 ± 0.1	4.4 ± 0.1	4.3 ± 0.1	3.2 ± 0.1	4.4 ± 0.1	4.5 ± 0.1	3.6 ± 0.1
2.0%, 48°C, 1.03 bar, 6.22 lpm	3.7 ± 0.0	3.5 ± 0.1	2.5 ± 0.1	4.4 ± 0.1	4.3 ± 0.1	3.3 ± 0.1	4.2 ± 0.2	4.2 ± 0.2	3.4 ± 0.1
2.0%, 48°C, 4.83 bar, 6.22 lpm	4.0 ± 0.1	3.8 ± 0.2	2.8 ± 0.2	4.4 ± 0.2	4.5 ± 0.1	3.3 ± 0.2	4.5 ± 0.1	4.5 ± 0.1	3.6 ± 0.1
5.0%, 22°C, 1.03 bar, 0.22 lpm	3.2 ± 0.1	3.7 ± 0.1	2.6 ± 0.1	4.2 ± 0.1	4.6 ± 0.1	3.1 ± 0.2	4.5 ± 0.0	4.5 ± 0.0	3.6 ± 0.0
5.0%, 22°C, 4.83 bar, 0.22 lpm	3.5 ± 0.2	3.6 ± 0.1	2.6 ± 0.1	4.3 ± 0.1	4.6 ± 0.1	3.1 ± 0.2	4.5 ± 0.1	4.5 ± 0.1	3.6 ± 0.1
5.0%, 22°C, 1.03 bar, 6.22 lpm	3.6 ± 0.2	3.7 ± 0.1	2.7 ± 0.1	4.6 ± 0.0	4.5 ± 0.1	3.5 ± 0.0	4.5 ± 0.2	4.5 ± 0.2	3.6 ± 0.1
5.0%, 22°C, 4.83 bar, 6.22 lpm	3.7 ± 0.1	3.5 ± 0.1	2.5 ± 0.1	4.3 ± 0.1	4.7 ± 0.1	3.1 ± 0.1	4.5 ± 0.1	4.5 ± 0.1	3.6 ± 0.1
5.0%, 48°C, 1.03 bar, 0.22 lpm	3.5 ± 0.1	3.7 ± 0.1	2.7 ± 0.1	4.3 ± 0.0	4.5 ± 0.1	3.2 ± 0.0	4.5 ± 0.1	4.5 ± 0.1	3.6 ± 0.1
5.0%, 48°C, 4.83 bar, 0.22 lpm	3.6 ± 0.1	3.7 ± 0.1	2.7 ± 0.1	4.3 ± 0.1	4.5 ± 0.1	3.2 ± 0.2	4.5 ± 0.1	4.6 ± 0.1	3.6 ± 0.0
5.0%, 48°C, 1.03 bar, 6.22 lpm	3.7 ± 0.0	3.6 ± 0.1	2.6 ± 0.1	4.4 ± 0.1	4.5 ± 0.1	3.2 ± 0.1	4.4 ± 0.1	4.4 ± 0.1	3.5 ± 0.1
5.0%, 48°C, 4.83 bar, 6.22 lpm	3.5 ± 0.2	3.9 ± 0.1	2.8 ± 0.1	4.4 ± 0.2	4.6 ± 0.0	3.2 ± 0.3	4.6 ± 0.1	4.6 ± 0.1	3.7 ± 0.1

