DISSERTATION

GROWTH AND CONTROL OF *ESCHERICHIA COLI* O157:H7 IN PROCESSING ENVIRONMENTS AND FRESH BEEF PRODUCTS AND OF *LISTERIA MONOCYTOGENES* ON PROCESSED MEATS DURING HOME STORAGE AND THAWING

Submitted by

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ABSTRACT OF DISSERTATION

GROWTH AND CONTROL OF *ESCHERICHIA COLI* O157:H7 IN PROCESSING ENVIRONMENTS AND FRESH BEEF PRODUCTS AND OF *LISTERIA MONOCYTOGENES* ON PROCESSED MEATS DURING HOME STORAGE AND THAWING

This dissertation delineates a set of studies designed to examine the fate of *Escherichia coli* O157:H7 on fresh beef and *Listeria monocytogenes* on processed meats during simulated processing and storage conditions in order to better understand the food safety implications associated with each scenario. The outer fat layer of beef carcasses may be pulled and torn away from underlying lean tissue during mechanical hide removal creating pockets where bacteria and liquids such as decontamination fluid may collect. Due to the continued potential for the presence of *E. coli* O157:H7 in ground beef, a large-scale investigation of the fate of *E. coli* O157:H7 in slaughter-floor fluids during carcass chilling would help to identify potential reservoirs of *E. coli* O157:H7 contamination. Therefore, one study evaluated: (i) the efficacy of different residual slaughter-floor decontamination runoff fluids against natural contamination and *E. coli* O157:H7; (ii) the ability of naturally occurring contamination and inoculated *E. coli* O157:H7 to survive in such decontamination fluid residues during beef carcass chilling;

and, (iii) the efficacy of post-chilling decontamination treatments against pathogen cells that survive in decontamination fluids during the chilling process. To do so, water was collected as it ran off of several beef carcasses sprayed with 82°C water, and fresh beef brisket tissue was excised from beef carcasses (n = 27), at a commercial beef packing facility. Pieces of beef tissue were prepared by dissecting the fat from the lean tissue to create a pocket. Runoff fluid pH was adjusted to 3, 5 or 7 using lactic acid and then adjusted to 25, 45 or 65°C. Prepared residual runoff fluids were inoculated (4 log CFU/ml) with a six-strain *E. coli* O157:H7 composite and poured into tissue pockets; the tissue pockets were then covered with aluminum foil and stored at 4°C for 48 h to simulate beef carcass chilling. At 0, 12, 24, 36 and 48 h of storage, fluid in pockets was analyzed for pH and microbial survivors. In addition, after 48 h at 4°C, fluid that had been adjusted to pH 3, 5 or 7 and to 25°C was removed from the pockets and inoculated (1.5 log CFU/cm^2) onto fresh beef carcass fat. Inoculated fat was sprayed (25°C, 1.38 bar 3 sec) with water (control), 5.0% lactic acid (LA), 0.5% cetylpyridinium chloride (CPC) or 0.02% peroxyacetic acid (PAA) to determine the efficacy of post-chilling decontamination treatments against cells that survived the chilling process in residual decontamination runoff fluids in tissue pockets. Microbial reductions, following exposure to runoff fluid residues were pH and temperature dependent. No decontamination treatment was capable of reducing natural contaminants or inoculated *E. coli* O157:H7 populations from 5.5 and 4.0 log CFU/ml, respectively, to below the level of detection following enrichment. Adjusting runoff fluid to pH 3 and to 65°C did reduce total aerobic bacteria and *E. coli* O157:H7 to below the detection limit (0.0 log CFU/ml) by spread plating. Adjusting runoff fluids of pH 5 or 7 to 45 or 65°C reduced total aerobic

bacteria and pathogen counts by 1.1 to 3.2 and 0.0 to 1.3 log CFU/ml, respectively. Microbial populations associated with ambient temperature runoff fluids adjusted to pH 5 or 7 did not change. Exposure to heated runoff fluids resulted in immediate microbial reductions but did not have a lasting effect on survivors during storage (4°C, 48 h). Adjusting fluids to pH 3 did suppress the growth of natural contamination during storage, indicating that cells that survive slaughter-floor decontamination treatments may survive during beef carcass chilling even under low pH conditions. Post-chilling decontamination treatments reduced *E. coli* O157:H7 populations (1.6 log CFU/cm^2) on beef fat by 0.1 to 1.3 log CFU/cm^2, and level of reduction decreased in order of CPC > LA > PAA = water. Treatments were generally more effective against cells derived from pH 7 than pH 5 or 3 runoff fluids with pathogen reductions ranging from 0.4 to 1.3 compared to 0.1 to 0.9 log CFU/cm^2, respectively. These data indicate that *E. coli* O157:H7 entrapped in defects on carcass surfaces can evade slaughter-floor decontamination treatments and survive the carcass chilling process, and that survivors may contaminate other carcasses or the surrounding environment during fabrication. These data also indicated that approved fabrication-level interventions were only moderately effective against *E. coli* O157:H7, and less effective against acid-stressed cells.

Biofilms in food processing environments typically exhibit increased resistance to otherwise lethal interventions and may act as recurring sources of contamination. The second set of studies examined the effects of fresh meat contact-surface material types, pre-conditioning of their surface, inoculation substrate, presence or absence of fluid during incubation, presence of air at the liquid-surface interface, incubation substrate, and incubation time on the attachment of *E. coli* O157:H7 cells and subsequent biofilm

formation. The materials examined were selected following visual inspection of beef fabrication facilities and verbal communication with fabrication equipment manufacturers, sales personnel and purchasers who identified them as those most commonly used to construct hooks, knives, cutting tables, conveyor belts, storage containers and employee safety equipment. These materials included stainless steel, acetal, polypropylene, and high-density polyethylene (HDPE) surface materials were acquired and maintained in new, unblemished condition. A six-strain rifampicin-resistant *E. coli* O157:H7 composite was used to inoculate (6 log CFU/ml, g or cm^2) tryptic soy broth (TSB, pH 7.29), beef fat/lean homogenate (FLH, pH 5.66), fresh conveyor belt-runoff fluids containing dilute Inspexx 200^{TM} (CBRF, pH 4.6), fresh ground beef (pH 5.76), or fresh beef fat (pH 6.03). These substrates were then used to inoculate dry, sterile stainless steel (Type 304, #2b finish), acetal, polypropylene or HDPE coupons (2x5 cm). Coupons were also pre-conditioned by submerging them in CBRF or beef fat homogenate (FH) for 45 min before inoculation. Attachment of *E. coli* O157:H7 was influenced by type of surface material when liquid inoculum was used to transfer cells onto coupons. However, when inoculated beef fat was used to transfer cells onto coupons, there were no significant differences in level of attachment onto different surface materials. Beef fat was also the most effective ($P < 0.05$) inoculation substrate followed by ground beef, FLH, and TSB. In general, pre-conditioning surfaces did not appear to influence attachment of *E. coli* O157:H7, regardless of inoculation substrate. As an exception, greater pathogen attachment was observed on pre-conditioned compared to dry HDPE coupons when a liquid inoculum was used; no difference was observed when fat was used as the inoculation substrate. To determine the effect of production

cycles on biofilm cells, inoculated coupons were incubated (15°C) in dry sterile tubes, in tubes containing 20 ml of diluted CBRF (dCBRF), or in tubes containing 20 ml of dCBRF for 8 h and then in dry tubes for 16 h to simulate a normal beef processing facility production cycle. Coupons from each treatment were analyzed at 24, 48 and 96 h of incubation and at each sample collection interval greater survival was observed on constantly hydrated coupons, followed by coupons that were rotationally hydrated and then dried. No *E. coli* O157:H7 survivors were observed on dry coupons after 96 h of incubation. To determine the effect of air at the incubation fluid-surface interface on biofilm formation, fat-inoculated coupons were placed in tubes containing either 20 ml (partially-submerged) or 30 ml (fully-submerged) of fresh CBRF (pH 3.07) or fresh beef fat homogenate (FH; 1:10 dilution in sterile distilled water, pH 4.21) for up to 16 d at 15°C. To determine the effect of incubation substrate on biofilm formation, fat-inoculated stainless steel and acetal coupons were partially-submerged (20 ml) in uninoculated TSB, FLH or CBRF for up to 10 d at 15°C. Incubation in FLH allowed the pathogen to survive and grow on both surface materials, although TSB was the most optimal incubation substrate. Maximal biofilm formation was observed between 2 and 8 d of storage at 15°C, and was dependent on the presence of air and characteristics of the incubation media. In general, the presence of air at the liquid-surface interface allowed pathogen populations to grow during incubation while growth was inhibited on fully-submerged coupons. These results indicate that the process of fabricating beef carcasses may be conducive to the attachment of *E. coli* O157:H7 onto meat-contact surfaces and subsequent biofilm formation. Furthermore, substrates found in beef fabrication settings

should be incorporated into future studies designed to investigate *E. coli* O157:H7 biofilms in such environments.

