CHARACTERIZING DIFFERENCES IN SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC) ATTACHMENT TO PRE-RIGOR AND CHILLED BEEF CARCASS SURFACES

by

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Abstract

The USDA declared seven STEC serotypes to be adulterants in raw, non-intact beef products due to their severe health implications. STEC contamination of carcasses is most likely to occur during hide removal. This study evaluated the efficiency of a mixed STEC-7 inoculum to attach to raw beef carcasses (predominantly lean muscle and adipose tissue), and compared the efficacy of 4.5% lactic acid (LA) to a water (W) spray to reduce STEC populations. Four carcass contamination scenarios, representing potential points whereby STEC could come into contact with raw beef surfaces during slaughter operations, were evaluated: (A) pre-rigor surface STEC inoculated (ca. 7 log cfu/cm^2), 30-min ambient temperature attachment, spray with LA or W; (B) pre-rigor inoculated, 24-h chilled attachment, spray; (C) tissue chilled 24 h, inoculated, 30-min attachment, spray; and (D) tissue chilled 24 h, rewarmed to 30°C, inoculated, 30-min attachment, spray. Predominantly lean muscle and adipose tissue were collected from four fed cattle immediately after harvest and assigned to the four scenarios for STEC inoculation, followed by a post-inoculation water (control) or LA spray. Tissue excision samples were collected pre- and post-treatment and analyzed to enumerate STEC-7 populations. Data were collected in a completely randomized design and analyzed using a mixed-model ANOVA. Pairwise comparisons of treatment means were made at $\alpha = 0.05$ with p-values adjusted using Tukey-Kramer. Initial STEC attachment levels to predominantly lean muscle and adipose tissues were not significantly different across all scenarios. Scenarios C and D showed greater STEC attachment compared to scenarios B and A. The LA spray reduced STEC levels more effectively than water across all scenarios. A significant treatment by tissue type interaction was observed for STEC reductions. A greater STEC reduction was observed for adipose tissue than for predominantly lean muscle when lactic acid spray was applied. A significant treatment by

scenario interaction was observed for STEC reductions. Scenarios A and B presented greater log reductions (1.77 ± 0.27 and 1.85 ± 0.25 log CFU/cm^2, respectively) than scenario C (1.04 ± 0.10 Log CFU/cm^2). LA spray presented the same level of effectiveness when applied to pre-rigor warm tissues and chilled tissues for reducing STEC. Greater post-LA spray reductions were observed when STECs were inoculated onto pre-rigor meat surfaces and submitted to a 24 h chill cycle, suggesting that cold storage temperatures (~2 °C) may stress or injure the STEC cells prior to subsequent antimicrobial spray applications to chilled surfaces. For laboratory studies, consideration must be given to when inocula are applied to tissue surfaces to accurately determine and/or compare the effectiveness of antimicrobial treatments. These findings provide insight to beef processors and researchers regarding inoculation protocols for comparative validation studies, and potential impacts on microbiological results from application of antimicrobial interventions at different points during raw beef processing.

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Dedication

I want to dedicate this thesis to my parents Iris and Inacio Schwan, who have always supported and encouraged me to pursue my dreams.

Chapter 1 - Introduction

The Centers for Disease Control and Prevention (CDC) has reported Shiga toxin-producing *Escherichia coli* (STEC) as major pathogens associated with beef products, mainly ground beef in the past 25 years in the U.S. Annually, more than 175,000 illnesses, 3,600 hospitalizations and 30 deaths are caused by STECs (CDC, 2015). *Escherichia coli* O157:H7 is estimated to cause 63,153 illnesses followed by 112,752 cases of non-O157 STEC strains (O26, O45, O103, O111, O121, O145) every year in the U.S. (CDC, 2015). However, the true number of foodborne illnesses caused by non-O157 STEC may be even higher because detection and isolation of those strains is time consuming and costly (Brooks et al., 2005). The annual healthcare expenses related to STECs foodborne illnesses are estimated to be $ 478 million (Economic Research Service - USDA, 2009).

It is well documented that the consumption of beef products contaminated with STEC have caused illnesses ranging from mild diarrhea to severe hemolytic uremic syndrome (Rivas et al., 2006). Ruminants, particularly cattle, are the major reservoir of STECs, and research has shown that these pathogens are spread to the carcass from the hide and feces during beef harvesting, thus resulting in the entry of these pathogens into the food chain (Small et al., 2005; s et al., 2003; Desmarchelier and Fegan, 2003; Barkocy-Gallagher et al., 2003; Elder, 2000; Smeltzer et al., 1980). In 1994, in response to a large foodborne illness outbreak that resulted in several deaths and numerous hospitalizations from the consumption of undercooked ground beef contaminated with *E. coli* O157:H7, the Food Safety and Inspection Service (FSIS) of the U. S. Department of Agriculture (USDA) declared *E. coli* O157:H7 to be an adulterant in raw, non-intact beef products (FSIS, 1999). Multiple foodborne illness outbreaks involving six non-O157

STECs (O26, O45, O103, O111, O121, O145) led the FSIS to later declare these strains also as adulterants in non-intact beef products (FSIS, 2012).

STECs pose a significant threat to consumers' health and can negatively impact the economy of the beef industry. Since attachment is one of the first steps that results in bacterial contamination and multiplication, the understanding of bacterial attachment mechanisms to different meat tissues is extremely important in the development of strategies to control and remove these pathogens from the meat surface during and after slaughter operation processes (Li and McLandsborough, 1999; Ofek and Doyle, 1994). Additionally, the use of antimicrobial interventions is beneficial and widely used by the beef industry as one of the control steps that assist in the development of mandated Hazard Analysis and Critical Control Points (HACCP) regulation by FSIS (CFR, 1996).

Because many studies have proven that the hide is the main source of STEC contamination of dressed (dehided) beef carcasses, and bacterial removal is easier before cells firmly attach to beef tissue surfaces (North American Meat Institute, 2015; Small et al., 2005; Nou et al., 2003; Barkocy-Gallagher et al., 2003; Elder, 2000; Smeltzer et al., 1980), numerous antimicrobial interventions have been used in the beef industry, and most of them are focused at the pre-rigor stage of slaughter operations. Physical and thermal interventions, acid and oxidizer antimicrobials, non-thermal interventions and multi-hurdle strategies are recognized as the most effective and promising mechanisms to reduce and control pathogens in the beef industry (Wheeler et al., 2014). Regardless of all the interventions cited above, the selection of the best intervention depends on several factors such as cost, effect on the food, and the legal limit of its use (Wheeler et al., 2014; Arthur et al., 2008; Small et al., 2005; Nou et al., 2003; Barkocy-Gallagher et al., 2003; Bell, 1997).

Acid interventions have been widely studied as antimicrobial agents against STECs. Lactic acid is the most common and widely used organic acid in the beef industry because of its effectiveness and low cost, when compared to other organic acids (Ransom et al., 2003). Several studies have demonstrated the efficacy of lactic acid in reducing STEC populations ranging from 1 to 4 log cycles, using either spray or immersion methods. The amount of reduction in the bacterial population using lactic acid depends on several factors: bacterial attachment/contact time to the meat surface, irregularities and characteristics of the meat surfaces (fat, uneven surface, small cuts), carcass temperature, moisture content of meat, and concentration, volume, temperature and exposure time of acid used (Dubal et al.,2004; Ransom et al.,2003; Ramirez et al.,2001).

Thus, this research was conducted to better understand the process of bacterial attachment to different types of beef tissues (predominantly lean muscle and adipose surfaces) when the STEC contamination occurs to pre-rigor (warm) and post-rigor (chilled) beef surfaces, and to study the effect of 4.5% lactic acid treatment on STECs population reduction on these different meat surfaces.

Chapter 2 - Research Questions

1. Are there any differences in the initial Shiga toxin-producing *Escherichia coli* (STEC) attachment levels to lean and fat beef tissues across all the pre-treatment samples for all scenarios when evaluated using the log CFU/cm^2 counts?

2. Are there any pre-treatment differences in attachment levels between pre-rigor, chilled or re-warmed surfaces (a scenario utilized by some researchers to represent pre-rigor beef conditions when conducting inoculated antimicrobial intervention validation studies in the laboratory), when evaluated using the log CFU/cm^2 counts?

3. Are there any differences in the recovery of STECs post-treatment across all scenarios when evaluated using log CFU/cm^2 reductions?

4. Do STECs attach differently when applied to pre-rigor warm tissue surfaces compared to chilled beef surfaces, considering beef tissues separately?

5. Does inoculating re-warmed post-rigor tissue after carcass chilling provide comparable levels of attachment to initially inoculated pre-rigor beef surfaces?

6. Does the application of water or 4.5% lactic acid provide effective and/or different STEC reductions on pre-rigor or chilled beef tissues?

Chapter 3 - Literature review

3.1. *Escherichia coli*

Escherichia coli are the most intensively studied microorganisms in the *Enterobacteriaceae* family, as well as in the overall bacterial community. Most strains of *E. coli* are harmless to human health, and some even are beneficial to their host by helping in absorption of nutrients and balancing the intestinal flora (Yang and Wang, 2014; Kaper et al., 2004). However, several strains of *E. coli* have adapted to survive various types of environments and conditions, and act as human and/or animal pathogens (Bari and Inatsu, 2014).

Strains of *E. coli* are classified as Gram negative, rod-shaped, non-sporeforming, peritrichous, motile and facultatively anaerobic organisms (Batt, 2014; Eden, 2014). Water activity (a_w), temperature and pH are the most important environmental factors that determine *E. coli*'s growth, survival and multiplication (Table 3-1; Astridge et al., 2013; ICMSF, 1996).

Table 3-1 Growth conditions for *Escherichia coli* when other factors are near to optimum*

Factors	Minimum	Optimum	Maximum
Temperature (°C)	7-8	35-42	45
pH	4	6-7	10
Water activity (a_w)	0.95	0.995	_

*Data obtained from ICMSF 1996; Astridge et al., 2013.

Escherichia coli pathotypes tend to be clonal and can be serologically differentiated based on the detection of specific antigens: O (somatic), H (flagellar) and K (capsule). While the 'O' antigen identifies the serogroup, the 'H' antigen identifies the serotype of an *E. coli* strain. In the process of strain identification, for instance during an outbreak, only the 'O' and 'H' antigens

are determined during the serotyping process. A total of 167 'O', 53 'H' and 74 'K' antigens have been identified and documented (Montville, Matthews, and Kniel, 2012; Kaper, Nataro, and Mobley, 2004).

The evolution of *E. coli* strains has resulted in the development of specific virulence factors, which allow the bacteria to easily adapt and survive adverse conditions, and cause a broad spectrum of diseases (Kaper et. al., 2004). Different virulence attributes, clinical syndromes, mechanisms of pathogenicity, and specific O:H serotypes are elements used to characterize pathogenic *E. coli* in specific categories (Montville et. al., 2012; Janda and Abbott 2006). Among the diarrheagenic *E. coli*, there are six well-described categories discussed (Yang and Wang, 2014; Montville et. al., 2012; Janda and Abbott, 2006; Kaper et. al., 2004) as follows:

1. **Enteropathogenic *Escherichia coli* (EPEC)**:

EPEC strains were first recognized in 1945 as the major source of infantile diarrhea in the United Kingdom; however, the high percentage rate of positive cases involving EPEC is still mostly reported among in-developing countries. Later on, researchers pointed to humans as important EPEC reservoirs. Typical infection symptoms are vomiting, watery diarrhea without gross blood, and fever. Currently, documented O serogroups linked with this infection type are O55, O86, O111ab, O119, O125ac, O126, O127, 128ab and O142. A typical characteristic associated with EPEC infections is an attaching and effacing (A/E) mechanism of adherence to the intestinal cells, which is one of the mechanisms responsible for intestinal inflammation and consequently diarrhea.

2. **Enterohemorrhagic *Escherichia coli* (EHEC)**:

Even though there are close to 400 serotypes of EHEC, not all of them have been identified or related to human illness. EHEC can be found in soil, water, food and gastro-

intestinal flora of humans and warm-blooded animals. The virulence factors of EHEC are known as Vtx (verotoxins), or sometimes Stx (Shiga-toxins), because of their similarity to toxins produced by *Shigella dysenteriae*. Because *E. coli* O157:H7 has been associated with numerous foodborne illness outbreaks, it is the most commonly recognized STEC and the most intensively studied. Additionally, non-O157 STECs have emerged as important and concerning foodborne pathogens. More than 200 virulent strains have been isolated from infected patients. The most common strains associated with human illness in the non-O157 STEC group are O26, O45, O103, O111, O121 and O145, also known as the "Big Six" STEC. Among the Big Six STEC group, the serogroups most frequently associated with foodborne illnesses are O111, O103 and O26.

Virulence factors vary among various STEC strains. Nevertheless, STEC strains produce either Shiga toxin 1 (Stx1) or Shiga toxin 2 (Stx2), or both. Shiga toxins cause illness by one of the following mechanisms: attacking the cells, interfering with protein synthesis, or attaching and entering the host cells, causing inflammatory reactions. Different levels of toxicity between Shiga toxins have been reported, with Stx2 having toxicity 1000 times greater than Stx1. Shiga toxins are produced in the colon and have the capacity to cause local damage, or even travel through the bloodstream to the kidneys, causing renal inflammation. In serious cases, this inflammation can lead to a life threatening condition called Hemolytic Uremic Syndrome (HUS), which results in acute kidney injury and failure (Figure 3-1).

STEC infections involve *E. coli* adherence to intestinal epithelial cells. The virulent *E. coli* attacks through the mechanism known as an 'attaching and effacing (A/E) lesion' on intestinal epithelial cells. Moreover, virulent STECs have a pathogenicity island known as the locus of enterocyte effacement (LEE), which provides proteins necessary for the formation of

A/E lesions. The locus of enterocyte effacement helps in the delivery of virulent factors into the host enterocytes, using a type III secretion apparatus (molecular syringe structure).

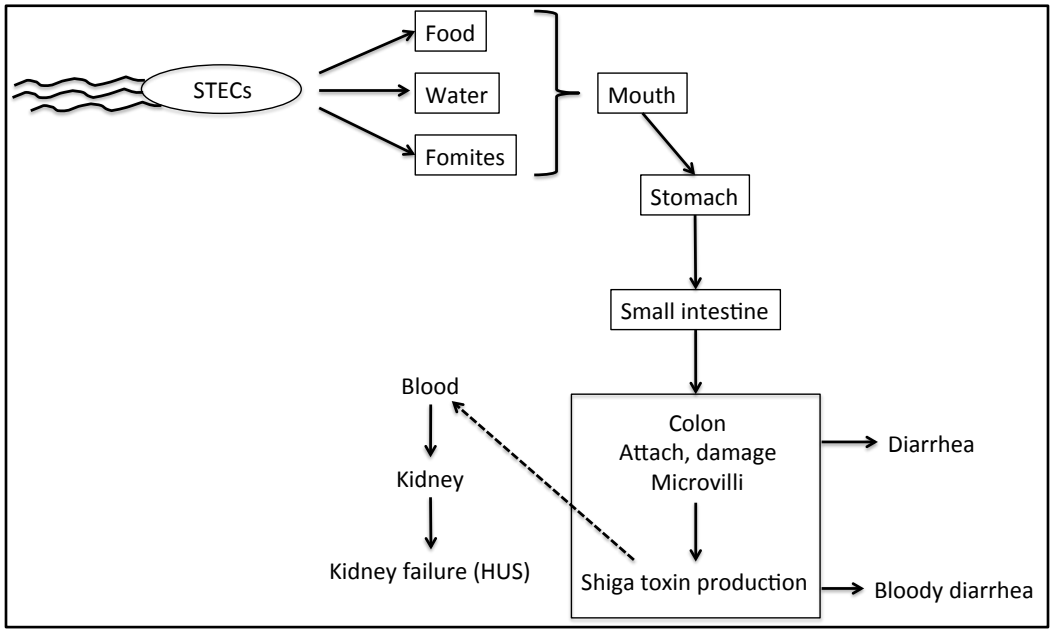

Figure 3-1 Brief overview of disease causing mechanism of STECs - Adapted from Bari & Inatsu, 2014

Shiga toxin-producing *E. coli* can be classified into the five main groups (A through E) based on their reported incidences of human disease, linkage with outbreaks versus sporadic infections, association with severe outcomes (such as HUS), and the presence of virulence factors (Duffy, 2014; Kaspar and Doyle, 2009):

A) Strains have high relative incidences, are commonly linked with outbreaks, and are associated with severe illness (such as HUS). Most common serotypes are O157:H7 and O157:NM.

B) Serotypes present moderate relative incidences, are uncommonly linked with outbreaks but can be associated with severe illness and HUS. Examples of this group are serotypes O26:H11, O103:H2/NM, O111:NM, O121:H19 and O145:NM.

C) Serotypes have low relative incidences, are rarely linked to outbreaks, but can be associated with severe illness and HUS. STECs involved in this group are O91:H21, O113:H21 and O104:H21.

D) Serotypes have low relative incidences, rarely associated with outbreaks, and there is no association with severe disease or HUS.

E) Serotypes are not associated with human disease.

3. Enterotoxigenic *Escherichia coli* (ETEC):

ETEC strains were first recognized in early 1970s in Calcutta, India. Even though the positive cases linked with ETEC have decreased, developing nations are still the major affected areas. Clinical symptoms of ETEC infections are often described as nausea, low fever, abdominal cramps and watery diarrhea. The natural reservoirs of ETEC are mostly humans, and infection is usually acquired by consumption of contaminated water or food. Common ETEC 'O' serogroups associated with illnesses are O6, O8, O15, O20, O25, O27, O63, O78, O85, O114, O128ac, O148, O159 and O167. Enterotoxigenic *E. coli* have the ability to colonize the small intestine utilizing fimbriae. Additionally, the production of heat-stable and heat-labile enterotoxins is closely related to the cause of infection, mainly diarrhea.

4. Enteroaggregative *Escherichia coli* (EAEC):

EAEC serotypes were initially associated with acute and severe diarrhea in children and adults globally. These EAEC serotypes were identified and differentiated from other diarrheagenic *E. coli* by their property of aggregative adherence to HEp-2 cells, which resembles a 'stacked-bricks' configuration. Humans are identified as the main reservoir of Enteroaggregative *E. coli*. Common symptoms of EAEC infections are mucoid and watery diarrhea along with low-grade to no fever. Typical 'O' serogroups linked with EAEC infections

are O3, O15, O44, O77, O86, O93, O111 and O127. Additionally to this group, in July of 2011, German authorities reported the biggest foodborne outbreak to date involving non-O157 STEC. *E. coli* O104:H4 sickened a total of 3,842 people and 53 died. The source of the outbreak was fenugreek sprouts imported from Egypt (CDC, 2011b; Astridge et al., 2013).