LA: lactic acid; lpm: liters per minute
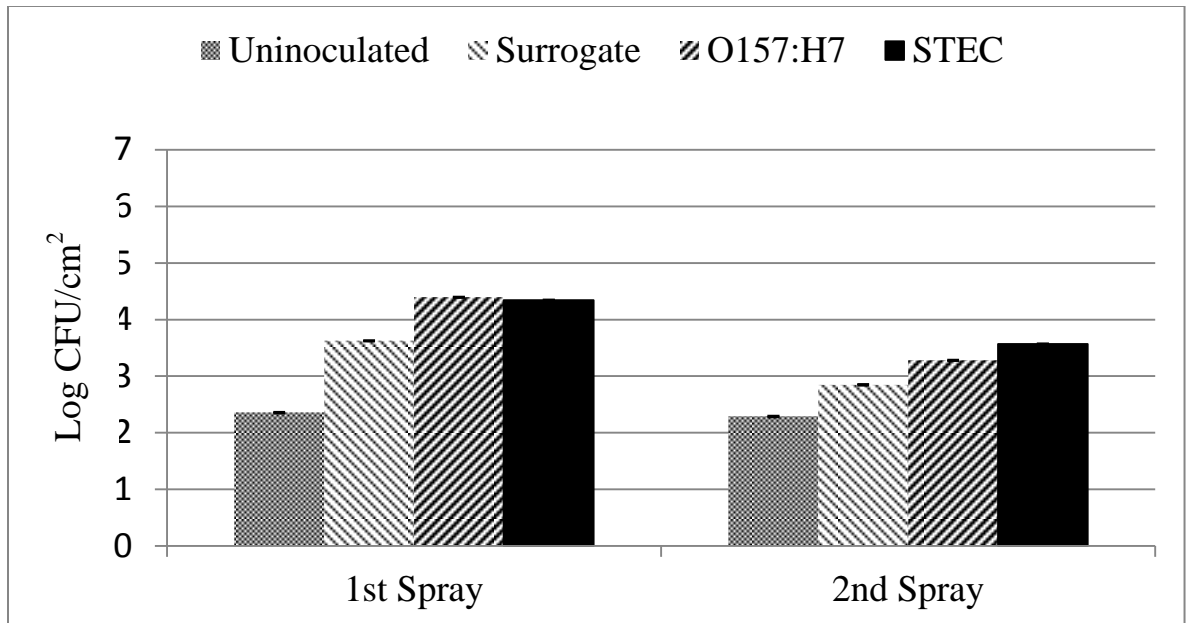
Figure 5.1. Differences in after treatment total plate counts between inoculants following first and second lactic acid application on inoculated chilled beef subprimals -starting counts 6.0 log CFU.cm^2.

Evaluation of BoviBrom as a Carcass Intervention for *Escherichia coli* O157:H7,

Non-O157 STEC, and Surrogate Biotype 1 *E. coli*

SUMMARY

Studies characterizing bacterial reductions when chemicals are applied to beef carcass tissue would be useful in validations of HACCP systems. The objective of this study was to validate use of BoviBrom as a hot carcass intervention during beef slaughter processes. Beef cutaneous trunci muscle segments (100 cm^2 of exposed surface lean) were assigned to four groups: 1) inoculated with 4 log of a 5-strain *Escherichia coli* O157:H7 cocktail, 2) inoculated with 4 log of a 12-strain, non-O157 shiga toxin-producing *E. coli* (STEC) cocktail, 3) inoculated with 4 log of a 5-strain non-pathogenic *E. coli* and *Salmonella* surrogate cocktail, and 4) not inoculated. Application of BoviBrom occurred in a custom-built spray cabinet at four temperatures (40, 80, 100, and 120°F), three pressures (15, 90, and 120 psi), and three doses (0.5, 1.0, and 1.5 ml/cm^2), at a single concentration (225 ppm). At sampling time, 100 cm^2 sections were homogenized for 120 seconds in 100 ml of 0.1% buffered peptone water and serially-diluted (10-fold) in 0.1% buffered peptone water. Appropriate dilutions were spread plated (0.1 ml) onto non-selective media [tryptic soy agar (TSA)] and selective media [TSA plus rifampicin (100μg/ml)] to determine total plate count (TPC) and total inoculated count (TIC), respectively. Following initial BoviBrom application, varying

application parameters did not affect the after treatment counts, with the exception of pressure of application as TPC and TIC were far higher when BoviBrom was applied at 15 psi. No difference ($P > 0.05$) was observed between samples treated with different temperatures or doses. No difference ($P > 0.05$) was observed between samples enumerated immediately following treatment and samples enumerated at 24 hours post-treatment. TPC and TIC after treatment counts were lower on samples treated with BoviBrom when compared to samples treated with only water, showing a portion of the lower counts can be attributed to the chemical action of BoviBrom.