Negative consequences associated with pathogens on inadequately cleaned surfaces necessitate the identification of sanitizers which are effective on both clean and soiled surfaces. The third study evaluated the efficacy of approved food contact surface sanitizers and sanitizers which are unapproved for use on food contact surfaces, against *E. coli* O157:H7 cells in biofilms formed on surface materials used in beef fabrication facilities. The influence of different surface materials and biofilm age on sanitizer treatment efficacy was also examined. Sanitizers applied at manufacturers' minimum and maximum recommended concentrations for use on food contact surfaces included acidified sodium chlorite (ASC), sodium hypochlorite (SH), potassium peroxymonosulfate/sodium chloride (PP/SC), two commercial quaternary ammonium compounds containing a mixture of ammonium chloride compounds (QUAT-A) or cetylpyridinium chloride (QUAT-B), peroxyacetic acid (PAA), and a peroxyacetic acid/octanoic acid mixture (PA/OA). Coupons (2x5 cm) of stainless steel, acetal, and HDPE were inoculated (3.6 log CFU/cm^2) with a 6-strain rifampicin-resistant *E. coli* O157:H7 composite and incubated (15°C) in fresh, unsterilized beef lean/fat tissue homogenate (pH 5.66). At d-3 of incubation, attached cells (4.3 log CFU/cm^2) were challenged by submerging coupons in minimum and maximum recommended concentrations of each of the seven sanitizing solutions or water (control) for 1 or 10 min. Sanitizer treatments reduced *E. coli* O157:H7 on coupons by 0.0 to 2.2 log CFU/cm^2 and treatment efficacy decreased in order of ASC > PAA > PP/SC = PA/OA > QUAT-B > QUAT-A = SH. Maximum reductions and level of inactivation generally increased as

concentration and exposure time increased. Three sanitizers (SH, QUAT-A and PA/OA) were also applied on 0- and 7-d old biofilm cells at minimum and maximum recommended concentrations for 1 or 10 min. On d-0, 10 min exposures to maximum concentrations of PA/OA or QUAT-A reduced *E. coli* O157:H7 (3.6 log CFU/cm^2) by 1.5 or 0.8 log CFU/cm^2, respectively. On d-0, 1 min sanitizer treatments were generally ineffective against biofilm cells. On d-3 and d-7, 10 min exposures to the maximum recommended concentration of PA/OA reduced *E. coli* O157:H7 (3.7-4.3 log CFU/cm^2) by $\leq$ 0.9 log CFU/cm^2. In general, SH treatments were ineffective against *E. coli* O157:H7 biofilm cells. Surface material did not ($P \geq 0.05$) influence the fate of biofilm cells during sanitizing treatments. These data indicate that, while no sanitizer consistently reduced pathogen populations by more than 1 log cycle on soiled surfaces, approved concentrations of ASC and peroxyacetic acid-based sanitizers may be more effective against *E. coli* O157:H7 on inadequately cleaned surfaces than other sanitizers. Increased resistance in older biofilm cells also emphasizes the importance of thorough cleaning before sanitation and applying sanitizers at the highest allowable concentrations for extended exposure times.

Little information is available regarding the fate of *L. monocytogenes* during freezing, thawing and home storage of frankfurters even though recent surveys show that consumers regularly store unopened packages of frankfurters in home freezers. The fourth study examined the effects of antimicrobials, refrigerated storage, freezing, thawing method, and post-thawing storage (7°C) on *L. monocytogenes* on frankfurters. Inoculated (2.1 log CFU/cm^2) frankfurters formulated without (control) or with antimicrobials (1.5% potassium lactate plus 0.1% sodium diacetate) were vacuum-

packaged, stored at 4ºC for 6 or 30 d and then frozen (-15ºC) for 10, 30, or 50 d. Packages were thawed under refrigeration (7ºC, 24 h), on a countertop (23 ± 2ºC, 8 h), or in a microwave oven (2450 MHz, 1100 watts, 220 s followed by 120 s holding), and then stored aerobically (7ºC) for 14 d. Bacterial populations were enumerated on PALCAM agar and tryptic soy agar plus 0.6% yeast extract. Antimicrobials completely inhibited ($P < 0.05$) growth of *L. monocytogenes* at 4ºC for 30 d under vacuum-packaged conditions, and during post-thawing aerobic storage at 7ºC for 14 d. As product aged before freezing initial pathogen levels on control frankfurters increased to 2.1 and 3.9 (log CFU/cm^2) on days 6 and 30, respectively, while freezing reduced counts by <1.0 log CFU/cm^2. Thawing treatments had little effect on *L. monocytogenes* populations (<0.5 log CFU/cm^2), and post-thawing fate of *L. monocytogenes* was not influenced by freezing or by thawing method. Pathogen counts on control samples increased by 1.5 log CFU/cm^2 at d-7 of aerobic storage, and reached 5.6 log CFU/cm^2 at d-14. As indicated by these results, consumers should freeze frankfurters immediately after purchase, and discard frankfurters without inhibitory agents within 3 d of thawing and/or opening the package.

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DEDICATION

To my parents for their dedication to the person I became, and to my husband Kyle, for living with it.

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CHAPTER ONE

Overview of Dissertation

1.1 Justification

Existing estimates attribute 76 million illnesses and 5,000 deaths to foodborne diseases each year in the United States (Mead et al., 1999), and fresh and processed meat products continue to be associated with outbreaks of foodborne illness and pathogen contamination related recalls. Concern associated with *Escherichia coli* O157:H7 contamination in fresh beef was first established in 1982 (Riley et al., 1983), prompting massive research and developmental efforts designed to mitigate presence of this pathogen in beef products. As a result, numerous control strategies were identified and implemented by beef processors, with notable success (Simpson Beauchamp and Sofos, 2009; Sofos, 2004, 2005; Stopforth and Sofos, 2006). However, while progress has been made, beef products continue to be associated with *E. coli* O157:H7-related outbreaks and recalls. Each year roughly 105,000 such illnesses are classified as Shiga toxin-producing *Escherichia coli* (STEC) infections, 75% of which are caused by *E. coli* O157:H7, and in 2009, the annual cost associated with domestic STEC infections was an estimated $4.7 million, which averaged $6,500 per illness (http://www.ers.usda.gov/Data/FoodborneIllness/). Previous control efforts have been largely successful, but evidence of remaining contamination suggests that not all sources of such contamination have been identified.

Another important food borne pathogen, *Listeria monocytogenes*, is of most concern in ready-to-eat (RTE) food products which require post-lethality handling and are capable of supporting its growth. While there are significantly fewer cases of domestic listeriosis compared to other foodborne pathogens, about 2,500 each year, approximately 20% of these infections end in fatality (CDC, 2005). Several strategies have demonstrated the successful control of *L. monocytogenes* in processed meats, although it is imperative that the limitations of such strategies are well understood and addressed. For these reasons, *L. monocytogenes* and *E. coli* O157:H7, as foodborne pathogens, are the focus of this dissertation.

1.2 Objectives

The overall objectives of this dissertation were to increase the current knowledge of the behavior of *E. coli* O157:H7 and *L. monocytogenes* in response to common procedures and conditions encountered during beef fabrication and during home storage and preparation of processed meat products. The specific objectives of this dissertation were:

I. To determine the fate of aerobic bacteria and *E. coli* O157:H7 in slaughter-floor runoff fluids at 4ºC, and to validate or eliminate concerns associated with tears and cuts in the subcutaneous fat layer of beef carcasses that are created during mechanical hide removal and may harbor slaughter-floor decontamination fluids as well as bacteria.

II. To evaluate the ability of *E. coli* O157:H7 to form biofilms on surfaces of materials used to construct beef fabrication equipment and utensils, and the effects of different environmental factors associated with beef fabrication

processes and facilities on the formation of such biofilms. Thus, individual experiments were designed to:

i. Identify the inoculation method and substrates which facilitate the greatest level of *E. coli* O157:H7 attachment and/or transfer onto surface materials;

ii. Examine the potential for *E. coli* O157:H7 biofilm formation on materials used to construct cutting surfaces and equipment found in beef fabrication facilities;

iii. Examine the effect of surface preconditioning compared to clean, dry surfaces on attachment of *E. coli* O157:H7 onto meat contact surface materials;

iv. Measure the influence of an air-liquid interface (instead of complete submersion of the surface in fluid) on the formation of *E. coli* O157:H7 biofilms on meat contact surface materials; and,

v. Examine the effect of post-contamination hydration on the survival of *E. coli* O157:H7 attached to meat contact surface materials.

III. To investigate the efficacy of different sanitizing solutions against *E. coli* O157:H7 biofilm cells on stainless steel, acetal and high-density polyethylene, and the relationship between age of biofilm cells, type of surface material, and sanitizer efficacy.

IV. To evaluate the effects of freezing, thawing method, and post-thawing aerobic storage on the fate of *L. monocytogenes* on frankfurters formulated without or with potassium lactate (1.5%) and sodium diacetate (0.1%).

CHAPTER TWO

Review of Literature

2.1 *Escherichia coli* O157:H7

2.1.1 Introduction

Escherichia coli account for the majority of the facultative flora found in the human gastrointestinal tract (Falkow, 1996; Nataro and Kaper, 1998), which, once colonized, shed these organisms into the environment. *E. coli* have been shown to persist in both predictable and unusual locations for significant lengths of time, and their ability to survive nutrient starvation, exposure to low pH (≤ 4.5), and thermal or osmotic stresses has been documented (LeJeune et al., 2004; Stopforth et al., 2003a, 2003b). Early foodborne *E. coli* O157:H7 infections were primarily associated with raw, undercooked, and ready-to-eat (RTE) beef products (Bell et al., 1994; CDC, 1995; Griffin and Tauxe, 1991; Riley et al., 1983; Tilden et al., 1996; Tuttle et al., 1999). Additional foodborne outbreak vehicles include unpasteurized (raw) milk, butter and soft cheeses made from raw milk, cheese curds, yogurt and ice cream, unpasteurized apple juice and cider, melons, grapes, radish and alfalfa sprouts, coleslaw and bagged lettuce and spinach (Besser et al., 1993; CDC, 1997, 2000a; 2001; Karmali, 1989; Morgan et al., 1993; NIIDIDCD/MHWJ, 1997; Rangel et al., 2005; http://www.fda.gov/ola/2006/foodsafety1115.html; http://www.fda.gov/Food/ScienceResearch/ResearchAreas/SafePracticesforFoodProcesses/ucm091265.htm). As is the case for most foodborne bacterial pathogens, the very

young, the elderly, and the immuno-compromised generally contract the most severe types of infection (Souza et al., 2002). The incidence of foodborne *E. coli* O157:H7 infections has decreased over time due to considerable efforts to control its presence in food, and in 2009, the Healthy People 2010 goal of ≤ 1.0 *E. coli* O157:H7 infections per 100,000 U.S. residents was met (CDC, 2010a). Nevertheless, control efforts require continued dedication as this pathogen continues to be isolated from finished beef products during routine testing before and after their release into commerce.