5. Enteroinvasive *Escherichia coli* (EIEC):

In 1971, DuPont and coworkers initially recognized and described EIEC as having the ability to invade and multiply within intestinal epithelial cells, resulting in cell damage and death. EIEC and *Shigella* spp. are closely related as they present similar biochemical, genetic and pathogenic characteristics. Clinical symptoms include malaise, severe abdominal cramps, bloody diarrhea and fever. As no animal host has been identified, humans are likely to be the natural reservoir of Enteroinvasive *E. coli.* Typical 'O' serogroups linked with EIEC infections are O28ac, O29, O112, O124, O136, O143, O144, O152, O164 and O167.

6. Diffusely Adherent *Escherichia coli* (DAEC):

Scaletsky et al. first recognized diffusely adherent *E. coli* in 1984, by their particular diffuse attachment pattern and characteristic mild, non-bloody diarrhea in children older than 12 months of age. The attachment process occurs through fimbrial or afimbrial adhesins and invasins. DAEC normally do not produce high levels of Shiga toxin, or heat-labile or heat-stable enterotoxins. The O1, O2, O21 and O75 serogroups are commonly associated with DAEC illnesses.

Additional information of diarrheagenic *E. coli* infections, including infective dose and duration of symptoms are presented in Table 3-2.

Table 3-2 Properties of *E. coli* groups associated with gastroenteritis*

E. coli group	Infectious dose	Incubation Period (h)	Mean duration of illness (days)	Presence of mucus or blood in stool	
				Mucus	Blood
EPEC	10^6 - 10^{10}	9-19	3-14	+	-
EHEC	50 - 700	24-336	6-9	-	+
ETEC	10^8 - 10^{10}	3-166	4-7	-	-
EAEC	~ 10^{10}	14-46	3 to >14	+	-
EIEC	≥10^8	<24	1-12	+	+
DAEC	unknown	unknown	≤14	-	-

*Adapted from Janda & Abbot (2006)

3.1.2. E. coli O157:H7

Escherichia coli O157:H7 received its name because it expresses the 157th somatic (O) antigen and the 7th flagellar (H) antigen. In 1982, after being linked with two outbreaks of hemorrhagic colitis, *E. coli* O157:H7 was recognized for the first time as a human pathogen (Riley, 1983). Clinical symptoms were reported as abdominal cramps, bloody diarrhea and low-grade fever. A year later, scientists confirmed that these outbreaks were associated with *E. coli* O157:H7 that produce Shiga-toxins and cause hemolytic uremic syndrome, a life-threating condition that leads to acute renal injury and potential death (Bari and Inatsu, 2014).

According to the CDC (2015), *E. coli* O157:H7 is estimated to cause 63,153 illnesses annually in the U.S. It is mainly transmitted to humans through consumption of contaminated food and water, as presented in Table 3-3. Contaminated food has been implicated in 75% of the outbreaks linked to *E. coli* O157:H7, resulting in a 20,660 illnesses, 90 deaths and 530 HUS confirmed cases over a period of 29 years (1982-2011). Out of 131 outbreaks, 43 implicated ground beef, roasted beef and its sub- products. Fruits and vegetables were responsible for 25 outbreaks and 19 were linked to milk products (Bari and Inatsu 2014).

Table 3-3 Outbreaks involving *E. coli* O157:H7 from the period of 1982-2011*

Mode of transmission	Outbreaks	Illness	Death
Foodborne	131(56%)	75%	90
Waterborne	52(22%)	18%	51
Animals and environment	26(11%)	3%	01
Person to person	23(10%)	3%	06

*Adapted from Bari & Inatsu, 2014.

The biggest outbreak involving *E. coli* O157:H7 was reported in 1996 in Sakai City, Japan. From July through August of that year, 9,578 cases and 11 deaths were reported. Even though radish seeds and uncooked sprouts were suspected to be the primary sources of the outbreak, authorities never positively confirmed the source (Batt, 2014; WHO, 1996).

The incident that drastically revolutionized the food safety concerns of the United States regarding *E. coli* O157:H7 contamination was the 'Jack in the Box' fast food restaurant outbreak in 1993. Around 700 people were sickened and 4 children died from *E. coli* O157:H7 infections, and many others suffered long-term medical complications. The outbreak was linked to undercooked beef patties served at Jack in the Box restaurants in Washington, Idaho, California and Nevada (Batt, 2014; Eden, 2014; Golan, et al., 2004). This outbreak thrust foodborne illness onto the national stage as a real and present threat, leading to a drastic change in the way Americans, the U.S. regulatory agencies, food manufacturers and restaurants treated food safety issues. As a consequence, in September of 1994, the Food Safety and Inspection Service of the U.S. Department of Agriculture (USDA-FSIS) declared *E. coli* O157:H7 to be an adulterant in raw non-intact beef products (FSIS, 1999). As continuing measures to improve food safety, all meat and poultry plants are required to develop and implement a preventative system (United States, per 9 CFR 417) known as Hazard Analysis Critical Control Points (HACCP). Through

different sampling programs, FSIS ensures the effectiveness of the HACCP system to prevent biological, chemical and physical hazards from entering the food chain. Now the HACCP system is used as one of the vital tools to prevent and control *E. coli* O157:H7 contamination in the beef industry. Establishments where *E. coli* O157:H7 is reasonably likely to occur must implement measures to control contamination during slaughter and processing steps. Those measures include interventions such as trimming, hot water washes and organic acid sprays.

3.1.3 Non-O157 STEC – "Big 6"

Recently, non-O157 Shiga toxin-producing *Escherichia coli* (non-O157 STEC) have brought more attention and concern to food safety authorities due to their high rate of involvement in foodborne outbreaks (Luna-Gierke et al., 2014). Serogroups O26, O45, O103 O111, O121 and O145 (Big 6 STECs) are the most common non-O157 STECs linked with foodborne illnesses (Wang et al., 2012). Data from the CDC (2012) indicate that 168,698 non-O157 STEC infections occur each year in the U.S., and approximately 71% of these infections are caused by the Big 6 serogroups. Like *E. coli* O157:H7, the non-O157 STECs can also be found in normal gastro-intestinal flora of warm-blooded animals, soil, water and contaminated food. Symptoms vary from watery diarrhea to HUS, depending on the type of strain and the virulence factors they carry (bacteriophages, plasmids, pathogenicity islands and O-islands; FSIS, 2012). In June of 2012, FSIS declared the Big 6 STEC group to be adulterants in non-intact raw beef products. Therefore, routine verification testing of non-intact beef is also required for *E. coli* O26, O45, O103 O111, O121, and O145 in addition to *E. coli* O157:H7, in non-intact beef products (FSIS, 2012).

In July of 2011, German authorities reported the biggest foodborne outbreak to date involving non-O157 STEC. A total of 3,842 people were sickened by *E. coli* O104:H4 and 53 people died. The source of the outbreak was fenugreek sprouts (CDC, 2011b; Astridge et al., 2013) from Egypt. This particular serogroup had never been associated with foodborne illness, and thus raised big concerns about the surfacing of non-O157 *E. coli* serogroups as foodborne pathogens and regulating non-O157 for different food products other than beef (Luna-Gierke et al., 2014; Kaspar and Doyle, 2009).

3.1.4 STEC Pathogens and Their Relation to Public Health

Foodborne diseases are a major concern of public health for government authorities and as well as for the consumers. According to the CDC (2014), 1 in 6 Americans get sick annually due to the consumption of contaminated food or beverages, resulting in 128,00 hospitalizations and 3,000 deaths. Historically, more than 250 different foodborne diseases have been documented. Bacteria, parasites and viruses are majorly responsible for the high numbers of foodborne diseases. Since many microorganisms have the ability to spread in different ways, sometimes it becomes difficult to identify the source of contamination, which could be a serious problem when authorities are trying to control outbreaks (CDC, 2014). The World Health Organization (2008) defines an outbreak as the scenario of two or more people acquiring the same illness from the same contaminated source. In 2014, CDC released a list of the most prevalent pathogens responsible for causing the most illnesses, hospitalizations and deaths every year in the U.S. The top five pathogens responsible for the highest number of hospitalization are nontyphoidal *Salmonella*, Norovirus, *Campylobacter* spp., *Toxoplasma gondii* and *E. coli* O157.

Escherichia coli, as a species, is one of the most intensively studied microorganisms (Jay et al., 2005; Eisenstein and Zaleznik, 2000). *Escherichia coli* is mostly found in the normal gastro-intestinal flora of humans and warm-blooded animals. Despite the fact that most strains are harmless to humans, there are six recognized groups of pathogenic *E. coli* that have the potential to cause severe illnesses and even deaths. Virulence factors and mechanisms of pathogenicity vary between each group, and many are host specific. Shiga toxin-producing *E. coli* (STEC) are classified under the Enterohemorrhagic (EHEC) group, and are one of the most investigated groups due to their high virulence and their linkage to many food outbreaks (Yang and Wang, 2014). Symptoms of STEC infections can range from asymptomatic to hemolytic uremic syndrome (HUS), and HUS is a severe life-threatening illness that occurs in about 10% of the *E. coli* O157:H7 cases, with a fatality rate of 4.6% (Gould et al., 2009). Usually, groups that have an immunocompromised system such as the elderly and children, are more susceptible and more likely to develop more serious symptoms (Astridge et al., 2013; Gould et al., 2009). Because of STECs' low infectious dose (10-100 cells depending on the strain) and severity of illness, regulatory bodies, academia and the food industry are devoting tremendous efforts researching new techniques and strategies to control and reduce foodborne illnesses related with STEC (Yang and Wang, 2014; Adams and Motarjemi, 2006).

According to the CDC, STECs are the major pathogens associated beef products, mainly undercooked ground beef. Cattle are the main carrier of *E. coli* strains, and historically, significant numbers of outbreaks have been associated with *E. coli* contaminated ground beef (Wheeler et. al., 2014; Scallan et al., 2011; CDC, 1993). Although beef products are the most common cause of illnesses related to STECs, many outbreaks also have been linked to a wide variety of food items. Hazelnuts, fresh produce, cheese, juice, yogurt, dried salami, raw milk,

mayonnaise and raw cookie dough are examples of food matrixes that have been involved in STEC related illnesses (Table 3-4; Johannes and Römer, 2010; Rangel et al., 2005; Cody, 1999; Friedman et al., 1999). Annually, more than 175,000 illnesses, 3,600 hospitalizations and 30 deaths are caused by STECs. *Escherichia coli* O157:H7 by itself is estimated to cause 63,153 illnesses followed by 112,752 cases of non-O157 STEC strains (O26, O45, O103, O111, O121, O145) every year in the U.S. (CDC, 2015). However, the true number of foodborne illnesses caused by non-O157 STEC may be even higher because detection and isolation of these strains is time consuming and costly (Hughes et al., 2006; Brooks et al., 2005). The annual healthcare expenses related to STEC foodborne illnesses are estimated to be $ 478 million (Economic Research Service - USDA, 2009).

Table 3-4 Major foodborne outbreaks linked with STEC (cases and/or fatalities)*

Year	Strain	Total no. Cases (fatalities)	Food Matrix	Country
2014	O121	19	Raw clover sprouts	US
2013	O121	35	Frozen food products	US
2012	O26	29	Raw clover sprouts	US
2012	O145	18(1)	Not identified	US
2011	O157:H7	60	Romain lettuce	US
2011	O1O4:H4	3842 (53)	Fenugreek sprouts	Germany
2010	O145	26	Shredded romain lettuce	US
2009	O157:H7	80	Cookie dough	US
2006	O157:H7	205 (3)	Spinach	US
2006	O157:H7	71	Taco Bell restaurant	US
1996 - 1997	O157:H7	490 (20)	Cooked meat products	Scotland
1996	O157:H7	7966 (3)	White radish sprouts	Japan
1995	$O111^{-}$	161(1)	Fermented mettwurst	Australia
1993	O157:H7	731 (4)	Hamburgers	US

*Data obtained from Astridge et al., 2013; CDC, 2014, 2013, 2012, 2011a, 2011b, 2010, 2006

3.2 STECs Contamination in Beef

Escherichia coli O157:H7 and the Big 6 STEC group have raised major concerns for the beef industry, health agencies and consumers. In spite of all the efforts made to control these foodborne pathogens, recalls are still happening and outbreaks are frequently identified, indicating that more attention is needed to address the issue of STEC-7 (*E. coli* O157:H7 plus the Big 6) contamination of food products (Sofos, 2008; Bacon and Sofos, 2005). The most significant beef recall in the U.S. history happened in 1997 when 25 million pounds of ground

beef were recalled with potential pathogenic *E. coli* contamination (FSIS, 1998). FSIS reported that approximately 33% of the total beef recalls were due to STECs contamination during the years of 2010 and 2014 (Table 3-5).

Table 3-5 Number of STEC related beef recalls from 2010 to 2014.

Year	no. of STEC related beef recall	no. of beef recalls	lbs of STEC related beef recall
2010	12	28	2,313,423
2011	13	35	1,002,971
2012	5	19	63,467
2013	7	20	89,919
2014	5	22	1,840,533
Total	42	124	5,310,313

*The total number of beef recalls from 2010 to 2014 was 38,224,001 lbs.; FSIS (2015c; 2015d; 2015e; 2015f; 2015g).

Because cattle are the major reservoir of STECs serotypes, there is a high potential for these microorganisms to be transferred to the carcass surface from hides and feces during the slaughter process (Liao et al., 2015). Beef products not only can become contaminated with STECs but also support their growth if not processed adequately, and subsequently handled and cooked properly, resulting in a serious public health problem (Wheeler, et. al., 2014). Several researchers have indicated that the hide is the primary source of carcass contamination (Small et al., 2005; Barkocy-Gallagher et al., 2003; Desmarchelier and Fegan, 2003; Nou et al., 2003; Elder, 2000). The three major factors that play a big role in the risk of potential carcass contamination are: 1) the contamination level of pathogens on the hide; 2) techniques used to minimize pathogen transfer from the hide to the carcass; and 3) efficacy of interventions applied during various beef processing steps (Wheeler et al., 2014; Barkocy-Gallagher et al., 2003). Various efforts used in the beef industry to control STEC contamination during beef processing include: animal cleaning and post-stunning hide decontamination, knife trimming and steam vacuuming of defined carcass areas, whole-carcass hot water and chemical intervention washes

or sprays, and effective carcass chilling. Usually, these preventative methods are applied in combination as an attempt to increase the meat safety (Wheeler et al., 2014; Sofos, 2008).

3.3 Bacterial Attachment

Understanding of bacterial attachment is critical to determine microbial persistence and ability to contaminate food products during all commercial food manufacturing processes (Kumar and Anand, 1998). Different bacterial species, serotypes or strains can demonstrate different responses to various environmental conditions and food matrix characteristics (eg. carcass surfaces). Types of substratum, availability of nutrients, moisture content and pH are some conditions that determine how bacteria will survive and thrive in different environmental circumstances. Two major bacterial distinctions define how bacteria attach to the carcass surface: the planktonic group (where bacteria live as individual free-floating organisms) and the sessile group (where bacteria attach to surfaces in a complex network structure). Sessile bacteria are responsible for biofilm formation (Frank, 2001). Beyond this classification, bacterial attachment is influenced by cell and substrata hydrophobicity, cell surface charge, bacterial surface structures (flagella and fimbriae), extracellular polysaccharides, and intermolecular forces (Dickson and Koohmaraie, 1989; Dahlback et al., 1981; Notermans and Kampelmacher, 1974).

Hydrophobicity is an important factor that influences bacterial attachment. In biological systems these interactions define the strength level of how apolar cells, molecules or particles interact among each other when in contact with water (Oliveira, 2001).

Cell surface charge plays an important role in bacterial adhesion. Pathogenic bacterial cells are usually negatively charged (Carpentier, 2014; Ofek and Doyle, 1994; Loosdrecht et al., 1987; Verwey and Overbeek, 1948). Furthermore, adhesive substrata on animal cells and tissues exhibit negative surface charges. For adhesion between bacteria and substrata to occur, an energy

barrier must be overcome. Since bacterial adhesion occurs in different steps, bacteria need to first overcome the repulsive forces that separate bacteria from the substrata. This barrier is overcome through hydrophobic interactions that effectively weaken the repulsive forces (Sylvester et al., 1996; Ofek and Doyle, 1994; Verwey and Overbeek, 1948; Derjaguin and Landau, 1941).

Different bacterial structures impact the efficiency of attachment to meat surfaces (Rivas, Dykes, and Fegan, 2006; Otto et. al., 1999; Dickson and Koohmaraie, 1989). Flagella and fimbriae, which are bacterial surface structures, have been reported to be important factors in the attachment process. However, non-motile bacterial strains can attach similarly to motile strains. Nevertheless, motile strains attach more quickly to surfaces than non-motile (Bouttier et al., 1997; Fratamico, 1996; Piette and Idziak, 1992; Dickson and Koohmaraie, 1989; Dahlback, et al., 1981; Notermans and Kampelmacher, 1974). The magnitude of influence of these adherence elements is related to the growth conditions, physiological state of the cell and specific bacterial strains (Rivas et. al., 2006; Dickson and Koohmaraie, 1989).

Another mechanism that explains bacterial attachment is the presence of the glycocalix structure. In wild environments, bacteria are covered by a network of polysaccharide fibers, called the glycocalix. This structure helps the bacteria to better attach to surfaces, and is involved in the formation of biofilm structures. When the environment has low availability of nutrients, the glycocalix is an important structure that facilitates bacterial attachment, and consequently, avoiding starvation. However, when there are abundant nutrients available in the environment, bacteria prioritize energy expenditure towards the multiplication process, instead of towards synthetizing the polysaccharides necessary for glycocalix formation. Therefore, the absence or a low quantity of glycocalix may lead to the loss of the bacteria's ability to attach (Rivas, Dykes

and Fegan, 2006; Cabedo, Sofos and Smith, 1996; Ofek and Doyle, 1994). For this reason, laboratory results might not be comparable to bacterial attachment in wild environments.

3.3.1 Bacterial Attachment to Beef Tissues

Many strains of Shiga toxin-producing *E. coli* have been associated with foodborne outbreaks. It is well documented that the consumption of beef products contaminated with STEC has caused illnesses ranging from mild diarrhea to fatal hemolytic uremic syndrome (Rivas, 2006). Since beef cattle are the major reservoir of STECs, there is a potential for meat surface contamination during the slaughter process (mainly during hide removal), resulting in the entry of these pathogens into the food chain (Desmarchelier and Fegan, 2003). A critical and vital step in food contamination is the ability of bacteria to attach to different food surfaces and their potential to multiply. A good comprehension of the mechanisms that are involved in bacterial attachment is needed to prevent and remove attached microorganisms (Cabedo, Sofos, and Smith 1996). Bacterial attachment to different food matrixes, such as beef, is still not well defined and further research is warranted. There are several methods available to measure bacterial attachment and each has specific advantages and disadvantages (Rosenberg and Kjelleberg, 1986).