Keywords: Beef Carcass, Intervention, *E. coli* O157:H7, STEC

INTRODUCTION

Muscle tissues of healthy animals are considered sterile, but become contaminated with bacteria from the environment or hide of the animal during slaughter and dressing procedures (Sofos et al., 1999). Possible contamination could result in the presence of pathogenic bacteria including *Escherichia coli* O157:H7, which has been estimated to cause 2,138 hospitalizations and 20 deaths a year in the United States (Scallen et al., 2011). Non-O157 Shiga toxin producing *E. coli* (STEC) are also pathogenic bacteria of concern that cause an estimated 271 hospitalizations each year (Scallen et al., 2011). Both *E. coli* O157:H7 and non-O157 STEC have been isolated from beef cattle hides, which are a common source of carcass contamination (Bacon et al., 2000; Hussein, 2007).

Various physical and chemical decontamination processes exist for the elimination or reduction of spoilage or pathogenic bacteria at the pre- and post-harvest levels including live-animal cleaning, chemical dehairing, spot cleaning of carcasses, and the use of water or chemicals in spraying or washing applications (Belk, 2001).

Single interventions reduce bacterial population numbers; however, remaining bacteria remains a concern. The use of multiple sequential interventions has been shown to be more effective than individual interventions (Sofos and Smith, 1998). Because of this, many intervention systems employ multiple combinations of knife trimming, steam vacuuming, hot water washes and chemical sprays to take advantage of the additive effects. Cutter et al. (1997) showed that increased water temperature enhanced the effect of acid solutions. Lactic acid function is enhanced following treatments with hot water

(Castillo et al., 1998). Bacon et al. (2000) found that microbial contamination on carcasses decreased after each processing step including hide removal, final wash, and chilling.

On July 25, 1996, USDA-FSIS published the Pathogen Reduction: Hazard Analysis and Critical Contol Point (HACCP) Systems Final Rule. This regulation states each establishment is require to evaluate and validate the effectiveness of its HACCP system's ability to control food safety hazards. USDA-FSIS issued the Draft Guidance: HACCP Systems Validation document in spring 2010, which clarified the expectation of validation. This document addressed the importance of validating the entire HACCP system including prerequisite programs. This document also stipulated what was necessary for validation and defined validation as, "the process of demonstrating that the HACCP system as designed can adequately control identified hazards to produce a safe, unadulterated product." Validation had two elements:

1) Scientific or technical support for the HACCP system

2) Practical in-plant demonstration showing the HACCP system effective

Scientific journal articles, documented studies, data underlying published guidelines, or in-house data, provided the hazard is identified, the level of prevention is defined, the critical parameters or conditions are used, and what processing steps will be used to achieve the desired reduction or prevention. If the scientific documentation used defines a particular parameter, that parameter must be used for the process.

In addition to proving the HACCP system is theoretically sound, based on scientific support, the establishment must show its system able to reach the desired effect,

through in-plant demonstration. The first step of in-plant validation is defining critical

operational parameters including time, temperature, pressure, concentration or log

reduction. In-plant validation must provide data sufficient to show the process can

operate effectively on a daily basis.