2.1.2 Characteristics

E. coli are members of the *Enterobacteriaceae* family, along with other enteric gram-negative bacteria, such as *Salmonella* and *Shigella*, and are known for their ability to adapt and colonize a diverse array of reservoirs, including open-air environments and the gastrointestinal tracts of mammals and birds (Falkow, 1996; Nataro and Kaper, 1998). These organisms are capable of facultative respiration, in which citrate, NO_2 and NO_3 take the place of oxygen in the electron transport chain (Stewart, 1988), and can also synthesize all metabolic requirements from glucose (Sussman, 1997). *E. coli* are mesophilic organisms which replicate at temperatures of 7 to 45ºC. Under optimal temperature conditions (35-40ºC, 95-104ºF) they can replicate at a pH of 4 to 10, and in the presence of up to 8% sodium chloride. A minimum water activity of 0.95 is typically required for survival (ICMSF, 1980, 1996). The manipulation of one or more growth factors may influence the minimum or maximum values of other factors involved in microbial survival or growth.

2.1.3 Human Illness

While most strains of *E. coli* are not human pathogens, a small population of them is responsible for three types of human illness, including neonatal meningitis, chronic urinary tract infection, and gastroenteritis (Buchanan and Doyle, 1997; Johnson et al., 2002). The most common symptoms associated with human *E. coli* O157:H7-related gastroenteritis include loose, watery stools (diarrhea), and mild to severe abdominal pain and/or cramping. Less frequently observed symptoms include mild fever, headache, nausea, or vomiting. Illness is not common in healthy adults and if encountered may be mistaken for a mild case of viral gastroenteritis, more commonly known as the "stomach flu". An increased risk of infection in healthy adults may be attributed to genetic predisposition (i.e., blood type) or the use of oral contraceptives and antibiotics (Begue et al., 1998; Berger et al., 1989; Besser et al., 1999; Wittels and Lichtman, 1986).

Those with immature or otherwise impaired immune systems are at an increased risk of contracting both mild and severe illnesses, and are also more likely to sustain secondary infections and chronic health complications (Besser et al., 1999). Symptoms of severe *E. coli* O157:H7 infections may include bloody diarrhea (hemorrhagic colitis), renal (kidney) malfunction and failure, thrombocytopenia (inadequate platelet count), or microangiopathic hemolytic anemia (lysis of red blood cells); patients with hemolytic uremic syndrome (HUS) exhibit all of these symptoms (Bacon and Sofos, 2003; Begue et al., 1998; McCrae and Cines, 2000). Persons with O157-Shiga toxigenic *E. coli* (STEC) infections are also more likely to experience bloody diarrhea and develop HUS than individuals infected with non O157-STEC strains (CDC, 2007). Classic HUS is generally

observed in children less than five years of age, while adults are more likely to develop a different form of HUS characterized by a lack of prodromal diarrhea (D-HUS) (McCrae and Cines, 2000). D-HUS is often misdiagnosed as thrombotic thrombocytopenic purpura (TTP), a similar, yet unrelated, disease which can lead to extensive and irreversible neurological damage (Boyce et al., 1995). In some cases, seizure, stroke, herniated bowels or chronic renal malfunction may occur (Buchanan and Doyle, 1997). Dehydration and malnutrition due to decreased nutrient uptake by damaged intestinal lining may also be observed in patients with prolonged or chronic cases of diarrhea (http://www.cfsan.fda.gov/~ebam/bam-4a.html).

2.1.4 Mechanisms of Virulence

2.1.4.1 Colonization of host cells

E. coli cells continually exchange genetic material with related and unrelated species of bacteria. This process has allowed each class of diarrheagenic *E. coli* to acquire a unique array of virulence genes (Souza et al., 2002). Enterohemorrhagic *E. coli* (EHEC), including *E. coli* O157:H7, require the formation of attaching and effacing (A/E) lesions to colonize host intestinal cells (Torres et al., 2005). The genetic factors involved in the formation of these lesions are encoded at the locus of enterocyte effacement (LEE), also known as the pathogenic LEE island, which includes over 50 open reading frames. Several of the proteins encoded at this locus comprise the type III protein secretion system, which is integrally involved in the formation of A/E lesions. One major component of this system is the type III translocon (comprised of EspA, EspB, EspD) and Tir (or EspE), which locates target host cells and inserts Tir into the plasma membrane.

Tir then functions as protein receptor for the adhesin protein, intimin (eaeA) (DeVinney et al., 1999; Hartland et al., 1999; Isberg et al., 1987; Kenny et al., 1997; Taylor et al., 1998). The interaction of Tir and intimin initiates the signal cascade required for the degradation of host epithelial cell microvilli at the site of bacterial effacement, the reorganization of the host cell cytoskeleton, and pedestal formation (DeVinney et al., 1999; Knutton et al., 1998; Moon et al., 1983; Singleton, 2004). Host colonization is generally retarded in Tir^- strains, demonstrating the essential role of Tir in this process (Hartland et al., 1999; Kenny et al., 1997). Once the lesion is formed and the pedestal protrudes into the lumen, other Type III proteins and toxins are injected into the host cell, initiating a second signal cascade involving protein kinase C, tumor necrosis factor (TNF), and interleukin-8 (Moon et al., 1983; Knutton et al., 1998; Singleton, 2004). In humans, TNF induces a 10-100 fold increase in expression of Shiga toxin receptors on the surface of vascular endothelial cells (Van de Kar et al., 1992). Interleukin-8 drives the translocation of host neutrophils across the epithelium, which can damage host mucosal lining and result in fluid secretion (Zhou et al., 2003; Van de Kar et al., 1992).

2.1.4.2 Toxins

Shiga toxins (also known as Shiga-like toxins or verotoxins), similar to those produced by *Shigella dysenteriae* (Sandvig, 2001), are the primary virulence factors of STEC strains, including *E. coli* O157:H7 and O157:NM, and the principal cause of hemorrhagic colitis and HUS in humans (Dean-Nystrom et al., 2003; O'Brien and Holmes, 1987). The interaction of Shiga toxins with host cell receptors Gb_3 and Gb_4 interrupts protein synthesis and triggers cell death (Sandvig, 2001). The number of receptors expressed by

host renal cells decreases dramatically in humans over 5 years of age, which may help explain why young children are predisposed to EHEC and STEC infections, hemorrhagic colitis, and HUS (Lingwood, 1994). Two types of Shiga toxins exist, Stx_1 and Stx_2, and currently available data indicate that strains which produce only Stx_2 are more commonly associated with HUS infections than strains that produce both Stx_1 and Stx_2, or only Stx_1 (Louise and Obrig, 1995). This may be due, in part, to the cumulative effects of the higher level of Stx_2 production in species which only produce the one toxin, a greater affinity of human host cells for Stx_2 relative to Stx_1, increased cytotoxic activity of Stx_2-specific cell types, and/or multiple Stx_2 toxin variants which induce diarrheagenic symptoms with an increasing range of severity (Beutin et al., 1989; Friedrich et al., 2002; Louise and Obrig, 1995; Obrig et al., 1988; Schmidt and Karch, 1996).

Hemolysins target and lyse red blood cells and erythrocytes, impede cytokine release, and interfere with neutrophile function (Bauer and Welsh, 1996; König et al., 1994; Russo et al., 2005). Hemolysins (i.e., α- , ß- or entero-hemolysin) are characterized based upon biochemical differences, extracellular location, and different species of hosts (O'Brien and Holmes, 1987). While α-hemolysin is not a mandatory factor, it is involved in the majority of HUS infections. Increased systemic iron, a metabolic requirement of *E. coli*, is caused by the hemolytic activity of α-hemolysin, potentially explaining this association (Schmidt and Karch, 1996). Other significant, yet less common, toxins associated with EHEC infections include cytolethal distending toxin and cytotoxic necrotizing factor (Aragon et al., 1997; Marques et al., 2003).

2.1.4.3 Other virulence factors

The virulence plasmid, pO157, present in all strains of *E. coli* O157:H7 and O157:NM, encodes genes for adherence factors, toxins, a catalase-peroxidase, and other factors of unknown importance (Brunder et al., 1996; Nataro and Kaper, 1998; Schmidt and Karch, 1996; Tatsuno et al., 2001). Motility factors (flagellar [H] antigens or the *fliC* gene), may also be involved in pathogenicity. For instance, flagellin proteins have been shown to up regulate the release of interleukin-8, highlighting a possible relationship between flagellin proteins and the degree of host intestinal mucosa inflammation (Berin et al., 2002; Steiner et al., 2000; Zhou et al., 2003).