Hermansson et al. (1982) and Pedersen et al. (1980) reported on common methods to measure bacterial attachment. Bacterial adherence to hydrocarbons (BATH), hydrophobic interaction chromatography (HIC), and contact angle are the methods used to measure relative hydrophobicity of bacterial cells. The cell surface charge of bacterial cells is measured by electrostatic interaction chromatography (ESIC). Later on, Ofek et al. (2003) described additional methods used to quantify attached bacteria: a) Enzyme-linked immunosorbent assay (ELISA); b) light microscopy; c) fluorescence (immunofluorescence microscopy, fluorescent

DNA and RNA probes, luminometer, image analysis); d) scanning electron microscopy; e) measurements of metabolites (CO_2, free radical production); f) radiolabeled bacteria; g) viable counts (CFU); h) growth assay; and i) biochemical measurements.

In 1978, Firstenberg-Eden defined the first model to measure bacterial attachment to chicken and beef muscle surfaces. Through establishment of the S value [$S = \log_{10}$ (firmly attached bacteria) – $\log_{10}$ (loosely associated bacteria), a measure of the relative strength of bacterial attachment], it was possible to measure the difference between firmly attached bacteria and loosely associated bacteria. Farber (1984) used the same model to measure psychrotrophic bacterial attachment on beef muscle. An increase in the S value indicates an increase in the numbers of firmly attached bacteria. From S value determination principles, it is possible to calculate the S_R value, which represents the percentage of the total population of bacteria firmly attached to the surface of beef tissues [S_R= (firmly attached bacteria)/(firmly attached bacteria + loosely associated bacteria)] (Kirsch et al., 2014; Rivas et. al., 2006; Dickson and Koohmaraie, 1989). Results presented by Dickson and Koohmaraie (1989) showed high variability in bacterial attachment depending on the method used to measure it (BATH, HIC, contact angle or ESIC), indicating lack of precision between methods. Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Listeria monocytogenes*) had higher S_R values of attachment on both lean and fat tissues when compared to gram-negative organisms (*E. coli* O157:H7, *Salmonella typhimurium*, and *Serratia marcescens*) after a 5-minute attachment period. In the same study, researchers also found that the net negative charge on the bacterial cell was the main contributing factor to attachment to lean tissues. Since the sarcolemma of a muscle fiber is a complex of protein, collagen, fibronectin and mucopolysaccharide, both negative and positive charges would be expected on the muscle surface. For this reason, both attractive and repulsive forces are

expected between bacteria and substrata. Results from this study established bacterial cell surface charge as an important factor in attachment to meat tissues (Dickson and Koohmaraie 1989).

According to Rivas et al. (2006) and Cabrera-Diaz et al. (2009), S_R values were not significantly different among STEC isolates, including *E. coli* O157:H7. These findings were different from those of Li and McLandsborough (1999), where *E. coli* O157:H7 had lower S_R values on beef muscle when compared to other serotypes. However, no correlation between S_R value and the concentration of attached bacteria was found by several researchers (Kirsch et al., 2014; Cabrera-Diaz et al., 2009; Rivas et al., 2006; Dykes et al., 2001; Benito et al., 1996, 1997; Notermans et al., 1980). Nonetheless, a high S_R value could indicate firmly attached bacteria, and thus, lead to the development of a bacterial community with biofilm formation (Benito et al., 1996).

Some studies used scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to analyze the bacterial attachment process. The number of *E. coli* attached to beef tissues was highly correlated ($R^2 = 0.85$ to 0.99) to the inoculum concentration level applied to the beef surface (Li and McLandsborough, 1999; Dickson, 1991; Firstenberg-Eden, 1981; Butler et al., 1979). However, no statistical differences were found between *E. coli* attachment to lean and fat tissues (Cabedo et al., 1996; Fratamico, 1996; Dickson and Frank, 1993; Chung et al., 1989). Also, there were no differences in total attachment levels (loosely + firmly attached cells) between strains of *E. coli* O157:H7 compared to *E. coli* K12 (Fratamico, 1996). Attachment was observed within 1 minute of incubation and there was a significant difference in attachment levels when different concentrations of inoculum (2, 3, 5, 7 and 9 log CFU/mL) were applied. The greatest attachment, for both *E. coli* O157:H7 and E. coli K12, was observed when

the highest inoculum concentration was applied. Additionally, bacterial attachment (total attachment = loosely + firmly attached cells) to meat was greater after 30 min compared to just 1 minute incubation period (Fratamico, 1996; Chung et al., 1989). The level of attached *E. coli*, when stored at 19 °C for 18 hours, was significantly higher than the level of attached bacteria following storage at 4 °C for the same period of time (Fratamico, 1996; Mattila and Frost, 1988).

Research has been conducted to study the role of aggregative surface appendages regarding the mechanisms of STEC attachment (Cookson et al., 2002; Prigent-Combaret et al., 2000, 2001; Warriner et al., 2001; Otto et al., 1999; Vidal et al., 1998; Fratamico, 1996). Under a transmission electron microscope, it is possible to observe that STECs can express a curli, wiry and thin protein fiber measuring approximately 4-12 nm (Chen et al., 2007; Chapman et al., 2002). Usually, non-optimal growth conditions such as starvation, lower temperatures, and low osmotic conditions induce STECs to produce the aggregative surface appendages (curli, fimbriae), which facilitate *E. coli* attachment to host matrix proteins on meat surfaces. Several authors reported statistically significant differences in STECs' attachment among strains that express the curli fimbriae and strains that did not (Chen et al., 2007; Gophna et al., 2001; Sjöbring et al., 1994; Arnqvist et al., 1992; Chung et al., 1989; Butler et al., 1979), while others found similarity in attachment comparing curli and noncurli-expressing variant strains (Rivas, et al., 2006; Warriner et al., 2001; Otto et al., 1999; Fratamico, 1996).

Several studies reported connective tissue fibers to be an important factor to establish bacterial attachment to meat surfaces (Kim and Slavik, 1994; Walls et al., 1993; Benedict et al., 1991; McMeekin et al., 1984). It has been described that the presence of specific binding sites on collagen (fibronectin and laminin), as well the existence of specific bacterial receptors for such matrix proteins, increases the attachment between bacteria and meat surfaces, thus confirming

the findings by Fratamico et al., (1996), Prachaiyo et al., (2000) and Firstenberg-Eden et al., (1978) where *E. coli* O157:H7 appeared to bind to collagen fibrils on beef tissues (Schulze-Koops et al., 1993; Schulze-Koops et al., 1992; Benedict et al., 1991; Speziale et al., 1986). Studies conducted by Chagnot et al., (2013) showed greatest attachment of *E. coli* O157:H7 to collagen I and III at 25 °C when compared to 7 and 4 °C. For lower temperatures (7 and 4 °C), the attachment was minimum. This could be explained by the production of fimbriae at higher incubation temperature. Zulfakar et al., (2012) during a study *in vitro* found greater STEC attachment to fibronectin, collagen IV and laminin at 37 °C when compared to 4 and 25 °C after a 120 min incubation period. However, STEC attached best to the extracellular matrix protein collagen I at 4 °C.

Cabedo et al., (1996) calculated the attachment strength (IIA) of *E. coli* O157:H7 to different meat tissues (beef muscle and adipose tissue) as IIA = log C – log [A-(B-C)], where A is cells loosely attached to the meat surface and cells found in water droplets on the meat surface, B is cells loosely and firmly attached to meat surface plus the cells found in water droplets on the meat surface, and C is cells loosely and firmly attached to the meat surface. There were no differences in bacterial attachment strength between beef muscle and adipose tissue even after 20 minutes of immersion in the inoculum. Additionally, meat tissues held at 4 °C for 3 hours did not present additional growth and their attachment strength did not change between muscle and adipose tissues. Dickson and Frank (1993) found similar attachment of *E. coli* O157:H7 to beef muscle and adipose tissues.

Warriner et al., (2001) evaluated bacterial attachment by analyzing loosely, firmly and irreversibly attached cells. Loosely attached cells were removed from the meat surface by a running stream water wash, while the firmly attached cells were removed from the surface by

stomaching the rinsed samples. The irreversibly attached bacteria were determined by stomaching the loin sections and subsequently subtracting the initial counts from the inoculated loin sections. Rinsed samples had a greater bacterial recovery for *E. coli*, *Listeria monocytogenes* and *Salmonella* Typhimurium when compared to samples that were stomached. This suggested that most bacteria were only loosely attached to the loin surface.

Kirsch et al., (2014) evaluated the effects of chilling and post-inoculation storage on beef briskets inoculated with non-O157 STEC and *E. coli* O157:H7. They reported that beef chilling status is an important factor in bacterial attachment. Greater STEC attachment was observed when briskets were exposed to chilling temperatures of 5 °C when compared to non-chilled temperatures of 25 °C. Recovery of STEC cells from briskets was greatest at time 0 minutes compared to 30, 60, 90 and 120 minutes post-inoculation. However, many researchers (Chen et al., 2007; Lillard, 1985; Notermans and Kampelmacher, 1974) reported a linear increase in bacterial attachment when evaluating chicken skin during post-inoculation incubation. Others reported no positive relationship between incubation contact time and number of attached bacteria (Benito et al., 1996; McMeekin et al., 1984). The measured S_R value was highly influenced by the incubation time, storage temperature and the chilling effect. The S_R value was significantly higher for briskets stored at 5 °C than at 25 °C. After 120 minutes of contact time, the bacterial attachment strength was the highest when compared to 0 min, but it was not significantly different from 30, 60 and 90 minutes of incubation. These results are in agreement with the results found by Fratamico et al., (1996) and Firstenberg-Eden et al., (1978), where an increase in bacterial attachment strength of *E. coli* O157:H7 and *E. coli* K-12 to meat tissues was observed. Findings from Cabedo et al., (1997) are in disagreement because there was no difference in attachment strength of *E. coli* O157:H7 over 3 hours of contact time.

Several researchers reported difficulty in comparing their results with results found in the literature because of a lack of commonality between experimental designs, organisms, methods of analysis, and meat tissues (Kirsch et al., 2014; Warriner et al., 2001; Fratamico, 1996; Piette and Idziak, 1992; Mattila and Frost, 1988; Loosdrecht et al., 1987; Notermans and Kampelmacher, 1983; Firstenberg-Eden, 1981). More research is needed to quantify STEC attachment characteristics, such as time needed for STEC to adhere to beef tissues, the role of flagella/fimbriae, influence of storage temperature, influence of tissue type (muscle/adipose), and laboratory inoculation preparation and inoculation procedures. All of these characteristics are important to access how STECs attach to beef tissues and in determining the effectiveness of antimicrobial interventions to enhance beef safety.

3.4 Typical Carcass Dressing and Processing Steps in Commercial Beef Processing and Use of Intervention Technologies to Control STECs

3.4.1 Process Flow in Commercial Beef Processing

According to the USDA-FSIS (2015b), "Slaughter is the process whereby healthy, live animals are humanely stunned, bled, dehided, dehaired and eviscerated." The slaughter process may vary from plant to plant; however, large plants have a mechanized and similar process, as shown in Figure 3-2 (FSIS, 2015b). The activities conducted by slaughter floor personnel at each step in the process provide an opportunity for contamination of the carcass surfaces and/or fabricated beef cuts. Following are the sequential steps involved in the slaughter process commonly used in the beef industry:

Cattle Receiving and Holding: Cattle are received in the plant and held in pens prior to the slaughter. The animals are allowed to drink water prior to the slaughter, but are kept off feed

to avoid potential contamination and facilitate dressing procedures. To identify any diseases in the animals, which could affect human health, an ante-mortem inspection is conducted prior to slaughter (FSIS, 2015b; Hui, 2012).

Stunning: Captive bolt, which is a mechanical method, is largely used by the beef industry to render the animals unconscious and minimize any discomfort during stunning. Moreover, this method complies with the Humane Slaughter Act (FSIS, 2015b; Hui, 2012).

Hide Opening in the Neck Area and Sticking: In this step, a sharp blade is inserted into the neck of the cattle, and the carotid artery and jugular vein are cut open, resulting in exsanguination and death. This is the first step where cross-contamination could occur. As the knife blade penetrates through the hide to the inside of intact muscles, there is a potential of introducing harmful bacteria, such as STECs (FSIS, 2015b; Hui, 2012).

Dehiding - Opening, Skinning and Hide Removal: This process can be achieved by various methods. It can be done by hand, usually in small operations, or using electrical equipment. This step is considered critical regarding cross-contamination from the hide. Pathogen reduction intervention treatments are very common and recommended by FSIS after hide removal. Hot water wash, organic acid wash or steam vacuuming are some interventions used to reduce bacterial contamination on the carcass surface immediately after hideremoval (FSIS, 2015b; Hui, 2012).

Head Removal: At this step the head is removed and inspected, and the carcass moves to the next step of the process. After inspection, heads are typically washed with water, hot water, and/or an antimicrobial solution, with head and cheek meat trimmed to include in ground beef manufacturing.

Evisceration: This step consists of separating the internal organs from the carcass. It is extremely important to perform the evisceration step properly in order to avoid fecal and fluids contamination from the intestines and stomach because harmful bacteria such as STECs, *Salmonella* and *Campylobacter jejuni* are harbored in these organs. Processors commonly use intervention treatments after evisceration. There are many options available as cited under the dehiding step above. After the organs are separated from the carcass, a post-mortem inspection is done on the viscera with the purpose of detecting potential diseases in the animal and determining parts or organs that may be unacceptable for human consumption (FSIS, 2015b; Hui, 2012).

Splitting: The carcass is split vertically down the spinal column with a large powered saw to yield two matching carcass sides.

Trim Rail: At this step an inspection is conducted to detect visible contamination (feces, milk, hair) and issues for the carcass quality. When the inspection is not satisfactory or the inspectors have concerns related to quality aspects, a trimming procedure is done to improve carcass quality (FSIS, 2015b; Hui, 2012).

Final Wash: The carcass is washed with hot water (150 to 180 °F) for ~5 seconds, and this allows additional removal of any remaining contaminants, which were not successfully removed during the trim rail step. This is a key step to apply a whole carcass antimicrobial intervention technology, such as organic acid sprays, to reduce the risk of pathogens remaining on carcasses. After the final wash and before the carcass enters the cooler, an USDA inspector evaluates the carcass for any contamination or quality aspects. If the carcass passes the final inspection, it receives an USDA-FSIS stamp (FSIS, 2015b; Hui, 2012).

Chill: Chilling the carcass is a necessary and important step during the carcass conversion process. Many factors play a role during the chilling step. Temperature, humidity and airflow are factors that need to be controlled in order to maintain the safety of the product and preserve its quality (FSIS, 2015b; Hui, 2012). The chilling process will also impact the development of rigor mortis. After the animal is slaughtered, muscles lose their supply of oxygen because the blood circulation system stops. Consequently, the muscle turns into an anaerobic glycolysis process because of the unavailability of oxygen in the system. From this point on, the energy used to contract the muscles is supplied from anaerobic glycolysis. Besides the energy production, this process also results in lactic acid formation, which causes a reduction in the muscle pH. Once glycogen supplies are consumed, the muscle contraction stops and the filaments (actin and myosin) are bound together, resulting in a permanent contraction called rigor mortis (Hui, 2012; Hannula and Puolanne 2004; Gigiel et al., 1989). Low chilling temperatures result in a slower rigor mortis development, while higher chilling temperatures results in a faster rigor mortis development. The post-mortem muscle has a pH of ~7.0, and pH declines to ~5.6 during the rigor mortis process. Once again, chilling conditions would also influence the time frame of the pH curve (7.0 to 5.6). Faster chilling processes result in a slower pH drop, while a slower chilling process will result in a faster pH drop (Hui, 2012; Savell, 2012; Hannula and Puolanne, 2004; Gigiel et al., 1989).

Carcass Fabrication – Primals or Trimmings: When the conversion of muscle to meat is completed, the carcass is fabricated; and primals and trimmings are the resultant meat cuts.