To effectively demonstrate a HACCP system is working properly to control

pathogens, surrogate indicator organisms are essential. Surrogate organisms can include

Enterobacteriaceae, coliforms, or generic *E. coli*. The effective indicator organisms

would show the ability of the HACCP system to reduce pathogen populations without

artificial introduction of pathogens into the plant. Recently, non-pathogenic *E. coli*

Biotype 1 was described as a surrogate for *E. coli* O157:H7. Marshall et al. (2005)

evaluated bacteria collected from beef hides and identified 5 non-pathogenic *E. coli*

isolates as surrogates of *E. coli* O157:H7 growth and reduction following antimicrobial

intervention. These isolates were further evaluated during cooking, fermentation,

freezing, and refrigerated storage of meat.   Keeling et al. (2009) found no difference

between two of the isolates (BAA-1428 and BAA-1430) and *E. coli* O157:H7 during

frozen storage, which even survived at a slightly higher rate than *E. coli* O157:H7. Under

refrigeration, all five isolates were found to be similar to *E. coli* O157:H7. No difference

was found between the isolates and *E. coli* O157:H7 during fermentation and the isolates

survived at higher levels than *E. coli* O157:H7, adding an additional layer of security

with over prediction.

On September 20, 2011,   USDA-FSIS published the Proposed Rules for Shiga

Toxin-Producing *Escherichia coli* in Certain Raw Beef Products, declaring six strains of

non-O157 *E. coli* adulterants of non-intact raw beef products. Because of this, validation

of an antimicrobial intervention's ability to control these bacteria would be very important.

Although many methods exist to reduce bacterial contamination of beef carcasses, there is a continued need for novel methods, as concerns about selecting acid tolerant organisms and continued equipment corrosion exist (Smulders and Greer, 1998; Sofos et al., 1999). The objective of this study was to evaluate the effectiveness of BoviBrom, applied at various temperatures, pressures, and doses, in reducing populations of pathogenic *E. coli* O157:H7, pathogenic non-O157 STEC, non-pathogenic *E. coli* surrogates, and natural flora on beef carcass tissue.

MATERIALS AND METHODS

Bacterial strains and preparation of inocula. Three different inocula were used in this study; one comprised of five *E. coli* O157:H7 strains [ATCC 43888, ATCC 43895, C1-057, C1-072, C1-109 (isolated from bovine feces (4))], the second comprised of five strains of non-pathogenic *E. coli* that are surrogates for *E. coli* O157:H7, as described by Marshall et al. (8) (ATCC BAA-1427, ATCC BAA-1428, ATCC BAA-1429, ATCC BAA-1430, ATCC BAA-1431), and a third comprised of 12 strains of non-O157 STEC (i.e., two strains each of serotypes O26, O45, O103, O111, O121, and O145) described in Table 1. Rifampicin resistant cultures of each of the strains were developed, based on the method described by Kaspar and Tamplin (6), to allow selection and differentiation of inoculum populations from natural flora associated with meat. The rifampicin-resistant strains were individually cultured and subcultured (35°C, 24±2 h) in 10 ml of tryptic soy broth (Difco, Becton Dickinson, Sparks, MD) supplemented with

100 µg/ml rifampicin (Sigma-Aldrich Inc., St. Louis, MO). Cell cultures (10 ml) of each

strain were harvested individually by centrifugation (Eppendorf model 5810 R,

Brinkmann Instruments Inc., Westbury, NY; 4,629×g, 15 min, 4°C), washed with 10 ml

phosphate-buffered saline (PBS, pH 7.4; 0.2 g/liter KH_2PO_4, 1.5 g/liter $Na_2HPO4·H_2O$,

8.0 g/liter of NaCl, and 0.2 g/liter KCl), re-centrifuged, and suspended in PBS to obtain a

concentration of 8 log CFU/ml. Inocula were then prepared by adding 10 ml of the

respective strains within each inoculum type together, and vortexing vigorously for 2

min.