2.2 *Escherichia coli* O157:H7 in Beef

2.2.1 Introduction

In 1994, the USDA-FSIS reacted to the health concerns, extensive media coverage, and consumer unease that stemmed from a 1992-1993 outbreak of *E. coli* O157:H7 infections associated with consumption of undercooked contaminated ground beef patties (Bell et al., 1994). In response, a "zero tolerance" policy was established to regulate the removal of visible contamination (soil, feces, milk or ingesta) on beef carcasses during slaughter (http://www.fsis.usda.gov/oppde/rdad/fsisdirectives/6420.2.pdf). *E. coli* O157:H7 was also classified as an "adulterant" in ground beef, and later (1999) in all non-intact fresh beef cuts (http://www.fsis.usda.gov/oa/background/ec0902.htm). The mandatory adoption of hazard analysis critical control point (HACCP) programs for all establishments under federal inspection was also implemented (Sofos, 2002, 2004; Sofos and Smith, 1998; USDA-FSIS, 1996). This has led to increased exploration of

commercially applicable technologies which control microbial contamination and allow finished beef products to comply with regulatory requirements, as well as with exisiting purchase specifications (Koutsoumanis and Sofos, 2004; Sofos and Smith, 1998; Stopforth and Sofos, 2006). While such efforts have clearly been beneficial, the beef industry continues to be plagued by *E. coli* O157:H7-related outbreaks and recalls. Ongoing issues indicate the need for continued research and development of *E. coli* O157:H7 mitigation strategies.

2.2.2 Distribution

Woerner et al. (2006) found that the prevalence of *E. coli* O157:H7 on beef carcasses prior to evisceration increased as pre-harvest pen floor fecal incidence increased. Apart from pre-evisceration, post-evisceration, and pre-chilled statistics regarding the level of beef carcass contamination during the slaughtering process, the level of contamination at other stages during the slaughter, chilling and fabrication process are difficult to determine since the sequential antimicrobial interventions applied by different processors may vary (Leistner and Gould, 2002; Sofos and Smith, 1998). Sofos et al. (1999a) found that *E. coli* biotpe I counts were systematically reduced as carcasses progressed through slaughter-floor processes and antimicrobial interventions. *E. coli* biotype I counts were recovered from 0.3 to 3.0% of carcasses after the final intervention and prior to carcass chilling. In a second study, the same group found that *E. coli* biotype I counts recovered from 90.0 to 99.2% of the carcasses presented to chilling fell within an acceptable range, according to the U.S. Meat and Poultry Inspection Regulations (USDA-FSIS, 1996), and that ≥ 90.0% of carcasses were also within an acceptable range 24 hours post-chilling

(Sofos et al. 1999b). In another study, *E. coli* counts ranged from 1.2 to 3.1 log CFU/100cm^2 on carcasses which had passed through the final slaughter-floor intervention and were presented to chilling at eight commercial packing facilities (Bacon et al., 2000). The authors also found that post-chilling (24 h) *E. coli* counts on beef carcasses were below the level of detection (0.9 log CFU/100 cm^2) in 7 of 8 packing plants examined.

It is well established that levels of microbial contamination increase rapidly and are maintained high on cutting table and conveyor belt surfaces during fabrication (Bacon et al., 2002). In one study conducted at Colorado State University, the overall level of total coliforms was greater in samples isolated from beef subprimals and steak samples than in those taken from whole beef carcasses 24 hr after chilling (Kain et al., 1999), indicating additional contamination events during handling, fabrication, packaging and distribution (Kain et al., 1999; Sofos et al., 1999a, 1999b, 1999c). Zhoa et al. (2001) collected raw beef samples from retail venues across Washington DC, and found that 19.0% were positive for *E. coli* O157:H7. In a similar study, 1.1% of ground beef samples (n = 1750) purchased from various supermarkets in the Seattle, WA area were positive for *E. coli* O157. More recently, an outbreak of *E. coli* O157:H7 infections was eventually traced back to contaminated ball tip sirloins that were ground at the retail level and therefore received no antimicrobial intervention just prior to grinding (http://origin-www.fsis.usda.gov/News_&_Events/Recall_034_2009_Expanded/index.asp). Collectively, these studies indicate that additional measures are needed to control contamination associated with incoming raw materials used to prepare retail beef products, and to minimize contamination events that originate at the retail level.

2.2.3 Outbreaks and Recalls

In 1982, *E. coli* O157:H7 was first recognized and classified as a human pathogen after outbreaks associated with improperly cooked hamburgers served at fast-food restaurants in Oregon and Michigan (Riley et al., 1983). Ten years later, improperly cooked hamburgers were linked to another large outbreak, which resulted in 501 illnesses, 45 cases of HUS, and 3 deaths (Bell et al., 1994). Between 1995 and 2009, ground and mechanically tenderized beef products have been associated with multiple outbreaks of *E. coli* O157:H7 infections (CDC, 1996, 2002a, 2010b; USDA-FSIS, 2005a). Between 1994 and 1998, several additional outbreaks of *E. coli* O157:H7 infections were associated with dry fermented sausage consumption (Alexander et al., 1995; MacDonald et al., 2004; Tilden et al., 1996; Williams et al., 2000). Prior to the outbreaks, published data had demonstrated the inability of existing fermentation, drying, and/or storage methods to inactivate the equivalent of 4 log cycles of *E. coli* O157:H7 in such products (Glass et al., 1992). In 1996, the USDA-FSIS mandated that all processors implement a combination of treatments and/or processes capable of inactivating ≥ 5 log cycles of *E. coli* O157:H7 in dry fermented sausage before retail distribution (USDA-FSIS, 1996). In response to continued outbreaks associated with consumption of *E. coli* O157:H7 contaminated salami products, it has been suggested that cells which survive the fermentation process may not be as susceptible to ensuing heat (56°C) treatments during processing (Buchanan and Edelson, 1999; Ryu and Beuchat, 1998). While most of these products do not permit growth or support extended survival of *E. coli* O157:H7 (Simpson Beauchamp and Sofos, 2009). The pathogen is tolerant to acidic environments and may survive long enough to cause foodborne illness if contaminated sausages are consumed shortly after production

(Glass et al., 1992; Naim et al., 2004). Outbreak-related contamination events may also occur during post-processing handling at the retail level (e.g., slicing and repackaging).

Outbreaks do not account for all, or even the majority of *E. coli* O157:H7 infections, although the food processing factors implicated in outbreaks most likely represent the most serious causative factors, which should also lead to isolated cases of illness (Rangel et al., 2005). The five most significant "foodborne illness risk factors" are: (i) acquiring products from unsafe sources, (ii) poor personal hygiene, (iii) inadequate heat treatments, (iv) improper holding temperatures, and (v) contaminated processing equipment (http://www.cfsan.fda.gov/~dms/retrsk2.html). Between 1998 and 2002, 46% of foodborne outbreaks were associated with one or more of the aforementioned risk factors (CDC, 2006). Seasonal and geographical factors may also influence outbreak and foodborne illness statistics, although such details were often overlooked in early prevalence data. While some prevalence studies have not observed seasonal trends (Alam and Zurek, 2004), *E. coli* O157:H7 is mesophilic, and therefore should be expected to thrive in warmer months and be less problematic during colder seasons (Simpson Beauchamp and Sofos, 2009). Thus, it is not surprising that more foodborne *E. coli* O157:H7 outbreaks are generally reported to the Centers for Disease Control and Prevention (CDC) during the summer and fall months (Hedberg et al., 1997; Rangel et al., 2005). Higher levels of environmental contamination, an increased prevalence of contaminated food products, and a greater frequency of outdoor picnic- or grilling-type events may also contribute to this trend.

2.2.4 Control Efforts

The most comprehensive strategy for improving the microbiological quality and safety of meat (Simpson and Sofos, 2009) includes the application of technologies that: (i) minimize the incidence and level of contamination associated with the live animal; (ii) minimize cross-contamination events involving the exterior of the animal and the slaughter environment, and the carcass or meat; (iii) reduce levels of contamination transferred during unavoidable events; (iv) inactivate cells that are transferred; and, (v) prevent or minimize the growth of surviving cells. Thus, successful control measures require effort at each stage of beef processing. Control of contamination on the carcass may be achieved through proper animal and hide cleaning, carcass decontamination interventions and carcass chilling (Simpson and Sofos, 2009; Sofos and Smith, 1998). The objective when applying multiple interventions should be to optimize the effect of individual treatments in order to achieve an additive or synergistic effect that is greater than the sum of individual treatments (Stopforth and Sofos, 2006).

The level of contamination carried on beef hides correlates with the subsequent proportion of contaminated carcasses (Woerner et al., 2006), and strategies to minimize hide contamination continue to be investigated. By reducing the number of animals shedding the pathogen, hide contamination and/or carcass contamination events during hide removal and evisceration may be minimized. Measures to control the colonization of cattle by *E. coli* O157:H7 include the use of probiotics, prebiotics, or other antimicrobial compounds in feed rations, water chlorination, vaccination programs, behavioral management and feedlot pen maintenance (Brashears et al., 2005; Callaway et al., 2005;

Diez-Gonzalez, 2005; Stopforth and Sofos, 2006). Unfortunately, these programs have yet to provide repeatable, lasting results when applied under realistic industry conditions, and additional research and developmental efforts are needed in this area.