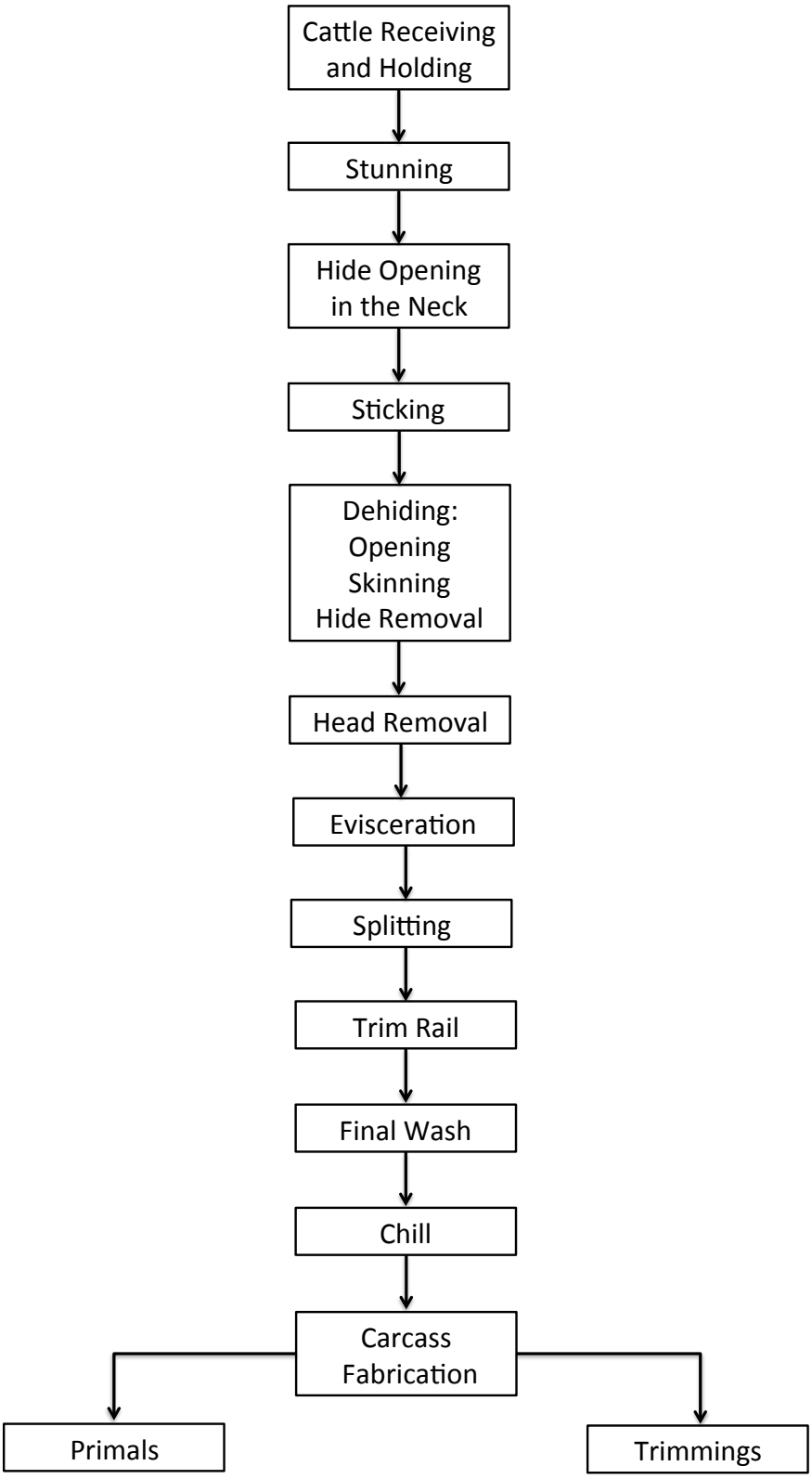

Figure 3-2 Beef slaughter and fabrication flowchart*

***FSIS, 2015b**

3.4.2 Intervention Treatments to Reduce/Control STECs

Disease outbreaks and recalls involving beef products cause regulatory authorities, the meat industry and consumers to focus on beef safety (Wheeler et al., 2014; Sofos, 2008). The most concerning group of bacteria for the beef industry is the STEC, including *E. coli* O157:H7 and the "Big 6" (Sofos, 2008; Koohmaraie et al., 2007). Antimicrobial interventions have been widely used to reduce contamination on raw beef, to minimize cross-contamination by inactivating microorganisms that have already entered the food chain, and to control/prevent the growth of microorganisms that contaminated the product but were not inactivated (Stopforth and Sofos, 2006; Juneja and Sofos, 2001; Sofos, 1994; Sofos 2005). Numerous interventions have been used in the beef industry, and most of them are focused on decontaminating carcasses at the pre-rigor stage (prior to chilling). Physical and thermal interventions, acid and oxidizer antimicrobials, non-thermal interventions and multi-hurdle strategies are recognized as the most effective and promising mechanisms to reduce and control pathogens in the beef industry (Wheeler, Kalchayanand and Bosilevac, 2014). FSIS has taken many actions to ensure meat safety, including: (a) implementation of sanitation standard operating procedures; (b) implementation and operation of HACCP programs; and (c) establishment of a sampling program for *E. coli* and *Salmonella* spp., as part of verification of the HACCP program (FSIS, 1996). The U.S. meat industry has improved its meat safety programs and has employed multi-hurdle strategies of decontamination interventions to provide safer beef products to consumers and to meet regulatory requirements (Sofos, 2005; Huffman, 2002; Sofos and Smith, 1998; Dorsa et al., 1997; Dickson and Anderson, 1992).

The effectiveness of antimicrobials has been studied in different food matrixes to control bacterial growth (Huffman, 2002). FSIS directive no. 7120.1 (USDA-FSIS, 2015a) describes the

use of antimicrobials in food and food products as follows: (a) type of antimicrobial (processing aids) added during processing, but it is removed or converted to normal food ingredients not leaving significant residuals; (b) secondary direct food additives, added during processing, but removed from the final product not leaving any technical effect from residuals; (c) direct food additives, added during processing providing technical effects into the final product. Antimicrobials used in the meat industry are allowed to be applied in fabricated meat products, however cannot exceed 0.5% in gained weight of the final product. The selection of the best intervention depends on several factors such as cost, effect on the food, and the legal limit of its use (Wheeler et al., 2014; Arthur et al., 2008; Small et al., 2005; Barkocy-Gallagher, et al., 2003; Nou et al., 2003; Bell, 1997). Most interventions are applied at the post-harvest level due to the hide being the primary source of carcass surface contamination (Wheeler et al., 2014; Small et al., 2005; Barkocy-Gallagher et al., 2003; Smeltzer et al., 1980).

Acid interventions have been widely studied as antimicrobial agents against STECs. Lactic, acetic and citric acids are widely used in the beef industry. Moreover, lactic acid is the most common organic acid that is being used due to its effectiveness and low cost (Ransom et al., 2003; Belk, 2001). Several studies have demonstrated the efficacy of lactic acid in reducing STEC populations ranging from 1 to 4 logs, using either spray or immersion methods (Schmidt et al., 2014; Kalchayanand et al., 2013, 2012; Geornaras et al., 2012; Ransom et al., 2003). The amount of reduction in bacterial population using lactic acid depends on several factors: bacterial attachment/contact time to the meat surface, irregularities and characteristics of the meat surfaces (fat, uneven surface, small cuts), carcass temperature, moisture content of meat, acid concentration, concentration of the undissociated molecule, pH, pKa, and temperature and

exposure time of acid used (Dubal et al., 2004; Ransom et al., 2003; Ramirez et al., 2001; Baird-Parker, 1980).

The mode of action of organic acids, such as lactic acid, is not completely understood, but many authors have described it as a combination of mechanisms between undissociated molecules and dissociated ions causing conflict with the trans-membrane proton gradient of the microbial cells, along with structures of the cell surface, outer membrane, and cytoplasmic membrane (Booth and Kroll, 1989; Corlett and Brown, 1980; Eklund, 1989). All of these changes can affect many vital processes of bacterial cells such as energy generation and nutrient transport, which will affect bacterial growth and multiplication. The low pH causes bacterial cellular damage leading to lethal injury of the cells (Wheeler et al., 2014). Currently, USDA-FSIS allows the use of lactic acid at a concentration level up to 5% in solution for livestock carcasses prior to fabrication (pre- and post-chill), offal, and variety meats. For beef and pork sub-primals and trimmings, a concentration from to 2 to 5% solution of lactic acid and not exceeding 55 °C is allowed (USDA-FSIS 2015a).

Ransom et al., (2003) evaluated different dip decontamination technologies (water at 25 °C, 10 ppm acidified chloride, 2% acetic acid, 2% lactic acid at 55 °C, 1% lactoferricin B, 5% peroxyacetic acid, 7% acidified sodium chloride, and 0.5% cetylpyridinium chloride) using different inoculation levels of *E. coli* O157:H7 on beef tissues. For both high (5 to 6 log CFU/cm^2) and low (3 to 4 log CFU/cm^2) inoculation levels of *E. coli,* a log reduction of up to 4 log CFU/cm^2 and 3 log CFU/cm^2 were observed for 0.5% cetylpyridinium chloride and 2% lactic acid at 55 °C, respectively. However, cetylpyridinium chloride is approved for use only on raw poultry carcass/parts, but not for meat products, while the use of lactic acid is allowed up to 5% in meat products (USDA-FSIS 2015a). Youssef et al. (2013) also evaluated the effect of 5%

lactic acid on high (4 log CFU/cm^2) and low (1 log CFU/cm^2) inoculation levels of *E. coli* isolated from commercially manufactured beef on membrane, fat and cut muscles surfaces. The greatest reduction (up to 4 log CFU/cm^2) was observed on high levels of inoculated membrane surfaces. A reduction of <1.2 log CFU/cm^2 was observed at low inoculation levels. Cut muscle surfaces presented similar reductions at both high and low levels of inoculation (>1 log CFU/cm^2 and ≤ 0.8 log CFU/cm^2, respectively). Similarly, Liao et al., (2015) evaluated the effectiveness of water at 21 °C, 5% lactic acid, 200 ppm hypobromous acid, and 200 ppm peroxyacetic acid on inoculated beef strip loin subprimals with high and low concentrations of *E. coli* O157:H7 and non-O157 STEC. Also, after the acid treatments, the effect of a vacuum storage (4 °C for 14 days) was evaluated. No significant reductions were observed among the acid-based treatments for either high or low STEC concentrations before the storage period. However, after the 14-day storage period, reductions of 2 and 1 log cycles of *E. coli* O157:H7 were observed among all the treatments for high and low concentrations of inoculum, respectively. Additionally, levels of non-O157:H7 STEC were reduced, in both high and low levels of inoculum, after the 14-day storage period when compared to the control. These results are in accordance to previous work by Cutter and Rivera-Betancourt (2000).

Wolf et al., (2012) tested the effectiveness 4.4% lactic acid dip and spray methods for reducing *E. coli* O157:H7, non-O157 STEC and *Salmonella* on beef trim and ground beef. Lactic acid dip was the most successful method and significantly reduced all three organisms in both beef trim and ground beef. Castillo et al., (2001) studied combinations of lactic acid concentrations (2, 4%), temperatures (55, 65 °C) and spray times (15 and 30 s) on inoculated (*E. coli* O157:H7) chilled beef surfaces. The combination of the three factors, which presented the greatest reduction (4.8 log CFU/cm^2) was 4% lactic acid solution at 55 °C sprayed for 30

seconds. This treatment, when combined with a pre-treatment water wash, was even more effective and resulted in a log reduction of 5.3 to 5.7 log cycles for both *E. coli* O157:H7 and *Salmonella* Typhimurium. These results are in agreement with several researchers that also tested a combined effect of a water wash followed by organic acid treatment on beef carcass surfaces (Bacon et al., 2002; Castillo et al., 1998, 2001; Dorsa, 1997; Dorsa et al., 1997; Gorman, 1995). Furthermore, Gill and Badoni (2004) observed a significant reduction (≥1.5 log CFU/cm^2) of *E. coli* on chilled meat surfaces when 4% lactic acid spray was applied and followed by chilling for 5 or 60 minutes at 7 °C. Zhao et al., (2014) observed similar results when 5% lactic acid spray was applied to a chilled beef surface at 4 °C. However, many researchers reported a non-significant bacterial reduction when ≤ 2% lactic acid was applied as a non-combined intervention (by itself) on cold beef surfaces (Gill and Landers, 2003; Bacon et al., 2002b ; Brackett, 1994; Acuff et al., 1987). This finding might be explained by the fact that temperature of the meat and the lactic acid are determinant factors that play a role on lactic acid effectiveness, which also was reported by several authors (Anderson and Marshall, 1989; Dickson and Anderson, 1992; Greer and Dilts, 1992).

Chapter 4 - Characterizing Differences in Shiga Toxin-Producing *Escherichia coli* (STEC) Attachment to Pre-Rigor and Chilled Beef Carcass Surfaces

4.1 Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are major pathogens associated with beef products, mainly ground beef, in the past 25 years in the U.S. Annually, more than 175,000 illnesses, 3,600 hospitalizations and 30 deaths are caused by STECs (CDC, 2015). *Escherichia coli* O157:H7 is estimated to cause 63,153 illnesses followed by 112,752 cases of non-O157 STEC strains (O26, O45, O103, O111, O121, O145) every year in the U.S. (CDC, 2015).

The consumption of beef products contaminated with STECs may cause illnesses ranging from mild diarrhea to severe hemolytic uremic syndrome (Rivas et al., 2006). Ruminants, particularly cattle, are the major reservoir of STECs, and research has shown that these pathogens are mainly spread to the carcass from the hide and feces during beef harvesting, posing a risk of their entry into the food chain (Small et al., 2005; Nou et al., 2003; Desmarchelier and Fegan, 2003; Barkocy-Gallagher et al., 2003; Elder, 2000; Smeltzer et al., 1980). In 1994, in response to a large foodborne illness outbreak that resulted in deaths of four children and numerous hospitalizations from the consumption of undercooked ground beef contaminated with *E. coli* O157:H7, the Food Safety and Inspection Service (FSIS) of the U. S. Department of Agriculture (USDA) declared *E. coli* O157:H7 to be an adulterant in raw, non-intact beef products (FSIS, 1999). Subsequent foodborne illness outbreaks involving six non-O157 STECs (O26, O45, O103, O111, O121, O145) led the FSIS to also declare these strains to be adulterants in non-intact beef products (FSIS, 2012).

STECs pose a significant threat to consumers' health and can negatively impact the economy of the beef industry. Since physical attachment is one of the first steps that results in bacterial contamination and multiplication, understanding bacterial attachment mechanisms to different meat tissue types is extremely important in the development of strategies to control and remove these pathogens from the meat surface during and after slaughter operations (Li and McLandsborough, 1999; Ofek and Doyle, 1994). Additionally, antimicrobial interventions are widely used by the beef industry as control steps in regulatory mandated Hazard Analysis and Critical Control Points (HACCP) programs (CFR, 1996). Their efficacy is partially dependent on the microbial population levels, and likely the strength of attachment, on the meat surfaces being treated.

Acid-based interventions have been widely studied as antimicrobial agents against STECs. Lactic acid is the most common and widely used organic acid in the beef industry because of its effectiveness and low cost, when compared to other organic acids (Ransom et al., 2003). Several studies have demonstrated the efficacy of lactic acid in reducing STEC populations ranging from 1 to 4 log cycles, using either spray or immersion methods. The magnitude of reduction in bacterial population using lactic acid depends on several factors: bacterial attachment/contact time to the meat surface, characteristics of the meat surfaces (fat, uneven surface, small cuts), carcass temperature, moisture content of meat, acid concentration, volume, temperature and exposure time (Dubal et al., 2004; Ransom et al., 2003; Ramirez et al., 2001). Therefore, this research was conducted to better understand the process of bacterial attachment to different types of tissues (predominantly lean muscle and adipose surfaces) when exposed to pre-rigor and chilled beef surfaces, and to study the effect of 4.5% lactic acid treatment on STEC population reduction on these meat surfaces.

4.2 Material and Methods

4.2.1 Overall Study Design

STEC attachment to different tissue types (predominantly lean muscle and adipose surfaces) and the effect of 4.5% lactic acid treatment on STEC population reduction on meat surfaces was studied. Pieces of beef carcass surfaces (representing predominantly lean muscle and adipose surfaces) were randomly assigned to four different carcass contamination scenarios (A, B, C and D; described in section 4.2.3) for STEC inoculation and treatment with either 4.5% lactic acid or water. Meat surface excision samples were collected after inoculation using a 30 min attachment period, and after each treatment to determine STEC attachment level achieved and log reductions after spray treatments. Four carcasses were utilized to conduct the experiment. Each piece of either predominantly lean muscle or adipose surfaces was inoculated and treated individually. The lowest order of each treatment combination of each carcass was considered as a replication (total 4 replications). Each scenario by tissue type was measured in duplicate to determine STEC attachment on each carcass. A fresh inoculum was used to inoculate the samples in each replication. Each scenario by tissue type by treatment was measured in duplicate to determine STEC log reductions for each carcass. STEC attachment and log reductions were analyzed assuming a completely randomized design.

4.2.2 Collection of Meat Samples from Carcasses

Two fed cattle (~400 kg of weight after dressing) were obtained locally from feedyards on two separate days (4 cattle total) and transported to the Kansas State University Biosecurity Research Institute where they were humanly slaughtered in compliance with USDA-FSIS approved procedures (FSIS, 2011). Warm, pre-rigor beef surface samples (representing scenarios

A and B described in section 4.2.3) were collected from the first beef carcass immediately after dehiding and carcass splitting (before any carcass washing). These carcass surface tissue sections were aseptically cut from both carcass sides using a 15.24 x 15.24 cm template (232 cm^2 surface area) at ~2.5 cm thickness. Sixteen samples were chosen from each carcass side to represent predominantly lean and sixteen to represent predominantly fat surface tissue (resulting in 32 samples of each tissue type from carcass 1). The samples were immediately placed into an insulated container after removal from the carcass and were transported to a walk-in incubator set at 42 °C where they were attached to 20 x 20 cm vinyl tiles (natural exterior tissue surface facing outward on the tile) using large binder clips. The 32 predominantly lean muscle or adipose tissue sections from carcass 1 were randomized within each group, and were assigned to lactic acid or water treatment. These samples were then transported to the microbiology laboratory for STEC inoculation (see section 4.2.5).

The second beef carcass was slaughtered and processed similarly to carcass 1, but was passed through a commercial hot water spray wash cabinet (~95 °C nozzle temperature for ~12 s at 50 psi) equipped with 22 oscillating spray nozzles on each side of the cabinet (Combination Carcass Wash, Hot Water Pasteurization and Chemical Spray Assembly, Chad Equipment LLC, Olathe, KS, USA). The average distance between nozzles and the beef carcass was 25 cm. After the hot water spray wash, the carcass was placed into a carcass chill cooler for 24 h; which utilized an initial 8 h of chilled water (2 °C) spraying for 1 min at 15 min intervals, followed by 16 h at 2 °C of dry chilling. After 24 h of chilled storage, 16 lean and 16 adipose surface tissues sections were collected from both carcass sides, as described for carcass 1. The 232 cm^2 chilled surface samples assigned to scenario C were inoculated chilled and treated (LA or water) using the same methodology as for scenarios A and B. Chilled surface samples assigned to scenario D

were re-warmed to ~30 °C in the 42 °C incubator and were treated using the same methodology as for scenarios A, B and C. The experiment was repeated using two additional fed cattle (carcasses 3 and 4) on a different day.

4.2.3 Carcass Contamination and Intervention Application Scenarios

Each beef surface tissue type (predominantly lean muscle and adipose surfaces) was assigned to one of the four scenarios (Figure 4-1):

(A) Warm (pre-rigor) beef surfaces inoculated shortly (45 min) after hide removal, and assigned to either the lactic acid or water control spray treatment.

(B) Warm (pre-rigor) beef surfaces inoculated shortly (45 min) after hide removal, chilled for 24 h at 2°C, and assigned to either lactic acid or water spray treatment.

(C) Beef surfaces chilled for 24 h at 2 °C, inoculated, and treated with either lactic acid or water sprays.

(D) Beef surfaces chilled for 24 h at 2 °C, re-warmed to ~30°C (simulating a pre-rigor carcass temperature) in an incubator, inoculated, and treated with either lactic acid or water.