Sample inoculation and treatment. This experiment evaluated use of BoviBrom

on beef cutaneous trunci muscle simulating application to the exterior surface of beef

carcasses. Four hundred forty-four 100 cm^2 pieces of cutaneous trunci were obtained

from beef carcasses prior to final wash at harvest and were shipped to the Center for Meat

Safety and Quality at Colorado State University within 2 hours of collection. The meat

pieces were kept at 35°C, the average carcass temperature at final wash, during

transportation. Samples were spot inoculated (100 µl of inoculant cocktail onto the

exposed surface from the carcass), to a target level of approximately 6 log CFU/cm^2, with

either the pathogenic *E. coli* O157:H7 mixture, the pathogenic non-O157 STEC mixture,

or the surrogate non-pathogenic *E. coli* mixture. Sterile PBS (100 µl) was placed on a

fourth set of sections assigned as the non-inoculated controls to mimic the inoculated

sections. Samples were placed at 30°C for 30 min to allow for bacterial cell attachment.

Uninoculated and inoculated sections were treated with BoviBrom (Elanco Food

Solutions, Greenfield, IN) in a custom-built spray cabinet (Chad Co., Olathe KS) at four

temperatures (4, 27, 38, and 49°C), three pressures (1.0, 6.2, and 8.3 bar), and three

doses (0.5, 1.0, and 1.5 ml/cm^2), at a single concentration (225 ppm). Concentrations of BoviBrom treatments were monitored at the time of application based on recommendations of the supplier. In addition to BoviBrom application, deionized water was applied at the same parameters to determine the washing effect of the spray application. Treated sections were placed in 20.3 x 35.6 cm vacuum bags (Cryovac, Duncan, SC), left unsealed and stored aerobically at 4°C for 18-24 h.

Microbiological analyses. Samples (100 cm^2) were enumerate before inoculation to determine initial total bacterial populations. Sections were placed into sterile filter bags (15 × 23 cm; Whirl-Pak, Nasco, Modesto, CA) containing 100 ml of 0.1% buffered peptone water (BPW; Difco, Becton Dickinson), followed by pummeling (Masticator, IUL Industries, Barcelona, Spain) for 2 min. Sample homogenates were serially diluted (10-fold) in 0.1% buffered peptone water (Difco, Becton Dickinson) and appropriate dilutions were surface-plated on tryptic soy agar (TSA; Difco, Becton Dickinson). After inoculation and prior to treatment with BoviBrom, sections were sampled likewise and surface-plated on TSA plus rifampicin (100 µg/ml) for enumeration of rifampicin-resistant *E. coli* inoculum populations; thus, determining inoculation level. Samples analyzed immediately following treatment, and following aerobic storage at 4°C were plated on TSA and TSA plus rifampicin (100µg/ml) for enumeration of total bacterial populations and rifampicin-resistant *E. coli* inoculum populations, respectively. Uninoculated treated sections were sampled likewise and surface-plated on TSA to enumerate total plate counts. Plates were incubated at 37°C for 18-24 hours prior to enumeration.

Statistical analysis. This experiment was conducted once with six individual samples per inoculum per treatment. Main effects of inoculum, temperature, pressure, dose, and the respective interactions were analyzed using the General Linear Model of SAS v. 9.2 (13). The chemical effect was also analyzed compared to the rinsing effect of the water only application. *P* values less than 0.05 ($P < 0.05$) were considered statistically significant.

Results and Discussion. Gorman et al. (1995) concluded that knife trimming alone significantly reduced microbiological contamination compared to the control, however, significant contamination remained following trimming. Steam vacuuming, an alternative method to knife trimming (FSIS – USDA, 1996), has also been shown to reduce microbial contamination on beef carcasses (Gorman et al., 1995). There is a concern with spraying/washing methods as the application of treatments "may cause penetration of bacteria into the meat or spreading and redistribution on the carcass" (Sofos et al., 1999). To combat this concern, methods have been developed to reduce/eliminate bacterial populations instead of solely relying on removal. Spraying/washing decontamination methods range from hot water and steam to chemical solutions including organic acids and other novel antimicrobial solutions. Hot water (Castillo et al., 1998; Gorman et al., 1995; Sofos et al., 1999) and steam (Gorman et al., 1995; Sofos et al., 1999) have been shown to effectively reduce bacterial populations on carcass surfaces while increasing the temperature of the carcass surface. Lactic and acetic acid have been shown to be effective when applied at varying concentrations, temperatures, and doses (Smulders and Greer, 1998; Sofos et al., 1999). Treatments of lactic, acetic, or citric acids, when applied at concentrations of 1-5%, have been shown to