When present, microorganisms can contaminate previously sterile inner tissue through cuts or tears in hides or outer tissues (Sofos, 2005; Sofos et al., 1999c; Woerner et al., 2006). Carcass tissue is sterile before hide removal, during which contamination may be transferred from the hide onto the carcass (Simpson Beauchamp and Sofos, 2009). The outer surfaces and trimmings from carcasses are generally intended for ground meat production in spite of the fact that these areas typically encounter the highest number of potential contamination events relative to other carcass locations (Gill, 2005). Thus, it is not surprising that the majority of fresh-meat related outbreaks and recalls involve ground meat (Rangel et al., 2005). Poor mechanical hide removal techniques can increase the incidence of hide-to-carcass transfer of pathogenic *E. coli*, as well as impacting the number of carcasses that fall to the floor from overhead rails and must then be diverted to areas reserved for trimming and additional FSIS inspection (Scanga, 2005). There may also be a greater incidence of hide and surface defects associated with carcasses processed using poor hide removal techniques (Scanga, 2005). The evisceration process may also generate high levels or increased incidence of contamination on carcasses if good practices are not used (Gill, 2005).

Employee behavior is critical in minimizing cross-contamination, and the most significant advances in process control almost certainly include those which address

worker education and hygiene (9 CFR 416.5). In-plant contamination issues may be reduced by continually reviewing the basic fundamentals of personal hygiene and hand washing, product and equipment handling, and appropriate behavior during illness (USDA-FSIS, 1999). Employee education and training courses are typically included in the prerequisite program portion of a HACCP program and should be designed to fit the needs of individual operations. Once proper behaviors are introduced and improper practices have been corrected, supervisors must provide access to and/or enforce the use of appropriate restroom and eating facilities, good hygiene practices, and behavior. The advent of mechanical devices as processing aids, which limit worker contact with food products, has also reduced cross-contamination events during processing (Scanga, 2005), although ineffective sanitation can nullify any potential benefit.

Knife trimming was implemented to remove localized visible contamination, and more specifically, to address the "zero tolerance" policy, which requires the removal of all visible soil, feces, ingesta, and milk contaminants from the surface of beef carcasses (USDA-FSIS, 1996). Even so, knives can easily transfer any contamination present (Bell, 1997). According to the Code of Federal Regulations (9 CFR 415.4) describing sanitary operations "all [direct and indirect] food contact surfaces including…utensils and equipment, must be sanitized as frequently as necessary to prevent the creation of insanitary conditions and the adulteration of product". In an effort to reduce the incidence of cross-contamination events between carcasses, knives may be rotated between consecutive carcasses (one knife is applied to a carcass, while the other knife is submerged in sanitizer) (Scanga, 2005). Other spot decontamination technologies include

steam pasteurization or steam vacuum units, which are used to remove visible contamination or to systematically treat the areas most likely to become contaminated during hide removal (Sofos and Smith, 1998). Attempts to remove contamination from the entire surface of every carcass using spot decontamination strategies are not feasible, especially at the chain speeds used to move carcasses in many domestic commercial facilities. Even so, spot decontamination efforts effectively reduce microbial counts and limit the spread of particulate matter over carcass surfaces during subsequent carcass washing steps (Gill, 2005).

The subcutaneous fat layer of beef carcasses may be cut or torn during mechanical hide removal. When carcasses are sprayed or rinsed with antimicrobial intervention fluids, these cuts or tears may allow fluids to collect in the areas where the outer fat layer has been pulled away from lean tissue. Resulting "pockets" of slaughter-floor fluids may remain in liquid form, or partially solidify during chilling, acting as harborages for spoilage and/or pathogenic bacteria. A recent study found that 0.5% (2/40) of such "pockets" were positive for *E. coli* O157:H7, although aerobic plate counts, total coliform counts, and *E. coli* Biotype I counts were all below the level of detection. These pockets are typically removed, along with the outer fat layer during fabrication, and are ultimately incorporated into ground product (Simpson et al., 2006). These harborages are easily eliminated, and in one plant, plastic film was tightly fastened over defects in the fat layer immediately after hide removal, before any decontamination fluids were applied (Simpson et al., 2006). Previous studies (Berry and Cutter, 2000; Brackett et al., 1994; Samelis et al., 2002; Stopforth et al., 2007) found that many spray interventions were

capable of significantly reducing levels of natural flora, while allowing the survival of acid-stressed *E. coli* O157:H7 during cold storage. Similarly, *E. coli* O157:H7 survived for 13 d in 2% lactic acid run-off fluid (Berry and Cutter, 2000; Samelis et al., 2004). One study also indicated that *E. coli* O157:H7 cells exposed to low but sublethal pH conditions during chilling (pH 4.89-5.22; 4°C) were increasingly tolerant to future acid exposures (Stopforth et al., 2007).

Carcass decontamination is incorporated into most HACCP programs to address microbial contamination introduced during slaughter. The decontamination of primals, subprimals and cuts is also being addressed in an increasing number of HACCP programs. Ideal raw meat decontamination strategies should not alter the organoleptic properties of the product, leave residues, damage the environment, harm plant personnel or consumers, or be of concern to consumers, public health officials, regulators or legislators. Such interventions should also be effective against multiple pathogens, be economical and simple to mix and apply, and should possess the ability to control pathogen growth without masking spoilage (Corry et al., 1995; Sofos and Smith, 1998). Countless technologies have been investigated in pursuit of the ideal antimicrobial intervention, as well as the most ideal sequence or combination of independently successful interventions (Sofos, 2002; Stopforth and Sofos, 2006). The majority of compounds which are approved for use in fresh meat decontamination are not considered "ingredients," and are typically referred to as antimicrobial interventions or processing aids. Interventions may be approved by the FSIS if they: (i) are generally recognized as safe (GRAS), (ii) do not lead to adulteration, (iii) do not create labeling issues (i.e. added

ingredients), (iv) are scientifically proven to be efficacious, and (v) do not pose human health issues to workers or consumers (USDA-FSIS, 2003a, 2008a). It is during the transition from hide-on carcasses to sides of beef that product contamination first becomes an issue. Thus, the majority of decontamination interventions are applied to beef carcasses after hide removal, before and/or after evisceration, before chilling, and to a lesser extent, following chilling and during fabrication (Stopforth and Sofos, 2006). Commonly used decontamination treatments applied during slaughter and dressing include rinsing carcasses (pre-evisceration) or carcass sides (post-evisceration) with an organic acid solution (lactic or acetic) or other chemical solutions, and thermal treatments involving hot water or steam.

Hot water is an economical and effective carcass decontamination solution although it lacks the residual antimicrobial activity associated with chemical treatments (Graves-Delmore et al., 1997; Ikeda et al., 2003; Koutsoumanis et al., 2004; Reagan et al., 1996). Castillo et al. (2003) examined the efficacy of a 9 second water wash, followed by a 30 sec water or ozone wash and found that aqueous ozone did not reduce populations of *S.* Typhimurium or *E. coli* O157:H7 on inoculated beef tissue more effectively than water washes alone. Other studies (Castillo et al., 1998; Cutter and Rivera-Betancourt, 2000; and Smith, 1992) investigated reductions of *Salmonella* spp. and pathogenic *E. coli,* including *E. coli* O157:H7, on inoculated beef tissue. When sprayed with hot water (72-95ºC) for 10 to 20 sec, *Salmonella* and *E. coli* O157:H7 populations in all experiments were reduced by ≥ 2 log CFU/cm^2. Level of reduction increased as water-spray temperature increased and hot water treatments applied at 72^{o}C, 80^{o}C, and 95^{o}C resulted

in ≥ 3.7, ≥ 3.0 and ≥ 2.0 log CFU/cm^2 reductions in *Salmonella* and pathogenic *E. coli*, respectively (Castillo et al., 1998; Cutter and Rivera-Betancourt, 2000; Smith, 1992). In general, hot water temperatures should be adjusted to induce the largest possible bacterial reductions while minimizing negative impact on carcass quality or appearance (Sofos and Smith, 1998). The use of steam as a thermal antimicrobial treatment is also approved for use in the U. S. In one study, a 6 sec steam pasteurization treatment reduced bacterial populations by 1 to 2 logs without inducing discoloration (Gill et al., 1998). In another study, Nutsch et al. (1998) found that the incidence of generic *E. coli* on beef carcass surfaces was reduced from 68% to 15% after the application of a patented steam pasteurization™ treatment (82.2ºC, 6.5 sec). Others have reported even greater pathogen reductions using steam pasteurization, although these treatments exceeded the 6 sec interval recommended to minimize quality defects (Gill et al., 1998; Phebus et al., 1997; Retzlaff et al., 2004).

The use of organic acid to reduce microbial contamination on beef carcasses and parts is widely practiced in the U. S. (Cutter and Rivera-Betancourt, 2000; Sofos, 2005; Sofos et al., 1999a, 1999b, 1999c, 2006). Organic acids are weak acids, which are less likely to dissociate and donate a proton to neighboring water molecules, and thus, organic acids enter cells in their undissociated state. Dissociation of the acid inside the cell forces the organism to expend energy as it attempts to remove excess protons and maintain homeostasis (Doores, 2005). Processes responsible for uptake of extracellular nutrients may also be shut down as cellular proteins, nucleic acids, and membrane components are affected by exposure to low intracellular pH (Beuchat, 1998; Langworthy, 1978).