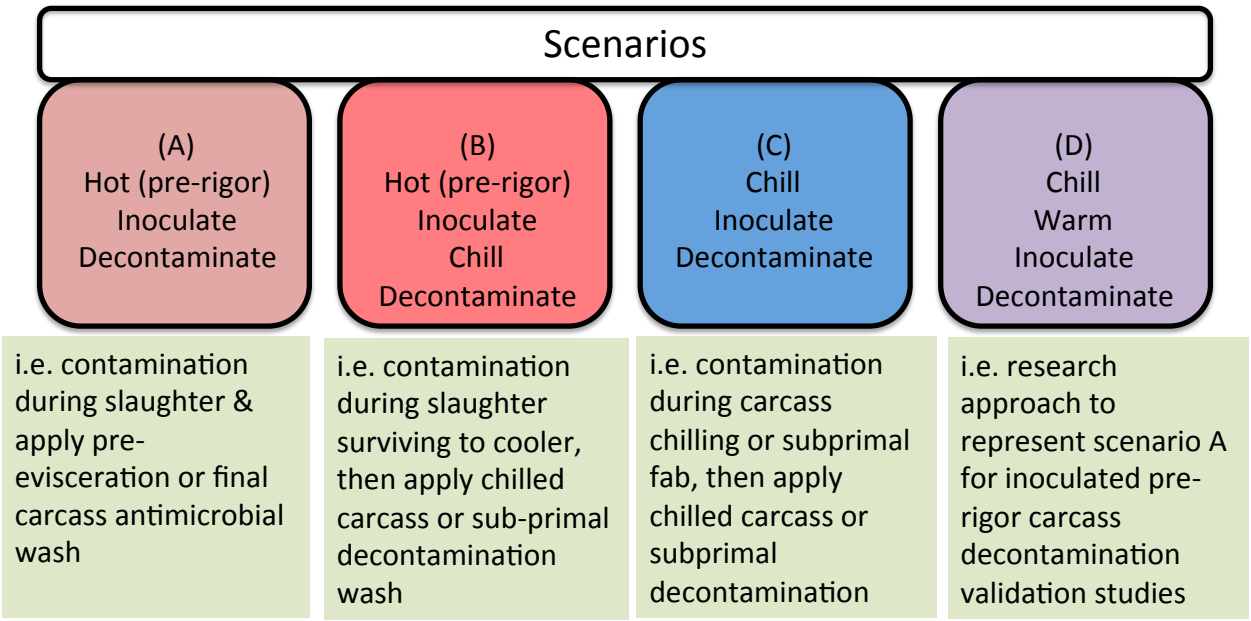

Figure 4-1 STEC contamination and antimicrobial treatment scenarios representing commercial beef industry processing.

4.2.4 Inoculum Preparation

Seven STEC serotypes, [O26 (H30, human isolate), O45 (CDC 96-3285, human isolate), O103 (CDC 90-3128, human isolate), O111 (JB1095, human isolate), O121 (CDC 97-3068, human isolate), O145 (83-75 human isolate) and O157 (ATCC 35150)] were streaked individually onto tryptic soy agar (TSA) plates (Difco, Becton Dickinson, Sparks, NJ, USA) and incubated at 35 °C for 24 h. After 24 h of incubation, one colony of each strain was inoculated into seven 10 mL tryptic soy broth (TSB; Bacto, Becton Dickinson, Sparks, NJ, USA) tubes and incubated at 35°C for 24 h. After 24 h of incubation, 5 mL of each TSB broth culture were combined into a single 50 mL conical tube, resulting in a concentrated STEC-7 cocktail (35 mL at ~10^8 CFU/mL). Individual strains and cocktail were plated on Petrifilm *E. coli*/coliform (ECC; 3M Corporation, Saint Paul, MN, USA) and incubated for 24 h at 35°C to confirm relative strain ratios in the cocktail and to confirm overall cocktail STEC-7 concentration. For master inoculum preparation, 25 mL of concentrated cocktail were diluted in 225 mL of 0.1%

peptone water in a sterile plastic bottle to achieve a STEC-7 master inoculum with ~10^7 CFU/mL concentration. Inoculum concentrates were stored at room temperature and used within 30 min. A new inoculum was prepared for carcasses 1 and 2, and for carcasses 3 and 4, since the chilled tissue inoculations (scenarios C and D) occurred the day following slaughter.

4.2.5 Meat Inoculation

Meat surface temperature was measured with an infrared type K thermometer (Fisher Scientific, PA, USA) immediately prior to inoculation. Meat sections were inoculated by pipetting 1 mL of STEC-7 master inoculum across the 232 cm^2 surface area and evenly distributing the inoculation fluid using a L-shaped spreader (Figure 4-2). Inoculated meat surfaces were left undisturbed for 30 min at room temperature to allow bacterial attachment, prior to the application of lactic acid or water sprays. STEC attachment was measured as the initial cell count (log CFU/cm^2) after the 30 min attachment period.

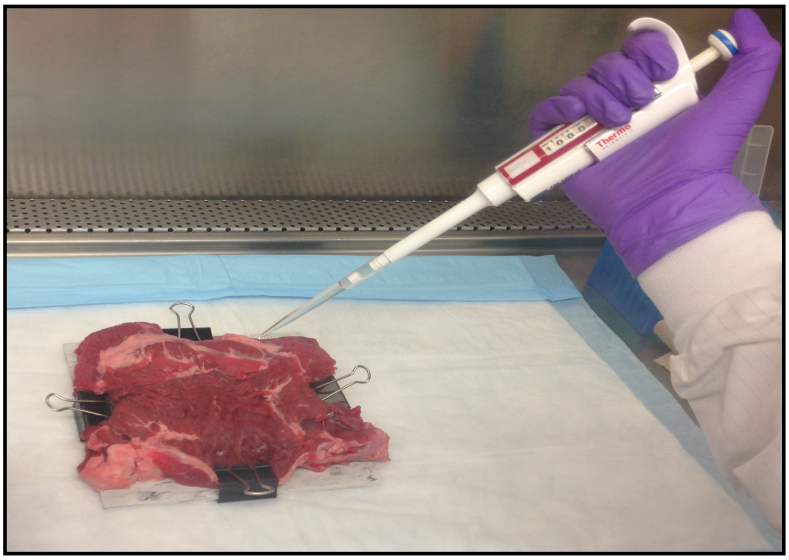

Figure 4-2 Meat surface inoculation

4.2.6 Lactic Acid Solution Preparation

Lactic acid solution (4.5%) was prepared by mixing 49.14 mL of concentrated lactic acid (88%; F.G^{TM}, Birko, CO, USA) with 1000 mL of deionized water. The concentration of lactic acid solution was confirmed by measuring the titratable acidity of the solution as described by Nielsen (2003). A sample (9 mL) was pipetted into a 100 mL titration flask and the pipette was rinsed with ~18 mL deionized water, and then titrated against 0.1 N sodium hydroxide (NaOH; Acros Organics, NJ, USA) using 0.5 mL phenolphthalein (Fisher Scientific, Pittsburgh, PA, USA) as an indicator. Titratable acidity was calculated using the following formula:

TA (% lactic acid) = mL of 0.1 N NaOH used × 0.1

4.2.7 Meat Surface Treatment

After the 30 min inoculum attachment period, the meat pH was measured (Exstil waterproof pH meter, Extech Instruments Corporation, NH, USA) using a surface contact probe before and after lactic acid or water treatment. For treatment of each meat surface, 15 mL of 4.5% lactic acid or water, which both were at room temperature, was sprayed onto the meat surfaces using a volume-calibrated hand-spray bottle (Figure 4-3), followed by a 3 min drip period before tissue excision samples were collected to enumerate residual STEC population levels. Two meat surface excision samples (9.1 cm^2 each) were collected using a sterile coring device prior to and after spray treatments (Figure 4-4). The two excised cores representing each sampling point were combined into a sterile filtered stomacher bag (Fisher Brand, Fisher Scientific, Pittsburgh, PA, USA) containing 75 mL of 0.1% of peptone water, stomached (Stomacher 400 Lab Blender, Seward Laboratory Systems Inc, FL, USA) for 60 s at 230 rpm, and plated on Petrifilm *E. coli*/coliform to determine the log reductions for STEC-7 after treatments.

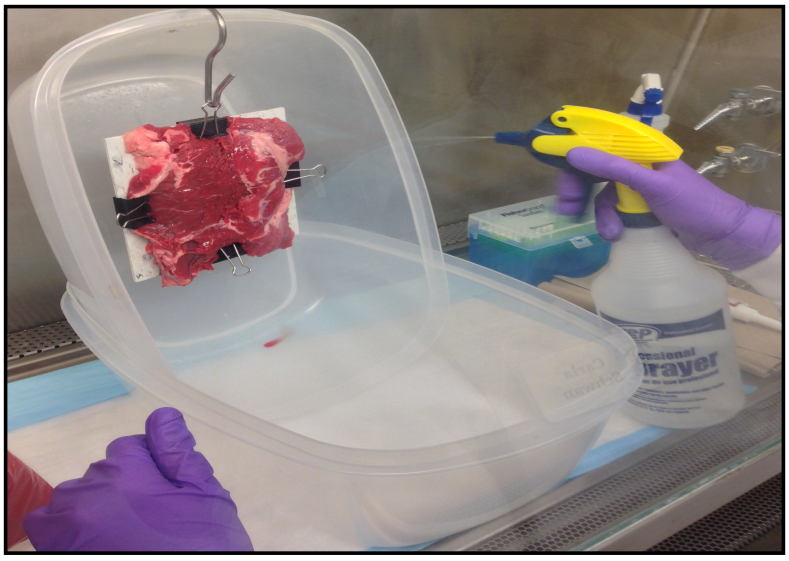

Figure 4-3 Application of treatments (4.5% lactic acid or water control) onto meat surfaces

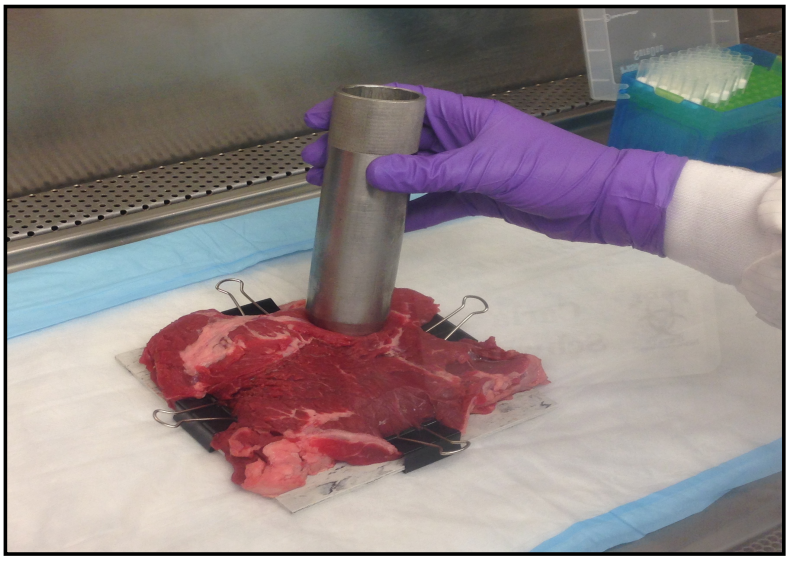

Figure 4-4 Meat surface excision for microbiological analysis

4.2.8 Experimental Design and Statistical Analysis

Bacterial attachment under the 4 STEC contamination scenarios (A-D) and log reductions resulting from 4.5% lactic acid or water sprays were analyzed assuming a completely randomized design. Bacterial attachment had a two-way factorial treatment structure with treatment factors with scenarios (A, B, C and D) and tissue type (predominantly lean muscle and adipose tissue), and each treatment combination was replicated four times. Log reductions had a three-way factorial treatment structure with four replicates per treatment combination, where treatment factors were tissue type, treatment (lactic acid or water) and scenarios. Both analyses were performed using the MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC). Residual homogeneity and normality were assessed graphically. The highest order interaction was evaluated first; if not significant, lower order interactions were evaluated subsequently. Main effects for treatment factors not involved in significant interactions were evaluated separately. The liner model for STEC-7 attachment contained main effects for scenarios and tissue type, and the two-way interaction. The model for log reduction included the main effects for scenarios, tissue type and treatment, as well as two- and three-way interactions. Least-squares means were calculated at the level at the highest order significant effects, and pairwise comparisons of treatment levels were performed using the Tukey-Kramer adjustment for multiple comparisons. Adjusted P-values were assessed at $\alpha = 0.05$.

4.3 Results and Discussion

4.3.1 Initial STEC-7 Cell Count (Attachment)

Initial STEC-7 cell concentrations in the master inoculum were 7.05 ± 0.21 log CFU/mL. The mean pH and temperatures (average for predominantly lean muscle and adipose tissue) of different scenarios at the time of inoculation are presented in Table 4-1.

Table 4-1 Surface tissue pH and temperature of different scenarios, averaged across predominantly lean muscle and adipose tissue.

	Scenarios			
	A	B	C	D
pH	5.66 ± 0.15	6.29 ± 0.19	6.06 ± 0.19	5.72 ± 0.05
Temperature (°C)	29.85 ± 0.96	34.53 ± 0.76	8.44 ± 0.48	30.51 ± 0.83

The interaction between inoculation scenarios and tissue type, and tissue type main effect were not significant (P>0.05; Table 4-2). Since the scenarios main effect was the only one significant, scenarios means were calculated by averaging over tissue type STEC-7 levels.

Table 4-2 ANOVA table of attachment

Source	P-value
Scenarios	<0.0001
Tissue type	0.1014
Scenarios*Tissue type	0.347

Initial STEC-7 cell counts between predominantly lean muscle (5.52±0.06 log CFU/cm^2) and adipose tissue (5.38±0.06 log CFU/cm^2) were not significantly different (P>0.05). There was no significant interaction between tissue type and scenario (P=0.34). However, the mean concentration of initial counts of STEC-7 cells on tissue type after 30 min attachment period was significantly different between scenarios (P≤0.05). The highest initial STEC counts were observed in scenarios C and D (5.85±0.05 and 5.55±0.07 log CFU/cm^2, respectively), which were simulating chilled stored beef surfaces (~8 °C) and rewarmed beef surfaces (~30 °C), respectively (Figure 4-5). However, scenarios A and D did not differ in initial STEC-7 cell counts (P=0.17). The lowest initial STEC-7 counts were observed for scenarios A and B (5.32±0.10 and 5.09±0.07 log CFU/cm^2, respectively), which were simulating contamination of pre-rigor beef carcass surfaces (~31 °C). Therefore, these results suggest that there might be

differences in the contamination levels depending upon the pre-rigor (warm) and post-rigor (chilled) surfaces, and the storage temperature.

Several studies reported connective tissue to be an important factor to establish bacterial attachment to meat surfaces (Kim and Slavik, 1994; Walls et al., 1993; Benedict et al., 1991; McMeekin et al., 1984). It has been described that the presence of specific binding sites on collagen (fibronectin and laminin), as well the existence of specific bacterial receptors for such matrix proteins, increases the attachment between bacteria and meat surfaces, thus confirming findings where *E. coli* O157:H7 appeared to bind to collagen fibrils on lean tissues (Schulze-Koops et al., 1993; Schulze-Koops et al., 1992; Benedict et al., 1991; Speziale et al., 1986). However, the current study did not evaluate STEC-7 attachment based on the collagen level or type associated with the meat tissues. STEC-7 attachment was measured in predominantly lean and adipose surfaces.

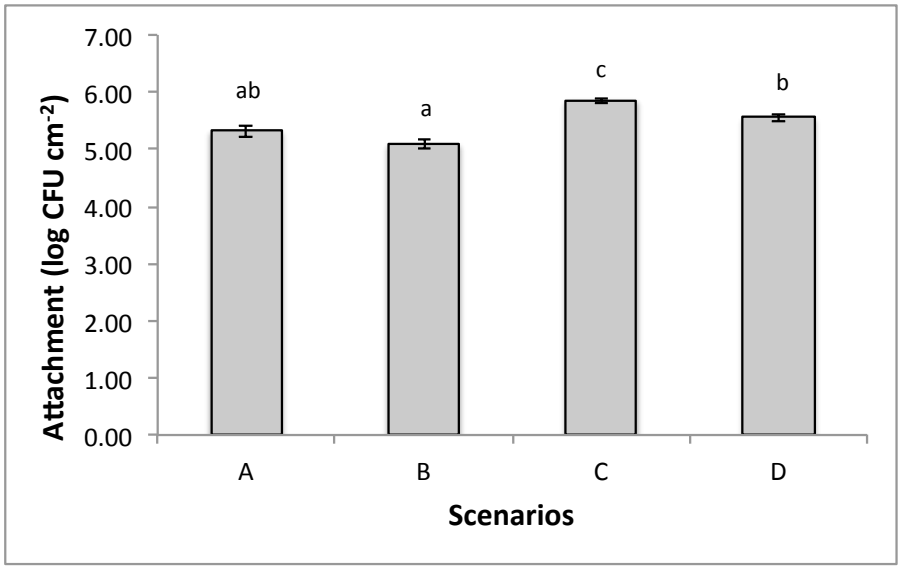

Figure 4-5 Shiga toxin-producing *Escherichia coli* attachment to 15x15 cm beef surfaces for different scenarios. a-c: microbial counts with different letters were significantly different ($P \leq 0.05$).

In agreement to results found in the current study, Cabedo et al., (1997) found similar *E. coli* O157:H7 attachment to predominantly lean muscle (5.48±0.09 log CFU/cm^2) and adipose tissue (5.31±0.08 log CFU/cm^2). Moreover, several researchers reported no differences in bacterial attachment between lean muscle and adipose tissue (Cabedo et al., 1997; Cabedo et al., 1996; Fratamico and Schultz, 1996; Dickson and Frank, 1993; Chung et al.,1989). Similarly, various studies have shown that other bacterial species such as *Salmonella arizonae*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Listeria monocytogenes, Serratia marcescens* and *Staphylococcus aureus* also did not differ in their attachment to lean muscle and adipose tissue (Benedict et al., 1991; Chung et al., 1989; Dickson and Macneil, 1991). However, some authors reported greater attachment levels on adipose tissue than lean muscle when evaluating STEC strongly attached cells, but there was no difference within loosely attached cells for both lean muscle and adipose tissue (Rivas et al., 2006; Foong and Dickson, 2004; Chung et al., 1989; Dickson and Koohmaraie, 1989). In the current study loosely and firmly attached cells were evaluated together as the total bacterial attachment.

Kirsch et al., (2014) reported that the beef chilling process is an important factor in bacterial attachment. They reported that STEC attachment was greater when beef surfaces were exposed to chilling temperatures of 5 °C (4.0 log CFU/cm^2) when compared to a non-chilled temperature of 25 °C (3.6 log CFU/cm^2). These results are in accordance with results found in our study, where the attachment was greater in post-chilled beef surfaces (~8 °C; scenario C) than to pre-rigor (~30 °C; scenario A) beef surfaces. However, Fratamico and Schultz (1996) found that a higher temperature (~19 °C) allowed better bacterial attachment when compared to a lower temperature (4 °C), which is in agreement to results found by Prendergast et al., (2007).