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reduce bacterial populations by 1-3 logs (Castillo et al., 1998; Cutter and Siragusa, 1993; Gorman et al., 1995).

The mean starting TPC for the uninoculated samples was 4.3 ± 0.1 log CFU/cm^2. Similarly, starting TIC were 4.4 ± 0.0 log CFU/cm^2 for all three artificial inoculated samples, for the *E. coli* surrogates, *E. coli* O157:H7, and non-O157 STEC inoculation groups.

After treatment with BoviBrom, TPC enumerated from the uninoculated samples averaged 3.3 ± 0.3 log CFU/cm^2 across all application parameters. TIC enumerated from the *E. coli* surrogates, *E. coli* O157:H7, and non-O157 STEC inoculation groups immediately after treatment were 3.4 ± 0.3, 3.4 ± 0.2, and 3.3 ± 0.3 log CFU/cm^2, respectively. BoviBrom was observed to have an effect on TPC and TIC that extended beyond a mere rising of the samples. When deionized water was applied in the same manner as BoviBrom, the TPC, averaged across application parameters, for the uninoculated samples were reduced from 4.4 ± 0.0 to 4.1 ± 0.1 log CFU/cm^2. Similarly, TIC after treatment with deionized water were 4.1 ± 0.1 for the *E. coli* surrogates, *E. coli* O157:H7, and non-O157 STEC inoculation groups. Although there was a significant difference between the uninoculated samples, *E. coli* surrogates, *E. coli* O157:H7, and non-O157 STEC, the range of the after treatment TPC was 0.2, a microbiologically meaningless difference as it consists of less than 1 log (NACAMF, 2009).

TPC and TIC enumerated immediately after treatment with BoviBrom were no different ($P > 0.05$) than TPC and TIC enumerated after treatment and 24 hours of aerobic storage at 4°C. Similarly, no difference ($P > 0.05$) was observed when

BoviBrom was applied at varying temperatures, or doses. Slight, significant, differences were observed with after treatment TIC when BoviBrom was applied at different pressures. Samples treated with BoviBrom at 1.03 bar averaged 3.7 ± 0.3 log CFU/cm^2, while samples treated at 6.21 and 8.27 bar were the same ($P < 0.05$), with TIC of 3.2 ± 0.1 log CFU/cm^2.

When evaluated on hot carcass tissue, *E. coli* O157:H7 and the non-O157 STECs responded similarly to BoviBrom. At an initial inoculation of approximately 4 log CFU/cm^2, the non-pathogenic surrogates are indicative of pathogenic *E. coli* (O157:H7 and non-O157 STEC) reductions when evaluated on hot carcass tissue.

Counts following treatment were observed to be greater than 1 log CFU/cm^2 lower than the starting inoculation level of approximately 4 log CFU/cm^2. These lower counts can be attributed to the chemical action of BoviBrom as opposed to merely a washing effect. During 18-24 h of refrigerated storage under aerobic conditions, the counts after treatment of BoviBrom did not change.