The pH at which equal proportions of dissociated and undissociated molecules are present in a solution is defined as the dissociation constant (pK_a) of an acid, and antimicrobial activity generally increases as pH conditions approach the pK_a (Stratford and Eklund, 2003). The partition coefficient (log P_{oct}) is a measurement of the preference of a compound for the octanol or water phase fraction of a biphasic test system, and positive log P_{oct} values are indicative of the lipophilicity of a compound (Leo et al., 1971). Correspondingly, the antimicrobial activity of organic acids is pH-dependent and also influenced by degree of water and lipid solubility (Davidson, 2001; Doores, 2005; Freese et al., 1973; Leo et al., 1971). Lipid soluble acids (e.g., sorbic and propionic) are superior antimicrobial agents, as they are able to penetrate the outer lipid membrane of microbial cells (Davidson, 2001; Freese et al., 1973), but are less effective in high fat products, as their increased affinity for the lipid phase limits the amount of acid present in the aqueous phase of these products. Sodium chloride may reduce the log P_{oct} of organic acids (Stratford and Eklund, 2003), allowing some manipulation in their range of applications.

Solutions (2 to 5%) of organic acids may be applied at temperatures ≥ 55ºC as water-based sprays, rinses or dips. Lactic and acetic acid are used more commonly than citric acid and their efficacy, alone and in combination with other interventions, is well established (Dickson and Siragusa, 1994; Hardin et al., 1995; Prasai et al., 1991, 1997; Sofos, 2005; Stopforth and Sofos, 2006). Lactic acid (CH_3- CH(OH)-COOH; log P_{oct} -0.620) is produced during fermentation by lactic acid bacteria (LAB) and exhibits

varying levels of activity against Gram-negative and -positive bacteria, and fungi (Sofos et al., 1998). The acid is characterized as a hygroscopic, syrupy liquid, with a moderately strong acidic taste (Doores, 2005; IOM, 2003; Leo et al., 1971) and daily intake of lactic acid is not limited (FAO/WHO,1966), although L- and DL-lactic acid are prohibited in infant formulas (Doores, 2003). Acetic or ethanoic acid (CH_3-COOH; log P_{oct} -0.319) is a monocarboxylic acid that occurs naturally in plant and animal tissues and is also a byproduct of ethanol oxidation by *Acetobacter*, *Gluconobacter* and other heterofermentative strains of LAB (Doores, 2003; Leo et al., 1971). Homofermentative LAB, gram-negative and gram-positive bacteria, yeasts and some mold species exhibit various degrees of sensitivity to acetic acid (Doores, 2005). Daily intake of acetic acid is not limited for humans, regardless of age, and acetic acid may be used in infant formulas (FAO/WHO, 1966, 1974). However, acetic acid is characterized by a distinctive flavor and odor profile, and thus is self-limiting in food applications.

The antimicrobial activity of organic acid decontamination treatments depends on temperature, pressure and duration of application, acid concentration, type of tissue being treated, and product storage conditions prior to and following treatment (Cords et al., 2005; Durán and Sofos, 2006; Hardin et al., 1995; Sofos et al., 2006). In one study, Cutter and Rivera-Betancourt (2000) estimated that 2% lactic or acetic acid sprays reduced levels of *E. coli* O157:H7 and *S.* Typhimurium DT104 populations by $\geq$ 2 log CFU/cm^2. Other groups have also reported similar results (Dickson, 1992; Ransom et al., 2003). By applying organic acids at higher pressure and at an elevated temperature (55ºC), additional microbial reductions may be achieved (Cutter et al., 1997; Hardin et al.

1995; Venkitanarayanan et al., 1999). In one study, post-evisceration spraying treatments (55ºC) of 1.0% lactic acid were more effective than pre-evisceration treatments in reducing microbial contamination on inoculated beef tissue (Prasai et al., 1991). Correspondingly, 1.5% lactic acid spray treatments were more effective in reducing microbial counts on beef strip loins when applied after storage, compared to pre-storage treatments (Prasai et al., 1997). When added to the water used to spray beef carcasses during the first 12 h of chilling, 2% lactic acid reduced acid-adapted and non-acid adapted *E. coli* O157:H7 populations by 2.3 and 4 log cycles after 48 hours, respectively, while counts on samples receiving a water-only spray were reduced by about 1 log after 48 h (Stopforth et al., 2004).

Sublethal organic acid treatments may facilitate the development of acid-resistant pathogens that are increasingly tolerant to ensuing control measures including sanitation, fermentation and cooking (Duffy et al., 2000; Davidson and Harrison, 2002; Samelis and Sofos, 2003). Therefore, the parameters associated with organic acid treatments in single or multiple hurdle strategies should be optimized to avoid sublethal exposures. Acids are generally more effective against a broader range of microorganisms when used in combination with additional antimicrobial agents, including compounds which interfere with cell membrane function and/or increase membrane permeability (Kadner, 1996) and other organic acids (Adams and Hall, 1988; Lueck, 1980; Malicki et al., 2004). Chapters and reviews dedicated to the characteristics and uses of organic acids, esters and salts are extensively available in the literature (Bogaert and Naidu, 2000; Chipley, 2005; Doores,

2005; Drosinos et al., 2006; Glass et al., 1999; Marshall et al., 2000; Sharma, 2000; Sofos, 2000; Stratford and Eklund, 2003; Wicklund et al., 2005; and Zhu et al., 2005).

Cetylpyridinium chloride (CPC) is a surface-active quaternary ammonium compound (QAC) commonly added to toothpaste, mouthwash, and cough drop formulations due to its ability to impede attachment of plaque-forming bacteria to tooth enamel (McDonnell and Russell, 1999; Oyarzabal, 2005). Available data also indicate that CPC (0.5-1.0%) is capable of significantly reducing pathogen populations on meat and poultry products encountered at multiple stages during processing (Belk, 2001; Huffman, 2002; Sofos and Smith, 1998; Sofos et al., 1999a). Safe Foods Corporation (North Little Rock, AR) has released a commercial patented CPC solution (pH 7.1) called Cecure™ (Safe Foods Corporation, 2008). Ransom et al. (2003) compared the ability of 0.5% CPC and other, approved intervention technologies to inactivate *E. coli* O157:H7 populations on inoculated beef carcass tissue samples and found that CPC induced greater reductions than 2.0% lactic acid, 0.02% acidified sodium chlorite, 2.0% acetic acid, 0.02% peroxyacetic acid, 1.0% lactoferricin B, or 0.001% acidified chlorine (reductions of 4.8, 3.3, 1.9, 1.6, 1.4, 0.7 or 0.4 log CFU/cm^2, respectively). Reductions in pathogen counts on CPC-treated lean beef tissue were also greater than other treatments (Ransom et al., 2003).

Trisodium phosphate (TSP) has been approved for use in the U. S. beef and poultry industries, has been shown to reduce bacterial contamination, and may also reduce bacterial attachment on carcass surfaces (Cabedo et al., 1996; Gorman et al., 1997; Sofos

et al., 1999a, 1999b, 1999c). In one study, Arrit et al. (2002) examined the ability of 0.5% CPC, 10.0% TSP or 0.1% acidified sodium chlorite (ASC) treatments (30 s to 10 min) to inactivate *Campylobacter jejuni* attached to poultry skin and found that CPC treatments were more effective than TSP or ASC. Ten-minute pre-inoculation treatments of 0.1% CPC at ambient temperature inhibited the attachment of *S.* Typhimurium to poultry skin, indicating the potential for very low concentrations of CPC to mitigate future contamination events (Breen et al., 1995). When added to the water used to spray beef carcasses during chilling, CPC treatments (0.1 or 0.5%, 30 sec spray, every 30 min for 10 h) reduced inoculated non-acid adapted and acid-adapted *E. coli* O157:H7 (5.1 log CFU/cm^2) on carcass tissue to below the level of detection after 10 and 24 h at 4°C, respectively; water-only spray treatments reduced populations by about 1 log CFU/cm^2 (Stopforth et al., 2004). When applied to RTE polish sausages inoculated with *Listeria monocytogenes* (3 to 7 log CFU/g), CPC (1%, 25ºC, 30 s, 20 psi spray) immediately reduced pathogen counts by 1 to 3 log CFU/g and inhibited survivors for 42 d (0 and 4ºC) without negatively impacting product color or texture (Singh et al., 2005).

Hydrogen peroxide (H_2O_2), also known as oxygenated or oxidizing water, occurs naturally inside mucous membranes and neutrophils, is a component of some foods, and may also be generated in food products during fermentation by LAB or during irradiation processes (Cords et al., 2005; Kuby, 1997; Lewis et al., 2002). Hydrogen peroxide dissociates into a single molecule of water and one hydroxyl radical, and does not generate carcinogenic or mutagenic compounds (Cords and Dychdala, 1993; Gleason et al., 1969). Hydroxyl radicals (also known as free radicals) are known to damage cell

membranes and DNA; significant amounts of damage can result in cell death or initiate cellular suicide mechanisms (Cords and Dychdala, 1993). Viruses, fungi, bacterial cells, and bacterial spores are all susceptible to hydroxyl radical-induced damage, and Gram-negative bacteria are more susceptible than gram-positive types (Cords and Dychdala, 1993). Optimal antimicrobial activity occurs at high temperatures, an acidic pH, and in the absence of organic material. A greater number of hydroxyl radicals are also generated in solutions that contain salts and/or metallic ions (Schumb et al., 1955). Antimicrobial activity is reduced at cold temperatures, or in the presence of alkaline pH, fats and proteins. Peroxyacetic acid (PAA) is another peroxide-based compound with an antimicrobial spectrum and mechanism of killing similar to that of hydrogen peroxide (Cords and Dychdala, 1993; FAO/WHO, 2004; Gagnaire et al., 2002). Optimal applications of PAA occur under acidic pH and in the absence of organic material. Ambient to cold temperature applications are effective even though greater efficacy is observed at higher temperatures. The presence of metallic ions (hard water) does not reduce antimicrobial activity (Cords et al., 2005; Gutzmann et al., 2000).