Reasons for such differences could include different experimental designs, STEC serotypes, methodologies used to inoculate beef surfaces, attachment period and variety of tissue types.

No significant difference in STEC attachment was found between predominantly lean muscle and adipose tissue in this study. Dickson and Koohmaraie (1989) reported a similar linear correlation between the negative charge on various bacterial cell surfaces (*Bacillus subtilis*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* Typhimurium, *Serratia marcescens*, *Staphylococcus aureus* and *Staphylococcus epidermidis*) and initial attachment to lean muscle and adipose tissue. However, Kim et al. (1996) did not find any correlation between surface charge on bacterial cell surfaces and initial bacterial attachment of *Salmonella* spp. to meat surfaces.

The bacterial attachment period has been reported to be an important factor to determine the final number of attached bacterial cells. In our study, STEC-7 recovery of attached cells was similar to attachment levels found by Kirsch et al., (2014). The 30 min (scenarios A, C and D) and 24 h attachment periods (scenario B) were selected to represent the actual time that a carcass could be exposed to bacterial contamination before entering the cooler (under typical commercial slaughter floor operations) and the approximate time that a carcass would be in the cooler before the fabrication process, respectively. Various studies have reported higher STEC attachment levels after a 30 min period when compared to 1 or 2 min (Fratamico and Schultz, 1996; Chen et. al, 2007). Kirsch et al., (2014) reported that although the initial attachment levels of STEC cells on beef briskets were higher at 0 min (4.2 log CFU/cm^2) compared to 30, 60, 90 and 120 min ($\geq$3.8 log CFU/cm^2) contact periods, the attachment strength of cells was greater at 60 min or greater contact time compared to 0 min. Differences in numbers of attached bacterial cells in various studies discussed above might be attributed to the elapsed attachment period.

4.3.2 STEC-7 Log Reductions After Antimicrobial Spray

The interactions between scenarios, tissue type and treatments, and scenarios and tissue type were not significant (Table 4-3). Since the tissue type (P=0.0652) and scenarios (P=0.0815) main effects were not significant, means were calculated averaging across tissue type and scenarios.

Table 4-3 ANOVA table for log reductions after lactic acid or water treatment.

Source	P-value
Scenarios	0.0815
Tissue type	0.0652
Scenarios*Tissue type	0.4745
Treatments	<0.0001
Scenarios*Treatments	0.0045
Tissue type*Treatments	0.0245
Scenarios*Tissue type*Treatments	0.7512

The STEC-7 log reduction achieved by spraying inoculated tissue sections with a 4.5% lactic acid solution or water after a 30 min attachment period was significantly affected by treatments ($P \leq 0.05$). The mean log reductions after lactic acid treatment ranged from 1.04 to 1.85 log CFU/cm^2, compared to -0.05 to 0.37 log CFU/cm^2 reductions for water treatment (Figure 4-6). Lactic acid spray was more effective than water for both predominantly lean muscle and adipose tissue. However, a greater log reduction was observed on adipose tissue (1.75±0.13 log CFU/cm^2) compared to predominantly lean muscle (1.29±0.15 log CFU/cm^2) when lactic acid treatment was applied (Figure 4-6).

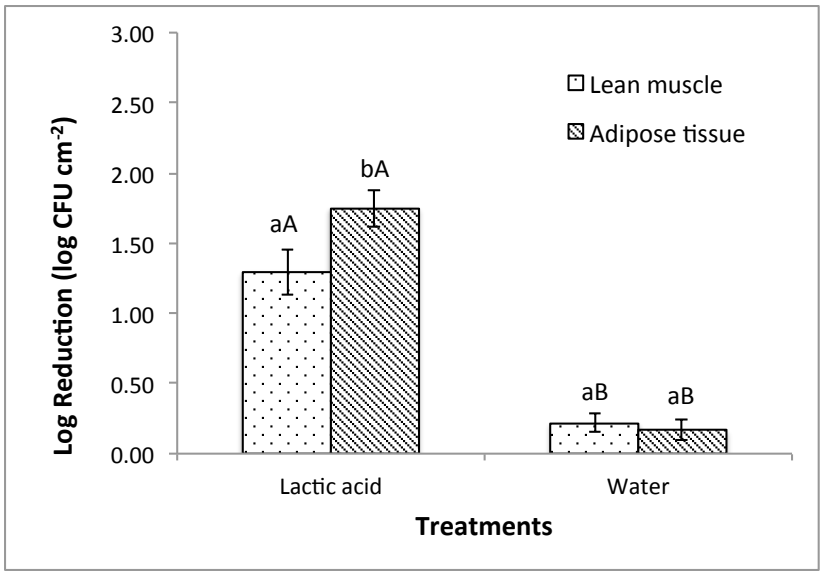

Figure 4-6 Shiga toxin-producing *Escherichia coli* log reductions on 15x15 cm beef surfaces under different treatments. a-b: log reductions within respective treatment with different letters were significantly different ($P \leq 0.05$). A-B: log reductions within respective tissue type with different letter were significantly different ($P \leq 0.05$).

Lactic acid spray reduced STEC-7 levels more effectively than water across all scenarios. A significant treatment by scenario interaction was observed for STEC-7 reductions when lactic acid was applied (Table 4-3). Scenarios A and B presented greater log reductions compared to scenario C (Figure 4-7). The mechanism of antimicrobial activity of lactic acid, which provided greater STEC-7 reductions, is reported to be by penetration into bacterial cells and interfering with energy generation and nutrient transport across the bacterial cell, and ultimately inactivating the cells (Wheeler et al., 2014; Booth and Kroll, 1989).

Lactic acid spray presented the same level of effectiveness when applied to pre-rigor carcasses and chilled beef surfaces for reducing STEC-7 (Figure 4-7). Greater reductions were observed on chilled meat surfaces when STECs were inoculated and subjected to a 24 h chill cycle, thus, indicating that cold temperatures (~2 °C) have an impact on stressing and injuring the STEC cells prior to antimicrobial exposure. No significant difference in log reductions was observed for STEC-7 when water was applied ($P>0.05$; Figure 4-7).

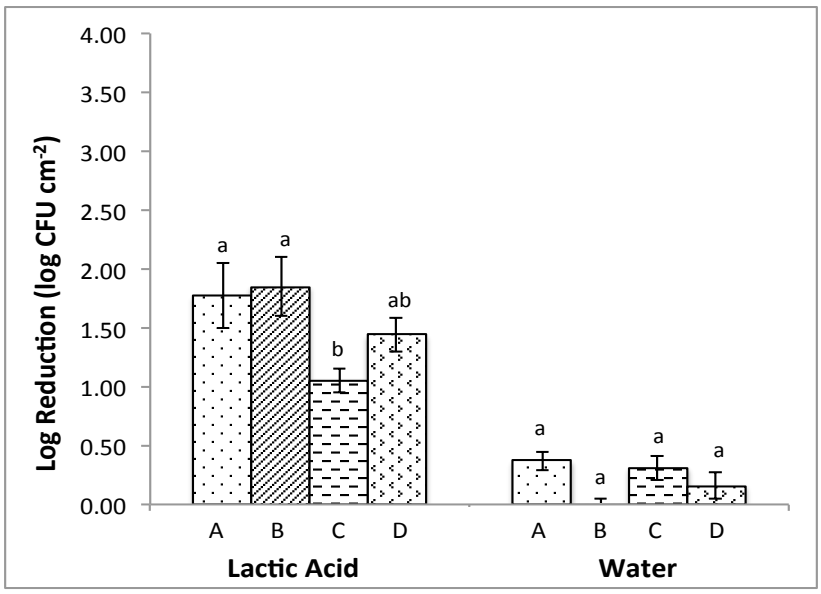

Figure 4-7 Shiga toxin-producing *Escherichia coli* log reductions on 15x15 cm beef surfaces under different scenarios after antimicrobial application. Scenarios followed by a different letter within antimicrobial treatments were significantly different ($P \leq 0.05$).

Several studies have demonstrated the efficacy of lactic acid in reducing STEC populations ranging from 1 to 4 log cycles, using either spray or immersion methods (Schmidt et al., 2014; Kalchayanand et al., 2013; Kalchayanand et al., 2012; Geornaras et al., 2012; Ransom et al., 2003). The reductions (up to ~1.85 log CFU/cm^2) found in the current study are smaller than the ones found by Ransom et al., (2003) when similar levels of inoculum were applied. A log reduction of ~3 log CFU/cm^2 was observed when 2% lactic acid dip was applied at 55 °C (Ransom et al., 2003). These differences in log reduction between Ransom et al., (2003) and our study might be explained by the differences in lactic acid temperatures and its method of application. Castillo et al., (2001) found that a combination of 4% lactic acid solution at 55 °C sprayed for 30 s was best for STEC reduction (>4.8 log CFU/cm^2) when various combinations of 2 or 4% lactic acid at 55 or 65 °C for 15 or 30 s were studied. Wolf et al., (2012) reported that a 4.4% lactic acid dip as the most successful method to reduce STEC and *Salmonella* spp. on beef trim and ground beef when compared to a spray method. However, many researchers reported a

non-significant bacterial reduction when ≤ 2% lactic acid was applied by itself as a non-combined intervention on chilled beef surfaces (Gill and Landers, 2003; Bacon et al., 2002; Brackett, 1994; Acuff et al., 1987). These observations are in agreement with the findings of the current study, where the highest reductions were observed when pre-rigor meat surfaces were inoculated and subjected to a 24 h chill cycle prior to application of lactic acid, thus, indicating that cold temperatures (~2 °C) have a synergistic impact on stressing and injuring the STEC cells when lactic acid is subsequently applied. These results might indicate that temperatures of both meat and lactic acid are the determinant factors playing a vital role in the efficacy of lactic acid treatment (Anderson and Marshall, 1989; Dickson and Anderson, 1992; Greer and Dilts, 1992). The differences in log reductions between the current study and studies published in the literature could be explained by differences in the methods used for lactic acid application, lactic acid concentration, and the differences in beef surfaces and lactic acid temperatures.

As observed in the current study, where the highest reduction was observed on the 24-h chilled beef surfaces, Dickson and Siragusa (1994) also found that log reductions in STEC population increased on acid-treated beef surfaces with an increase in storage time. Furthermore, Liao et al., (2015) reported increased STEC population reduction (~1.5 log CFU/cm^2) when a combination of 5% lactic acid treatment followed by a 14 day storage at 4 °C was used. Additionally, Gill and Badoni (2004) observed a significant reduction (≥1.5 log CFU/cm^2) of *E. coli* on chilled meat surfaces when 4% lactic acid spray was applied. Zhao et al. (2014) reported similar results when 5% lactic acid spray was applied to a chilled beef surface at 4 °C. This is further supporting evidence that beef surfaces at cold temperatures followed by lactic acid treatment have greater log reduction.

In our study, lactic acid was slightly more effective in reducing STEC-7 attached to adipose tissue (1.75 log CFU/cm^2) than on predominantly lean muscle (1.29 log CFU/cm^2). However, Youssef et al., (2013) found no difference in *E. coli* reductions between adipose tissue (~0.7 log CFU/cm^2) and cut muscles (~0.8 log CFU/cm^2) when 5% lactic acid was applied. The rupture of meat surface adipose cells and hence changes in the adipose coating on the meat surfaces commonly occurs at various stages of meat processing, which can change the hydrophobicity of the meat surface (Dickson and Koohmaraie, 1989). Therefore, variation in microbial reductions in Dickson and Koohmaraie (1989) and our studies could be due to the differences in post-harvest meat handling, which resulted in different adipose layer characteristics on meat surfaces, and ultimately affected the microbial attachment to the meat surfaces.

4.4 Conclusion

Our results indicate that there is no difference in total STEC-7 attachment (loosely + firmly attached cells) when comparing different tissue types (predominately lean muscle and adipose surfaces) under four (A-D) contamination scenarios. However, there were differences in attachment when STEC-7 mixed cocktail was exposed to different contamination scenarios. Chilled and re-warmed surfaces presented the greatest attachment levels, respectively. Inoculation of re-warmed post-rigor tissues after carcass chilling provided similar attachment levels when compared to pre-rigor beef surfaces.

Applying a 4.5% lactic acid spray at room temperature as a pre-evisceration and/or final carcass wash provided STEC reductions up to ~1.70 log CFU/cm^2, and lactic acid spray is also effective as a chilled carcass intervention when dealing with slaughter floor originating contamination. However, if STEC contamination occurs during carcass chilling or chilled

product processing, the application of 4.5% lactic acid is less effective as an intervention treatment. For future laboratory antimicrobial process validation studies, consideration must be given to when inoculum cultures are applied to beef surfaces and at what point in the slaughter to fabrication continuum chemical interventions are optimum in series.

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Appendix A - SAS Code Used for the Experiment

A.1 Analysis of pairwise comparisons of specific scenarios for STEC-7 attachment

```
PROC IMPORT OUT= WORK.beef
            DATAFILE= "C:\Users\Chris\Documents\KSU
Consulting\Carla_Schwan\6x6 study - reformated.xlsx"
            DBMS=xlsx REPLACE;
RUN;

ods rtf file='C:\Users\Chris\Documents\KSU
Consulting\Carla_Schwan\output_CRD_v1.rtf' style=journal;

ods graphics on;
data beef; set beef;
   pretrt="AB";
   if scenarios="C" then pretrt="C";
   if scenarios="D" then pretrt="D";
run;
```

A.2 Analysis of STEC-7 overall attachment

```
title 'Analyis of Pre-Treatment Log Counts';
proc mixed data=beef plots=all;
  class Reps Scenarios Lean_or_Fat LA_or_water;
  model pre_trt = Scenarios|lean_or_fat/ddfm=KR;
  random reps reps*Scenarios*lean_or_fat;
  lsmeans  scenarios /pdiff adjust=Tukey cl;
  estimate 'warm v. cold innoc' scenarios 1 1 -1 -1/divisor=2;
  estimate 'AB v. C' scenarios 1 1 -2 0/divisor=2;
  estimate 'AB v. D' scenarios 1 1 0 -2/divisor=2;
run;
```

A.3 Analysis of STEC-7 log Reductions

```
title 'Analyis of Post-Treatment Logreductions';
proc mixed data=beef plots=all;
  class reps scenarios lean_or_fat LA_or_water;
  model reduction = scenarios|lean_or_fat|LA_or_water;
  lsmeans scenarios*LA_or_water lean_or_fat*LA_or_water/pdiff adjust=Tukey;
run;

ods rtf close;
```

Appendix B - Data for the Experiment

B.1 Raw data of the four replicates

Reps	Scenarios	Lean or Fat	LA or Water	Pre trt	Pos trt	Reduction
Rep 1	A	Lean	LA	6.1	1.59	4.50
Rep 1	A	Lean	LA	5.9	3.35	2.57
Rep 1	A	Fat	LA	6.0	4.32	1.71
Rep 1	A	Fat	LA	5.1	3.05	2.08
Rep 1	A	Lean	Water	5.5	5.32	0.16
Rep 1	A	Lean	Water	5.6	5.50	0.09
Rep 1	A	Fat	Water	5.5	4.70	0.82
Rep 1	A	Fat	Water	5.4	5.41	0.00
Rep 1	B	Lean	Water	5.49	5.86	-0.37
Rep 1	B	Lean	Water	5.56	5.19	0.37
Rep 1	B	Fat	Water	4.44	4.78	-0.34
Rep 1	B	Fat	Water	5.12	5.12	0.01
Rep 1	B	Lean	LA	5.04	3.30	1.74
Rep 1	B	Lean	LA	5.48	2.97	2.51
Rep 1	B	Fat	LA	5.28	1.61	3.66
Rep 1	B	Fat	LA	4.63	2.10	2.54
Rep 1	C	Lean	LA	6.01	5.05	0.96
Rep 1	C	Lean	LA	5.53	5.20	0.32
Rep 1	C	Fat	LA	6.08	5.19	0.88
Rep 1	C	Fat	LA	6.23	4.83	1.40
Rep 1	C	Lean	Water	5.57	5.02	0.55
Rep 1	C	Lean	Water	5.47	5.34	0.13
Rep 1	C	Fat	Water	5.83	5.82	0.01
Rep 1	C	Fat	Water	6.52	6.04	0.48
Rep 1	D	Lean	LA	6.15	4.19	1.96
Rep 1	D	Lean	LA	6.11	4.54	1.57
Rep 1	D	Fat	LA	5.63	3.94	1.68
Rep 1	D	Fat	LA	5.73	4.16	1.56
Rep 1	D	Lean	Water	6.25	5.20	1.05
Rep 1	D	Lean	Water	6.11	5.51	0.60
Rep 1	D	Fat	Water	5.43	5.58	-0.15
Rep 1	D	Fat	Water	5.64	5.04	0.60
Rep 2	A	Lean	LA	3.04	3.49	-0.45

Rep 2	A	Lean	LA	5.38	4.04	1.35
Rep 2	A	Fat	LA	5.52	3.38	2.14
Rep 2	A	Fat	LA	5.85	3.91	1.93
Rep 2	A	Lean	Water	5.61	5.30	0.31
Rep 2	A	Lean	Water	5.99	5.56	0.43
Rep 2	A	Fat	Water	5.60	4.87	0.73
Rep 2	A	Fat	Water	6.28	5.28	1.00
Rep 2	B	Lean	Water	4.79	5.17	-0.38
Rep 2	B	Lean	Water	6.17	5.83	0.34
Rep 2	B	Fat	Water	5.63	5.85	-0.22
Rep 2	B	Fat	Water	4.49	5.05	-0.56
Rep 2	B	Lean	LA	4.74	3.02	1.71
Rep 2	B	Lean	LA	5.74	3.21	2.52
Rep 2	B	Fat	LA	4.47	1.57	2.90
Rep 2	B	Fat	LA	5.48	2.96	2.52
Rep 2	C	Lean	LA	6.29	4.61	1.67
Rep 2	C	Lean	LA	6.06	4.79	1.27
Rep 2	C	Fat	LA	5.83	5.05	0.79
Rep 2	C	Fat	LA	6.23	5.47	0.77
Rep 2	C	Lean	Water	5.83	6.27	-0.43
Rep 2	C	Lean	Water	6.30	5.46	0.84
Rep 2	C	Fat	Water	6.37	5.61	0.76
Rep 2	C	Fat	Water	5.58	6.06	-0.48
Rep 2	D	Lean	LA	6.12	5.12	1.01
Rep 2	D	Lean	LA	5.39	4.01	1.39
Rep 2	D	Fat	LA	5.89	3.65	2.24
Rep 2	D	Fat	LA	5.46	4.48	0.98
Rep 2	D	Lean	Water	6.04	5.49	0.55
Rep 2	D	Lean	Water	6.16	5.77	0.39
Rep 2	D	Fat	Water	5.10	5.05	0.06
Rep 2	D	Fat	Water	6.06	5.44	0.62
Rep 3	A	Lean	LA	4.98	4.58	0.40
Rep 3	A	Lean	LA	5.59	3.93	1.66
Rep 3	A	Fat	LA	5.31	3.20	2.11
Rep 3	A	Fat	LA	4.98	3.34	1.64
Rep 3	A	Lean	Water	5.61	5.41	0.20
Rep 3	A	Lean	Water	5.49	4.86	0.63
Rep 3	A	Fat	Water	5.07	4.50	0.56
Rep 3	A	Fat	Water	5.24	4.92	0.32
Rep 3	B	Lean	Water	5.08	5.10	-0.03
Rep 3	B	Lean	Water	4.52	4.42	0.10
Rep 3	B	Fat	Water	4.94	4.53	0.42
Rep 3	B	Fat	Water	4.49	4.81	-0.31