Although there were observed differences ($P < 0.05$) between the parameters of application, the difference was small enough not to affect microbial growth. Therefore, the recommended application of BoviBrom would be either (4, 27, 38, and 49°C), three pressures (1.0, 6.2, and 8.3 bar), and three doses (0.5, 1.0, and 1.5 ml/cm^2), at a single concentration (225 ppm). There was a slight washing effect when water was applied identically to the application of BoviBrom. BoviBrom successfully lower the counts of natural background flora and inoculated bacteria, including pathogenic *E. coli*. Because

of this, BoviBrom can be effectively used as a final wash strategy to lower bacterial
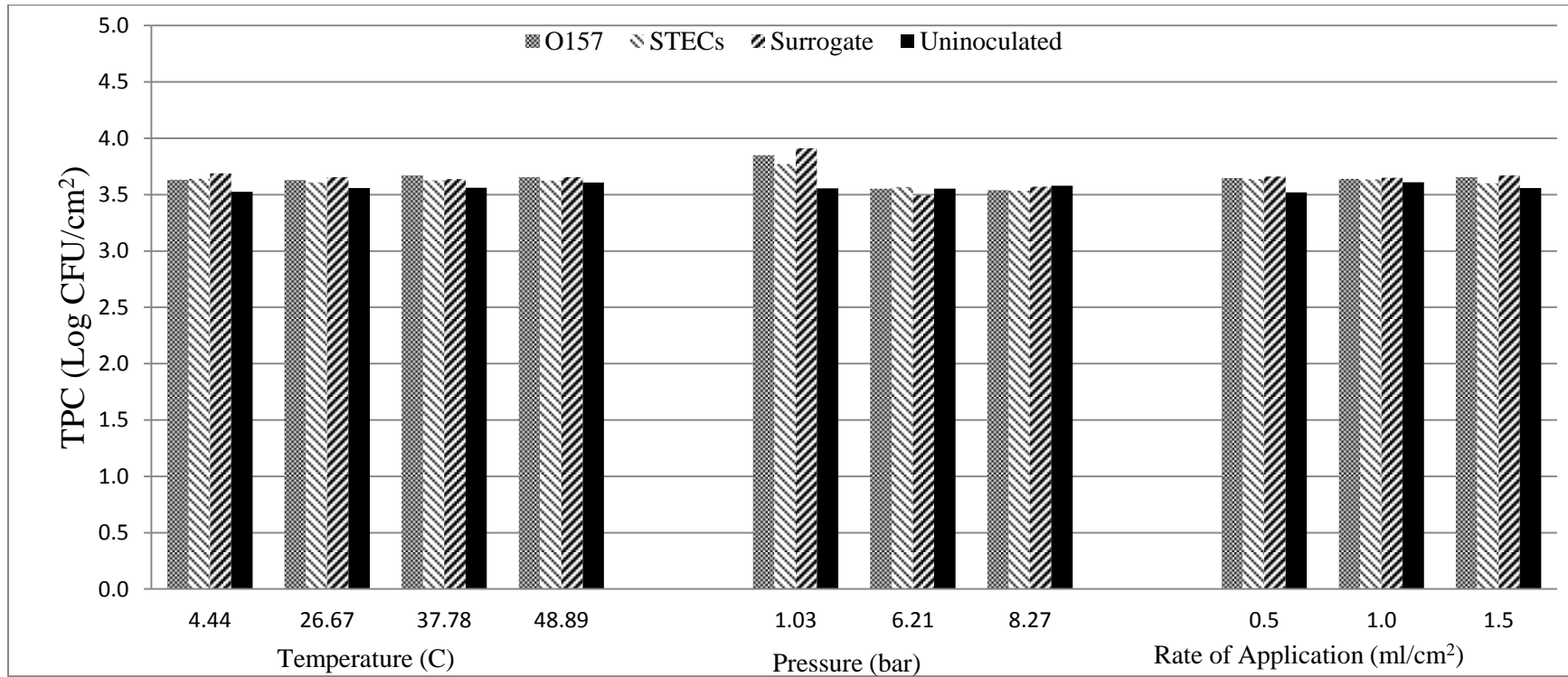
populations on hot beef carcass tissue.

Figure 6.1. Effect of application parameters on after treatment Total Plate Counts (log CFU/cm^2).

CHAPTER VII

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