Oxidizing agents used in decontamination solutions have been shown to cause visible bloating and/or bleaching of lean tissue, although this type of damage is observed less frequently when using PAA than hydrogen peroxide (Hilgren and Gutzmann, 2002). Corrosion of food processing equipment is also less problematic when using PAA (Bricher, 2005). The FDA has approved Inspexx 200^{TM}, a 2% peroxyacetic acid solution mixture manufactured by Ecolab Inc. (Saint Paul, MN) (Ecolab, 2008). The manufactures of Inspexx 200^{TM} recommend use of PAA-specific dispensing equipment in order to

optimize results. PAA has been proven as a successful antimicrobial intervention for the decontamination of fruits and vegetables (FAO/WHO, 2004; Parish et al., 2003; Park and Beuchat, 1999); however, the majority of published data indicate that PAA is not as effective as other chemicals against microbial contamination associated with meat products (FAO/WHO, 2004). Two recent studies showed that PAA treatments were less effective than 2% lactic acid in reducing *E. coli* O157:H7 on beef cuts and trimmings (Ransom et al., 2003; Ellebracht et al., 2005). In another study, 0.02% PAA was added to the water spray applied to beef carcass tissue (30 sec, every 30 min for 10 h) during simulated chilling (-1 ± 2ºC, 48 h), and after 48 h, *E. coli* O157:H7 counts on PAA-treated tissue were similar to non-treated and water-treated controls (Stopforth et al., 2004). The use of PAA as a post-chilling antimicrobial intervention for beef carcasses and trimmings was also examined. While PAA (1000 ppm, 43-55ºC, spray) reduced *E. coli* O157:H7 counts by 1.7 log CFU/cm^2, lactic acid treatments (4%, 55ºC, spray) resulted in significantly higher reductions (2.7 log CFU/cm^2) (King et al., 2005). Despite such findings, the beef industry continues to use PAA-based spray treatments to decontaminate beef products at the fabrication level. It would be valuable to better understand the basis behind the industry's adoption of post-chilling PAA treatments despite such poor performance in published trials.

Several chlorine compounds are approved for use as food animal carcass decontaminants at various levels of free available chlorine (Cl_2, HOCl, OCl^-), and are generally effective against Gram-positive, Gram-negative and acid-fast bacteria, fungi and viruses (McDonnell and Russell, 1999). The antimicrobial efficacy of most chlorine compounds

is diminished in the presence of excessive organic material, an alkaline pH and when mixed using a hard water source (Cords et al., 2005; Kaczur and Caulfield, 1994). Thus, for optimal efficacy, chlorine solutions should be replaced with a fresh solution on a regular basis (Lillard, 1980). When combined with a weak GRAS acid (i.e., citric acid or sodium acid sulfate), sodium chlorite dissociates to produce chlorous acid (HO_2Cl) and chlorine dioxide (ClO_2) (Warf and Kemp, 2001). The antimicrobial activity of acidified sodium chlorite (ASC) is optimized at pH ≤ 2.5 and in the absence of organic material (EFSA, 2008).

Chilling methods and facilities can directly influence the level and incidence of microbial contamination on chilled carcasses. Simpson et al. (2006) found that, as surface temperature decreased to 4ºC within 9.33, 11.0, or 21.7 h following slaughter and during carcass chilling, total coliform counts on carcasses at approximately 48 h decreased, did not change, or increased, respectively. Certain slaughter-floor interventions may also provide lingering antimicrobial activity throughout storage. In one study, dipping *L. monocytogenes*-inoculated beef pieces in 75ºC water, 2% lactic at 55°C, or 2% acetic acid at 55°C before storage at 4 or 10°C all reduced pathogen populations by ≥ 1.4 log CFU/cm^2; however, after 14 d of storage at 4ºC, populations exposed to acid dip treatments were maintained at much lower levels than water treated populations (1.6 to 2.8 log CFU/cm^2 versus 3.6 to 4.6 log CFU/cm^2, respectively) (Ikeda et al., 2003). At 4ºC, the stationary phase of acid-treated populations was also longer then the stationary phase of water treated populations (Ikeda et al. 2003). Özdemir et al. (2006) found that the application of a 1% or 2% lactic acid spray alone or followed by hot water (82ºC)

resulted in immediate *S.* Typhimurium reductions and continued to reduce populations throughout 5 d of storage; d-0 counts were 0.05 to 1.19 log CFU/cm^2 compared to 0.43 to 1.78 log CFU/cm^2 at d-5. Dickson and Siragusa (1994) reported contrasting results; while 1% lactic or 1% acetic acid washes immediately reduced *S.* Typhimurium, *E. coli* O157:H7, and/or *L. monocytogenes* Scott A populations on sterile beef tissue, but no measureable reductions were observed during simulated carcass chilling (spray- and dry-chilling; 5ºC for 3 d). Additional studies are necessary to evaluate the efficacy and residual antimicrobial activity of common "final intervention" techniques. In one such study, Samelis et al. (2001) examined the fate of *L. monocytogenes*, *E. coli* O157:H7 and *S.* Typhimurium in decontamination fluids when stored at 4 or 10ºC. All inoculated pathogens survived in nonacidic fluids, and *S.* Typhimurium populations increased by approximately 2 log CFU/ml at 10ºC (Samelis et al., 2001; Samelis and Sofos, 2003).

As previously indicated, the efficacy of any antimicrobial intervention depends on several factors. Surface treatments may be applied as spray, cascade, rinse, or deluge applications and parameters associated with each such treatment (temperature, pressure, duration) should be adjusted until optimal activity and efficiency are achieved (Cutter et al., 1997; Sofos et al., 1999b; Sofos and Smith, 1998). Specific parameters influencing efficacy include the size, range, and angle of spray droplets (Pordesimo et al., 2002; Sofos et al., 1999c). More nozzles delivering a high-pressure spray (1.4 to 2.1 kPa) should also result in a marked increase in efficacy (Pordesimo et al., 2002; Sofos et al., 1999c). Greater microbial reductions may be observed when the surface contact time of a given treatment is extended (Arrit et al., 2002; Russell and Axtell, 2005; White, 1992) or when applied at

higher temperatures (Hardin et al., 1995). Immersion and flood treatments are commonly used in the pork and poultry industries as part of scalding and chilling processes. Correspondingly, organic load and the subsequent renewal schedule of antimicrobial solutions can significantly impact the consistency and degree of microbial reductions used in immersion treatments (Lillard, 1980; Yang and Johnson, 2001).

2.3 Biofilms in Food Processing Operations

2.3.1 Biofilm Formation

Biofilms contain diverse communities of microorganisms and organic compounds from the surrounding environment, encased by a protective layer of polysaccharides (Carpentier and Cerf, 1993). Biofilms represent a natural state of existence for most microbes (Davey and O'Toole, 2000). Medical implants and prostheses, contact lenses, fluid and food processing operations, and ship-hulls are all examples of environments burdened by the formation and persistence of biofilms. Biofilms in these environments can lead to human illness and infection, cross-contamination, mechanical blockages, the impedance of heat transfer and the corrosion and/or degradation of metals and polymers (Chmielewski and Frank, 2003; Mittleman, 1998; O'Toole et al., 2000).

While many cellular behaviors are involuntary, microorganisms may also differentiate, proliferate, adapt, or die based on the chemical or environmental stimuli they encounter (Pollard and Ernshaw, 2004). Such stimuli trigger specific signal transduction pathways, which set into motion a number of cellular responses that alter the eventual fate of a cell. Some researchers attribute the initiation of biofilm formation to low nutrient availability

or starvation conditions (Brown et al., 1977; Mittelman; 1998). Other factors involved in initial cell attachment and biofilm formation include temperature, pH, osmolarity, oxygen, and the availability of iron (O'Toole et al., 2000). While the behavior and characteristics of biofilm cells are not well understood, the basic stages of biofilm formation have been well established. Cells first attach reversibly to a surface via van der Waals forces and electrostatic and hydrophobic cell-surface interactions (Chmielewski and Frank, 2003). Reversible attachment requires as little as 5 to 30 sec, and can be reversed by applying very little force (Chmielewski and Frank, 2003; Mittleman, 1998). "Irreversible" adhesion is mediated by the proteins responsible for anchoring cells and the production of protective polysaccharides (Davey and O'Toole, 2000). The interval between reversible and irreversible adhesion is unclear, although, in one study, polysaccharide production by gram negative bacteria was observed between 5 and 6 h after attachment (Allison and Sutherland, 1987). Drastic measures, which tear or break chemical bonds within appendages linking cells to a surface, are required to remove irreversibly attached cells (Chmielewski and Frank, 2003).

Surface properties of a substance can also impede or promote initial attachment. Hydrophilic surfaces, with high free surface energies (e.g., glass, stainless steel) tend to promote cellular attachment, while less hydrophilic surfaces (e.g., buna-N-rubber, Teflon, nylon) tend to retard biofilm formation (Blackman and Frank, 1996; Wong, 1998). The long-term significance of such differences has not been established. Surface hydrophobicity can be altered in the presence of a conditioning layer, a thin coating of compounds which temporarily modify the free energy, or other characteristics of a

substance (Chmielewski and Frank, 2003). Organic molecules and ions adsorbed to a surface may aid in bacterial attachment, as can the absorption of water molecules by a wet surface (Bryers 1987, McEldowney and Fletcher, 1986; Verran and Jones, 2000). The presence of additional microbial species may also influence cellular attachment and adhesion, and results appear to be highly specific, regarding the species in question, type of surface material, available nutrients, etc. (Hood and Zottola, 1995; Jeong and Frank, 1994; McEldowney and Fletcher, 1986). As the pili, flagellum, and outer surface proteins function much like Velcro® during cellular adhesion events, an increased number of blemishes, even if visually undetectable, will most likely provide sites for bacterial entrapment and elevate the incidence of established biofilms (Pratt and Kolter, 1998). The expression of such adhesion factors differs dramatically between microbial species, serotypes and strains, and is also influenced by cultivation conditions and stage of growth (Li and McLandsborough, 1999; Pratt and Kolter, 1998).