Rep 3	B	Lean	LA	5.28	5.04	0.24
Rep 3	B	Lean	LA	5.02	3.49	1.54
Rep 3	B	Fat	LA	5.81	3.28	2.53
Rep 3	B	Fat	LA	4.74	4.77	-0.03
Rep 3	C	Lean	LA	5.75	4.35	1.41
Rep 3	C	Lean	LA	5.75	5.35	0.41
Rep 3	C	Fat	LA	5.33	4.34	1.00
Rep 3	C	Fat	LA	5.50	4.42	1.09
Rep 3	C	Lean	Water	5.63	5.45	0.18
Rep 3	C	Lean	Water	5.87	4.94	0.93
Rep 3	C	Fat	Water	5.79	5.77	0.03
Rep 3	C	Fat	Water	5.82	5.40	0.42
Rep 3	D	Lean	LA	5.28	4.51	0.77
Rep 3	D	Lean	LA	5.49	4.76	0.72
Rep 3	D	Fat	LA	5.16	3.54	1.63
Rep 3	D	Fat	LA	5.55	4.37	1.19
Rep 3	D	Lean	Water	5.46	5.79	-0.33
Rep 3	D	Lean	Water	5.47	5.59	-0.12
Rep 3	D	Fat	Water	5.40	5.58	-0.18
Rep 3	D	Fat	Water	4.47	5.21	-0.74
Rep 4	A	Lean	LA	5.40	4.58	0.83
Rep 4	A	Lean	LA	5.06	3.79	1.27
Rep 4	A	Fat	LA	4.49	1.65	2.84
Rep 4	A	Fat	LA	4.67	2.89	1.78
Rep 4	A	Lean	Water	4.91	5.12	-0.21
Rep 4	A	Lean	Water	5.40	5.13	0.27
Rep 4	A	Fat	Water	4.70	4.39	0.32
Rep 4	A	Fat	Water	4.95	4.60	0.35
Rep 4	B	Lean	LA	5.15	4.54	0.61
Rep 4	B	Lean	LA	5.29	3.95	1.34
Rep 4	B	Fat	LA	4.97	3.45	1.52
Rep 4	B	Fat	LA	4.78	3.07	1.71
Rep 4	B	Lean	Water	5.33	5.88	-0.55
Rep 4	B	Lean	Water	4.65	4.59	0.06
Rep 4	B	Fat	Water	5.08	4.83	0.24
Rep 4	B	Fat	Water	5.25	4.77	0.48
Rep 4	C	Lean	LA	5.95	5.18	0.77
Rep 4	C	Lean	LA	5.82	4.68	1.14
Rep 4	C	Fat	LA	5.74	4.54	1.20
Rep 4	C	Fat	LA	5.95	4.28	1.67
Rep 4	C	Lean	Water	5.87	5.31	0.56
Rep 4	C	Lean	Water	5.49	4.95	0.53
Rep 4	C	Fat	Water	5.70	5.47	0.23

Rep 4	C	Fat	Water	5.64	5.50	0.15
Rep 4	D	Lean	LA	5.40	4.96	0.44
Rep 4	D	Lean	LA	5.07	3.66	1.41
Rep 4	D	Fat	LA	5.34	3.13	2.21
Rep 4	D	Fat	LA	5.48	3.17	2.31
Rep 4	D	Lean	Water	5.34	5.15	0.18
Rep 4	D	Lean	Water	5.41	5.34	0.08
Rep 4	D	Fat	Water	4.90	4.89	0.01
Rep 4	D	Fat	Water	5.19	5.23	-0.04

B.2 Temperature and pH measurements

Sample	Scenario	Treatment	Tissue type	pH	Temperature (°C)
1 pre-trt	A	LA	L	5.79	23.1
1 post-trt	A	LA		3.52	
2 pre-trt	A	LA	L	4.74	24.2
2 post-trt	A	LA		3.53	
3 pre-trt	A	LA	F	7.72	23
3 post-trt	A	LA		3.19	
4 pre-trt	A	LA	F	6.41	25.2
4 post-trt	A	LA		3.57	
5 pre-trt	A	Water	L	4.85	29.8
5 post-trt	A	Water		6.85	
6 pre-trt	A	Water	L	5.85	24.7
6 post-trt	A	Water		5.72	
7 pre-trt	A	Water	F	4.24	27.1
7 post-trt	A	Water		7.31	
8 pre-trt	A	Water	F	4.18	29
8 post-trt	A	Water		7.48	
9 pre-trt	A	LA	L	5.08	18.8
9 post-trt	A	LA		2.85	
10 pre-trt	A	LA	L	5.04	18.4
10 post-trt	A	LA		3.78	
11 pre-trt	A	LA	F	4.51	NA
11 post-trt	A	LA		3.84	
12 pre-trt	A	LA	F	4.43	NA
12 post-trt	A	LA		3.1	
13 pre-trt	A	Water	L	6.71	31.2
13 post-trt	A	Water		6.48	
14 pre-trt	A	Water	L	6.63	26.4

14 post-trt	A	Water		5.88	
15 pre-trt	A	Water	F	6.76	31.9
15 post-trt	A	Water		7.13	
16 pre-trt	A	Water	F	5.99	29.3
16 post-trt	A	Water		7.07	
17 pre-trt	B	Water	L	4.64	34.6
17 post-trt	B	Water		7.56	
18 pre-trt	B	Water	L	6.67	36.5
18 post-trt	B	Water		5.71	
19 pre-trt	B	Water	F	6.18	36.3
19 post-trt	B	Water		8.58	
20 pre-trt	B	Water	F	5.49	29.7
20 post-trt	B	Water		3.42	
21 pre-trt	B	Water	L	8.02	30.4
21 post-trt	B	Water		6.31	
22 pre-trt	B	Water	L	8.23	32
22 post-trt	B	Water		7.56	
23 pre-trt	B	Water	F	5.43	30.2
23 post-trt	B	Water		6.81	
24 pre-trt	B	Water	F	8.3	30.6
24 post-trt	B	Water		8.44	
25 pre-trt	B	LA	L	5.76	32.7
25 post-trt	B	LA		4.33	
26 pre-trt	B	LA	L	3.46	33
26 post-trt	B	LA		5.19	
27 pre-trt	B	LA	F	6.58	34.1
27 post-trt	B	LA		3.04	
28 pre-trt	B	LA	F	NA	33
28 post-trt	B	LA		NA	
29 pre-trt	B	LA	L	NA	34.6
29 post-trt	B	LA		NA	
30 pre-trt	B	LA	L	NA	30.9
30 post-trt	B	LA		NA	
31 pre-trt	B	LA	F	NA	31.5
31 post-trt	B	LA		NA	
32 pre-trt	B	LA	F	NA	29.4
32 post-trt	B	LA		NA	
33 pre-trt	C	LA	L	4.3	10.2
33 post-trt	C	LA		2.5	
34 pre-trt	C	LA	L	5.25	7.6
34 post-trt	C	LA		3.28	
35 pre-trt	C	LA	F	6.91	9.8
35 post-trt	C	LA		1.72	

36 pre-trt	C	LA	F	4.7	12
36 post-trt	C	LA		1.47	
37 pre-trt	C	Water	L	5.86	10.8
37 post-trt	C	Water		4.82	
38 pre-trt	C	Water	L	4.71	11.5
38 post-trt	C	Water		7.58	
39 pre-trt	C	Water	F	5.38	12.9
39 post-trt	C	Water		6.99	
40 pre-trt	C	Water	F	9.03	10.9
40 post-trt	C	Water		8.63	
41 pre-trt	C	Water	L	6.13	8.1
41 post-trt	C	Water		7.7	
42 pre-trt	C	Water	L	4.93	9.2
42 post-trt	C	Water		8.82	
43 pre-trt	C	Water	F	7.51	9
43 post-trt	C	Water		8.6	
44 pre-trt	C	Water	F	7.4	10
44 post-trt	C	Water		9.2	
45 pre-trt	C	LA	L	8.1	10.4
45 post-trt	C	LA		3.97	
46 pre-trt	C	LA	L	7.34	16
46 post-trt	C	LA		4.85	
47 pre-trt	C	LA	F	4.94	10.6
47 post-trt	C	LA		2.19	
48 pre-trt	C	LA	F	5.5	10.9
48 post-trt	C	LA		2.83	
49 pre-trt	D	LA	L	NA	26.4
49 post-trt	D	LA		NA	
50 pre-trt	D	LA	L	NA	24.9
50 post-trt	D	LA		NA	
51 pre-trt	D	LA	F	NA	26.7
51 post-trt	D	LA		NA	
52 pre-trt	D	LA	F	NA	26.6
52 post-trt	D	LA		NA	
53 pre-trt	D	Water	L	NA	26.5
53 post-trt	D	Water		NA	
54 pre-trt	D	Water	L	NA	25.2
54 post-trt	D	Water		NA	
55 pre-trt	D	Water	F	NA	27.4
55 post-trt	D	Water		NA	
56 pre-trt	D	Water	F	NA	26.7
56 post-trt	D	Water		NA	
57 pre-trt	D	LA	L	NA	26.6

57 post-trt	D	LA		NA	
58 pre-trt	D	LA	L	NA	26.5
58 post-trt	D	LA		NA	
59 pre-trt	D	LA	F	NA	26.9
59 post-trt	D	LA		NA	
60 pre-trt	D	LA	F	NA	25.3
60 post-trt	D	LA		NA	
61 pre-trt	D	Water	L	NA	26.2
61 post-trt	D	Water		NA	
62 pre-trt	D	Water	L	NA	26.4
62 post-trt	D	Water		NA	
63 pre-trt	D	Water	F	NA	27.1
63 post-trt	D	Water		NA	
64 pre-trt	D	Water	F	NA	27.6
64 post-trt	D	Water		NA	

B.3 Temperature and pH measurements of animals 3 and 4

B.3.1 Temperature profile for hot water carcass spray

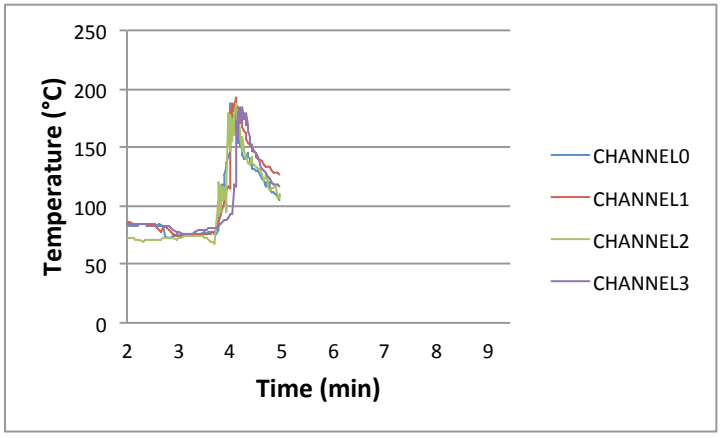

Figure 4-8 Temperature profile for hot water carcass spray measured by a data logger with different channels distributed along the beef carcass – Carcass 1, side 1.

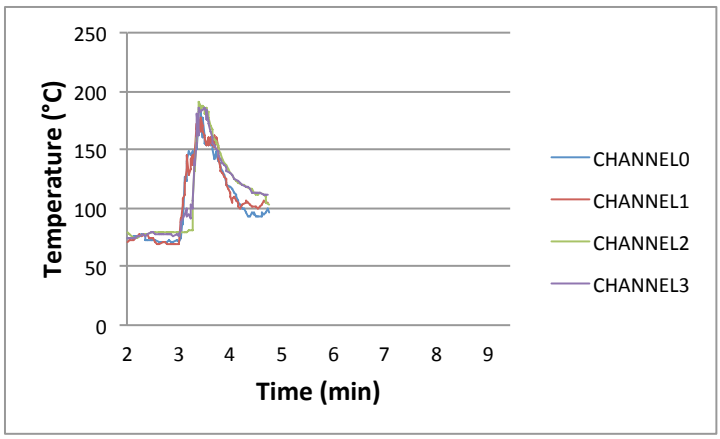

Figure 4-9 - Temperature profile for hot water carcass spray measured by a data logger with different channels distributed along the beef carcass – Carcass 1, side 2.

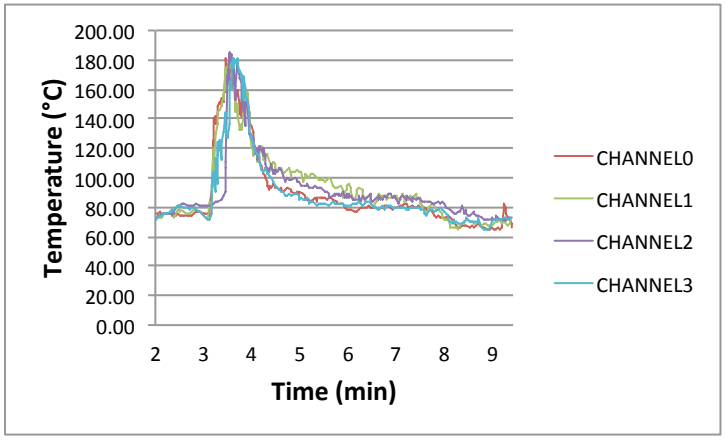

Figure 4-10 - temperature profile for hot water carcass spray measured by a data logger with different channels distributed along the beef carcass – Carcass 2, side 1.

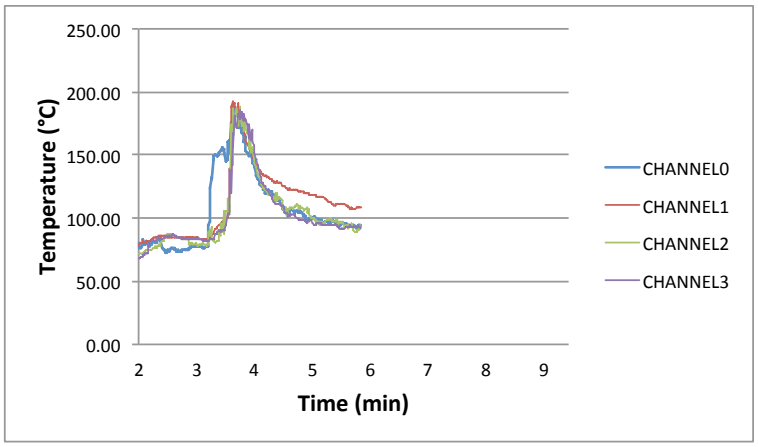

Figure 4-11 - Temperature profile for hot water carcass spray measured by a data logger with different channels distributed along the beef carcass – Carcass 2, side 2.

B.4 Summary table

Table 4-4 Summary table of the mean log reductions of Treatments (lactic acid and water) and Scenarios (A, B, C, D) average across tissue type.

Treatments	Scenarios			
	A	B	C	D
Lactic Acid	$1.77^{aA} \pm 0.27$	$1.85^{aA} \pm 0.25$	$1.04^{aB} \pm 0.10$	$1.44^{aAB} \pm 0.14$
Water	$0.37^{bA} \pm 0.08$	$-0.05^{bA} \pm 0.09$	$0.31^{bA} \pm 0.10$	$0.16^{bA} \pm 0.11$

$^{a-b}$ Means (n=32) ± SE with different superscripts within a column differ ($P \leq 0.05$).

$^{A-B}$ Means (n=32) ± SE with different superscripts within a row differ ($P \leq 0.05$).

Appendix C - Residual homogeneity assessment

C.1 Residual homogeneity assessment for bacterial attachment

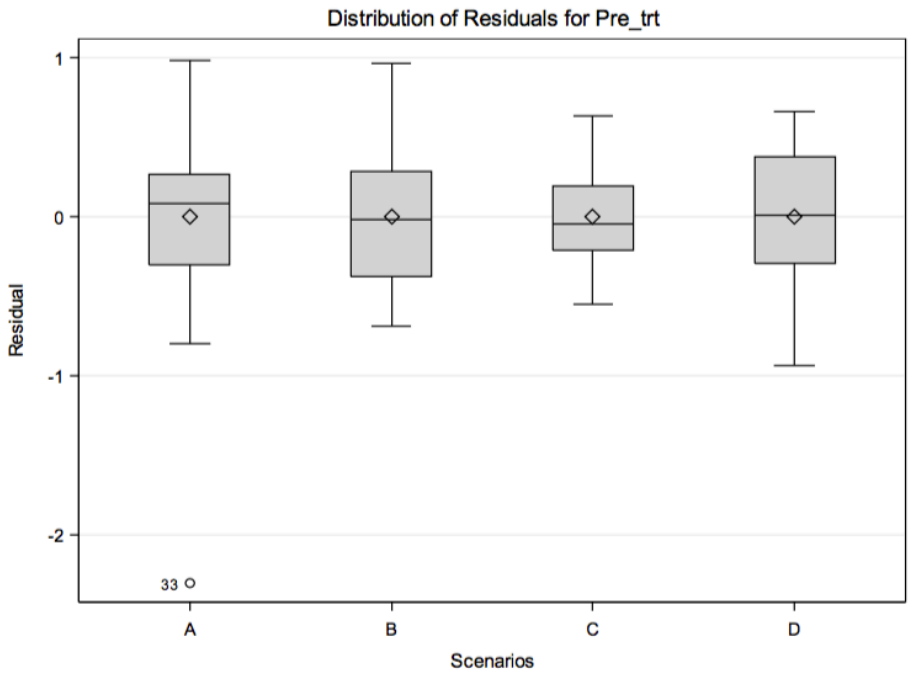

Figure 4-12 Scenarios residuals homogeneity assessment of bacterial attachment.

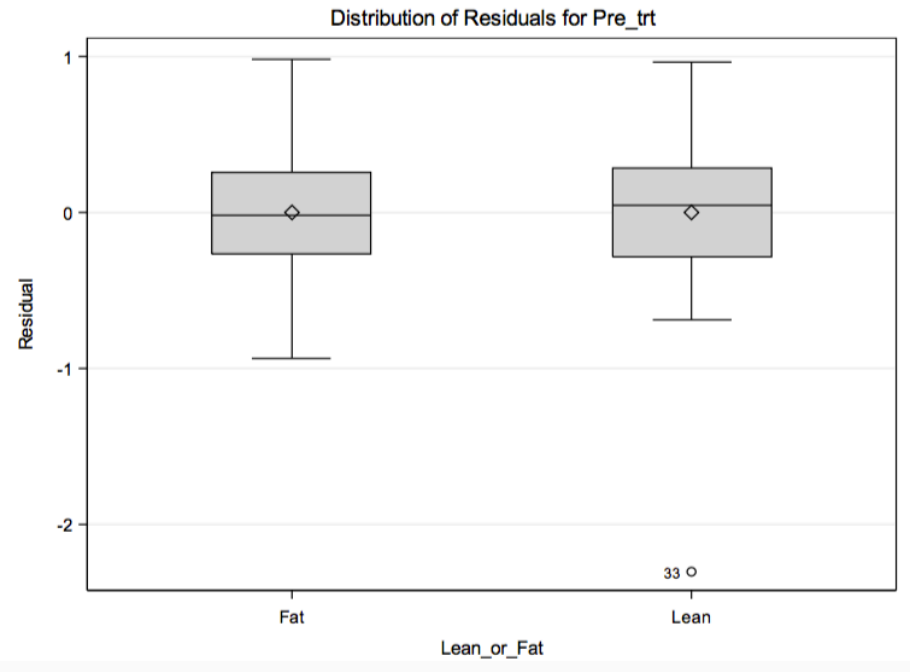

Figure 4-13 Tissue type residuals homogeneity assessment of bacterial attachment.

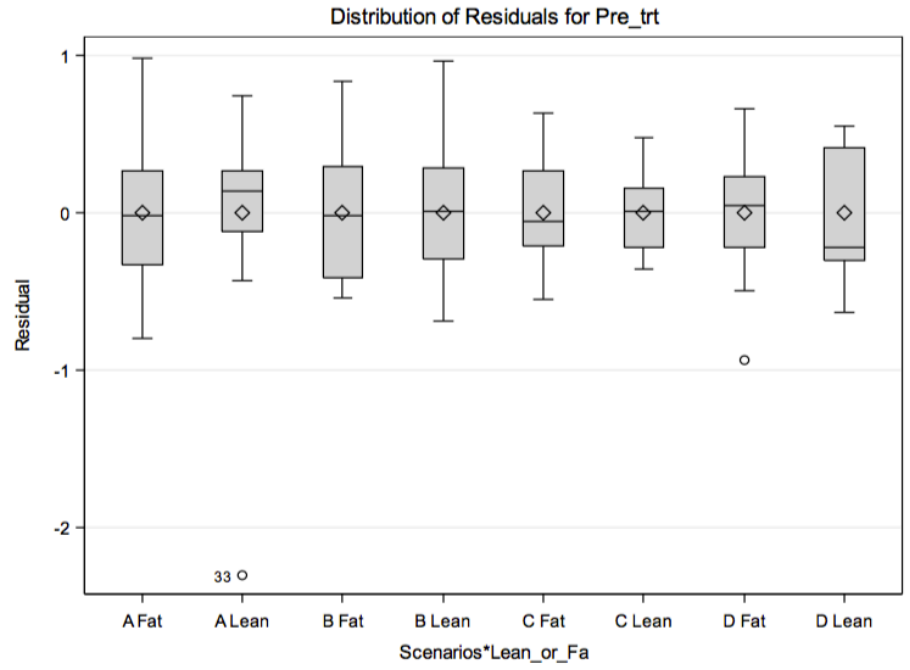

Figure 4-14 Two-way interaction (scenarios and tissue type) residuals homogeneity assessment of bacterial attachment.

C.2 Residual homogeneity assessment for log reductions

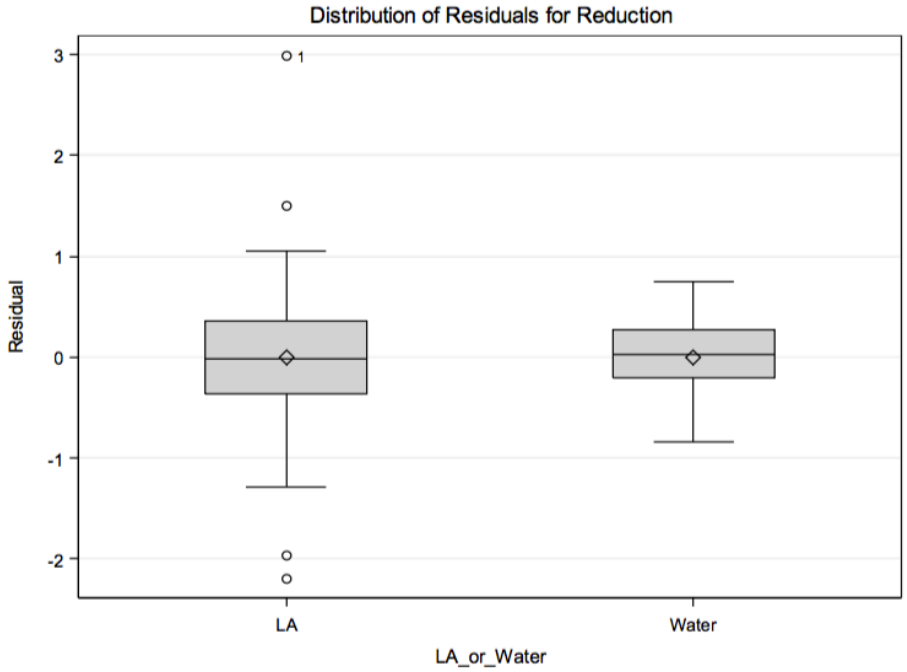

Figure 4-15 Treatment residuals homogeneity assessment of log reduction.

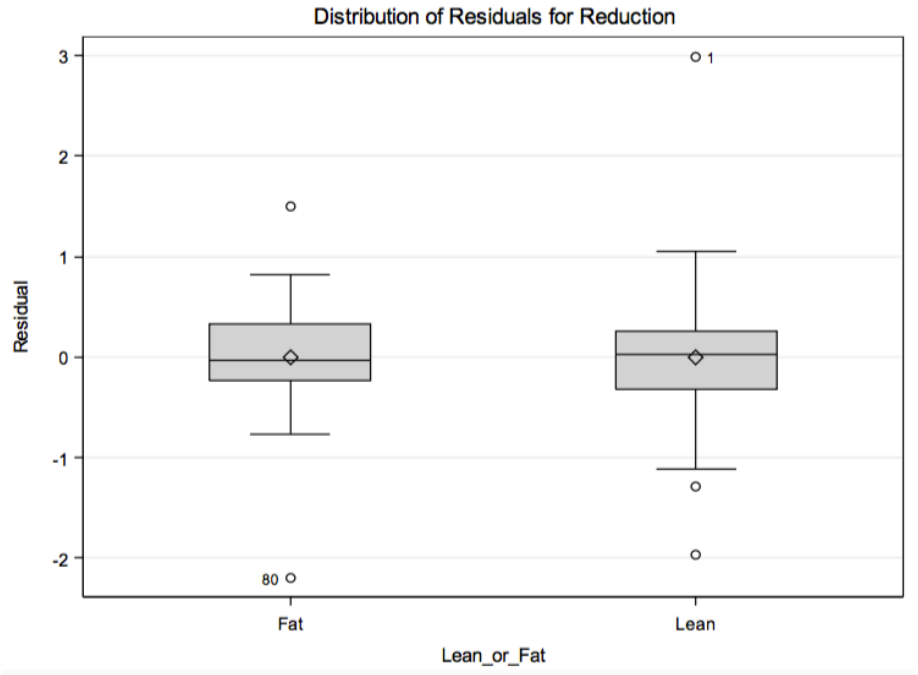

Figure 4-16 Tissue type residuals homogeneity assessment of log reduction.

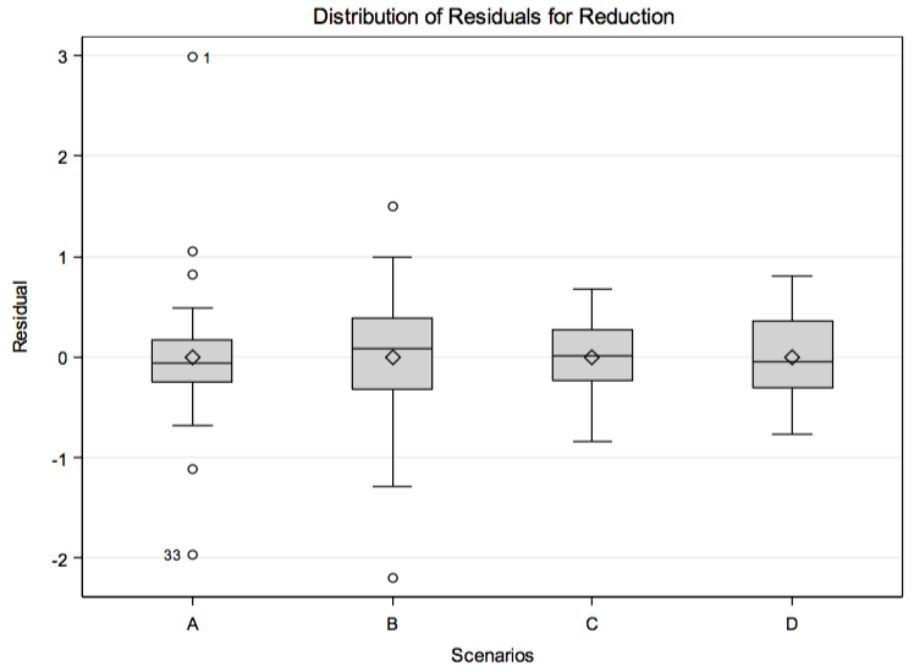

Figure 4-17 Scenarios residuals homogeneity assessment of log reduction.

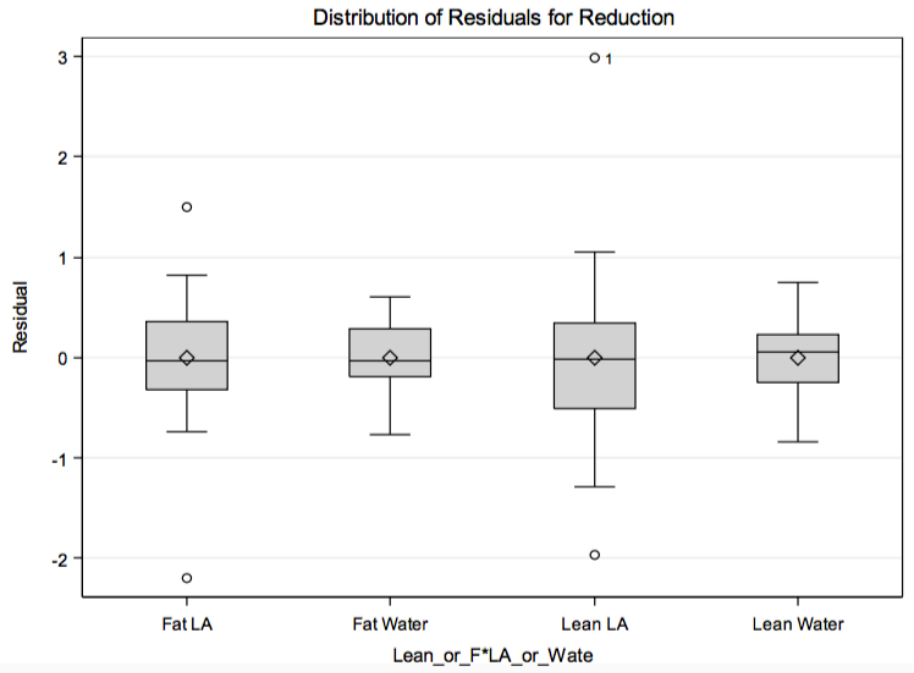

Figure 4-18 Two-way interaction (tissue type and treatment) residuals homogeneity assessment of log reduction.

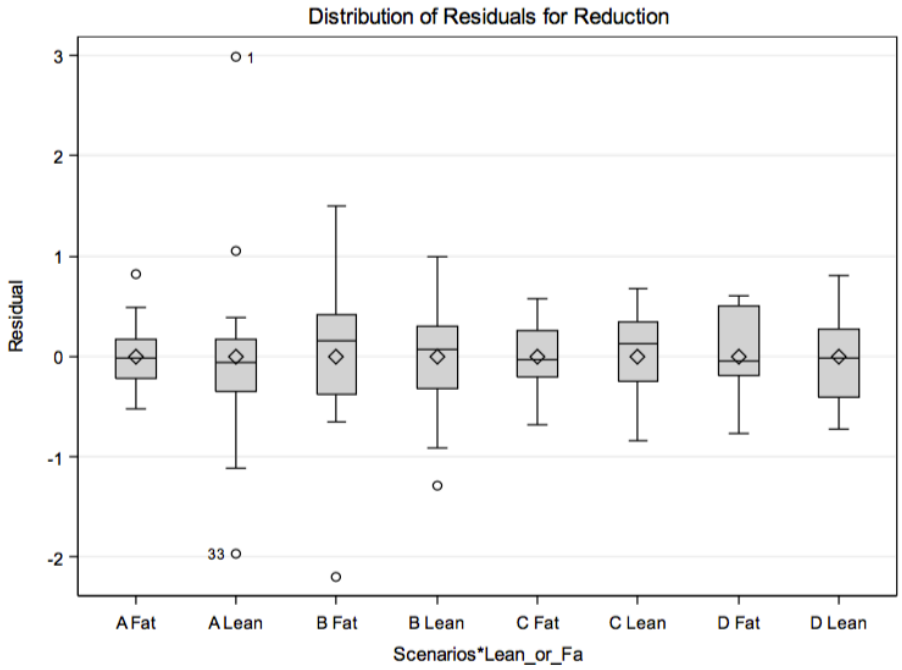

Figure 4-19 Two-way interaction (scenario and tissue type) residuals homogeneity assessment of log reduction.

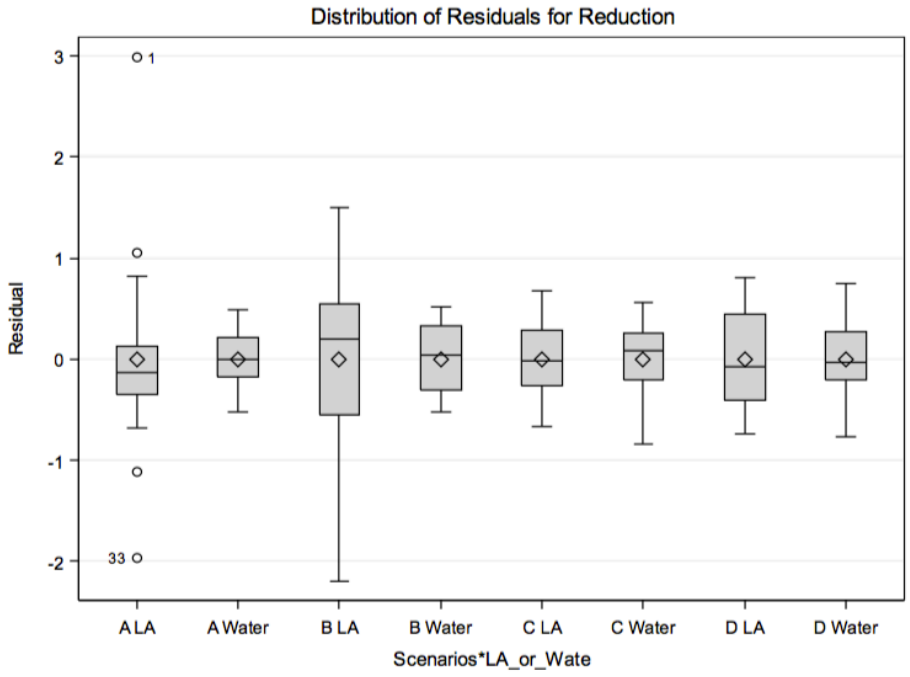

Figure 4-20 Two-way interaction (scenario and treatment) residuals homogeneity assessment of log reduction.

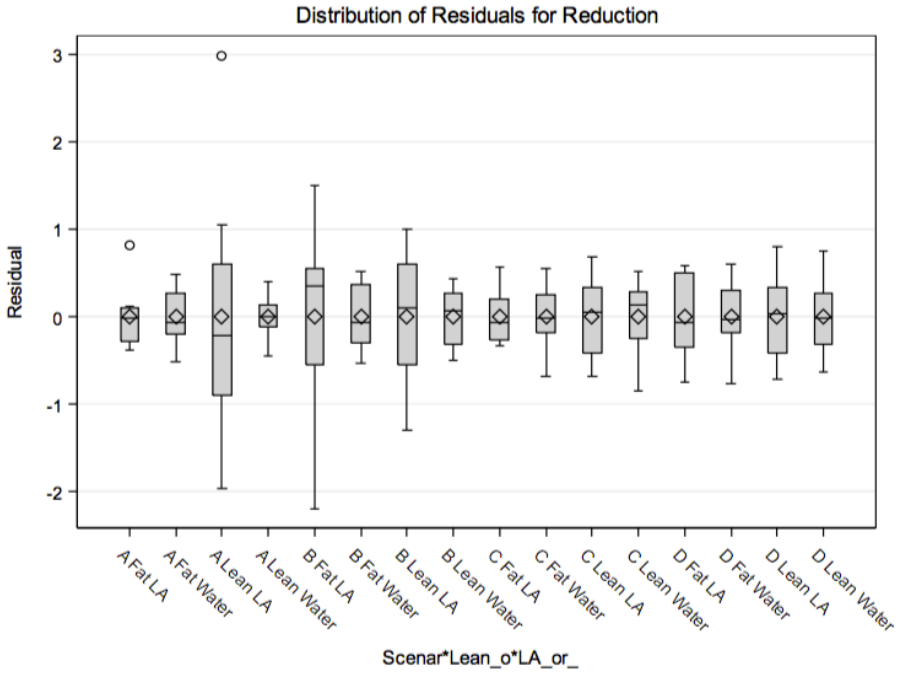

Figure 4-21 Three-way interaction (scenario, tissue type and treatment) residuals homogeneity assessment of log reduction.