Once established, biofilms generally exhibit increased resistance to antimicrobials. Data indicate a greater resistance in "older" versus "young" (i.e., < 24 h) biofilm cells (Anwar et al., 1990; Lee and Frank, 1991). It is unclear, however, if resistance increases over time, or if cells are simply surrounded by an increasing number of dead cells and other organic matter which acts as a protective barrier against such antimicrobials. In one study, *L. monocytogenes* biofilm cells showed increased resistance to peroxide, quaternary ammonium compounds and chlorine sanitizers as they aged. However, when cells in older biofilms were detached from surface materials and exposed to sanitizing solutions planktonically, they exhibited the same level of resistance as control cells,

indicating that increased resistance was due to the presence of the extracellular biofilm matrix and not a phenotypic or genotypic cellular change (Pan et al., 2006). In either case, an increased resistance to previously lethal interventions only exacerbates the consequences associated with biofilms. Older biofilms are also increasingly prone to the sloughing off of outer cell layers, which leads to the re-distribution of cells and the potential establishment of new biofilms or cross-contamination of foods (Chmielewski and Frank, 2003).

2.3.2 *Escherichia coli* O157:H7 Biofilms in Food Processing Facilities and on Meat.

The ability of *E. coli* to form biofilms *in vivo* and *in vitro* is documented (Van Houdt and Michiels, 2005). In general, flagellum-mediated motility is required for biofilm formation by *E. coli* cells (Van Houdt and Michiels, 2005). Other genetic determinants for "biofilm formers" include the expression of curli, fimbriae, and/or conjugative pili, as well as the production of cellulose, autotransporter proteins, and/or exopolysaccharide (Van Houdt and Michiels, 2005). While the absence of one or more of these factors may not affect the ability of a cell to attach onto a material, subsequent biofilm formation may be impaired (Ryu et al., 2004a, 2004b). The ability of *E. coli* O157:H7 to form biofilms varies by strain; however, even those strains which are poor biofilm formers may become integrated into heterogeneous biofilms established by natural flora (James et al., 1995). Some cells may be more tolerant to inhibitory factors even after being incorporated into a biofilm, and in one study *E. coli* O157:H7 biofilm cells which were positive for exopolysaccharide production and curli expression exhibited an increased resistance to

chlorine, as compared to *E. coli* O157:H7 biofilm cells that did not express/produce these factors (Ryu and Beuchat, 2005).

The attachment of bacteria, specifically *E. coli* O157:H7, to muscle and adipose tissue has also been a topic of investigation, and appears to be mediated by the factors described above; surface charge, hydrophobicity, cell-surface structures, and conditions during microbial cultivation and attachment (Bouttier et al., 1994; Butler et al., 1979; Chung et al., 1989; Dickson and Koohmaraie, 1989; Li and McLandsborough, 1999; Rivas et al., 2006). In one study, the ability of STEC isolates, cultivated as planktonic or as sessile cells, to attach to beef muscle and adipose tissue was examined (Rivas et al., 2006). Sessile cells attached more strongly than planktonic cells to both types of tissue, and while some STEC strains attached to either tissue with equal affinity, the majority of strains, regardless of cultivation conditions, attached more strongly to beef adipose tissue (Rivas et al., 2006). Dickson and Koohmaraie (1989) found that the major contributing factor to lean tissue attachment was the net negative charge of the microbe, and speculated that the hydrophobic properties of adipose tissue also encouraged microbial attachment. When the preference of different species of bacteria for muscle versus adipose tissue was examined, *E. coli, L. monocytogenes, Serratia marcesens* and *Staphylococcus aureus* exhibited a greater affinity for adipose, while *S.* Typhimurium and *Staphylococcus epidermidis* attached more strongly to lean tissue, while *Bacillus subtilus* attached to both types of tissue in equal ratios (Dickson and Koohmaraie, 1989).

Aside from attachment to carcass tissue, common biofilm reservoirs in food processing operations include floors, walls, waste water pipes, bends in pipes, crevices, Buna-N rubber, Teflon seals and other gaskets on processing equipment (Blackman and Frank, 1996; Brightwell et al., 2006; Fletcher, 1985; Kumar and Anand, 1998; Mafu et al., 1990; Rivera-Betancourt et al., 2004). The adhesion of *E. coli* appears to be independent of hydrophobicity, but highly dependent on charge of a surface, and adheres more readily to surfaces with low negative charge (van Loosdrecht et al., 1987). *E. coli* O157:H7 has been shown to attach to stainless steel (Rivas et al., 2007; Ryu et al., 2004a, 2004b) and survive for 28 d at 4°C (Wilks et al., 2005), while copper and copper alloys were bactericidal. Even in light of such findings, the functionality of stainless steel minimizes the likelihood of its replacement in food processing facilities. Pathogens, including *E. coli* O157:H7, have also been recovered from fabrication-floor conveyor belt food contact surfaces during preoperational and mid-shift inspections (Rivera-Betancourt et al., 2004). Others have recovered viable bacteria from conveyor belt and carcass breaking and cutting surfaces, and inadequately sanitized food contact surfaces have been implicated in multiple outbreaks of foodborne illness (Chmielewski and Frank, 2003; Gill and Landers, 2004; Lindsay et al., 1996). Despite such findings, attachment and biofilm formation by *E. coli* O157:H7 strains on the plastic polymers used to construct the conveyor belts used during beef fabrication has not been thoroughly investigated.

The presence of *E. coli* O157:H7 in beef processing facilities, its ability to form biofilms on food contact surfaces, and the resistance of biofilm cells to traditional pathogen control measures are well documented. The combination of these factors has

undoubtedly contributed to the persistence of environmental and foodborne *E. coli* O157:H7 contamination and subsequent outbreaks of foodborne illness. However, the fate of *E. coli* O157:H7 biofilm cells in beef fabrication facilities is unclear. According to Chmielewski and Frank (2003), the significance of biofilms in food processing is not well understood because of the lack of direct observation of biofilms in this environment and a lack of research using model systems that closely simulate the food system environment. It would be useful to investigate the potential by different strains of *E. coli* O157:H7 recovered from beef cattle feces, beef products implicated in outbreaks, and human clinical cases to form biofilms on fabrication equipment and under realistic conditions encountered in commercial fabrication facilities. The efficacy of approved food contact surface sanitizers against the resulting *E. coli* O157:H7 biofilm cells should also be examined to establish the potential for biofilm persistence in these settings.

2.3.3 Biofilm Removal and Control

Attempts to remove irreversibly adhered biofilm cells from surfaces must first address an outer, highly resistant layer of polysaccharides. When cleaning plastics, Lewis et al. (1980) recommends the use of alkali or nonionic solutions, applied at temperatures between 40 and 90°C as determined by degree of soiling. Manual scrubbing should be incorporated into cleaning programs although most cleaning agents suggest effectiveness without scrubbing. Even highly effective cleaning protocols, which remove the vast majority of contamination, are not designed to inactivate detached cells, which may be translocated and adhere to other surfaces, and sanitizers are used to inactivate microorganisms which survive and/or are translocated during cleaning processes. The

most common sanitizing solutions used to decontaminate meat processing equipment and facilities include halogens, peroxygens, acids and QACs, and their effectiveness is influenced to different degrees by the presence of soil and organic material, water hardness, temperature of solution during its application, and the duration of contact with microbial cells (Chmielewski and Frank, 2003; Gibson et al., 1999; Kim and Frank, 1995).

QACs are commonly added to personal hygiene, pharmaceutical, food industry, and environmental sanitation products (IPCS, n.d.). They are characterized as surface active agents, or surfactants, due to amphiphilic regions of QAC molecules, which interact with and disrupt/damage cell membranes, ultimately resulting in the loss of action potential across the plasma membrane and eventual death (Oyarzabal, 2005). Viruses, fungi and bacteria are susceptible to QAC activity, although gram-negative bacteria are less susceptible than gram-positive types (Maxcy et al., 1971). QACs are more effective at higher temperatures and at acidic pH values (Cords et al., 2005), although optimal pH during application is generally microorganism-specific. Activity is reduced in the presence of organic matter (especially at low temperatures), in the presence of metallic ions (hard water source), or anionic surfactants, detergents or soaps. QACs form a film with residual antimicrobial activity, and thus, are not appropriate for use in facilities that use fermentative lactic acid cultures (i.e., yogurt, fermented sausages) (Chmielewski and Frank, 2003). Maxcy et al. (1971) examined acquired QAC-tolerance in *E. coli* and found that tolerant cultures grew more slowly in growth medium and were more sensitive to QACs and other inhibitory factors than parental cultures, and therefore, unlikely to persist

in the environment. The development of QAC-tolerance was dependent upon the structure and degree of affinity of each compound for microbial cells, as determined by adsorption, filtration and elution of remaining QACs after treatment (Maxcy et al., 1971).

Several chlorine derivatives are available to clean and sanitize food processing facilities. Sodium hypochlorite (NaOCl) is the most popular chlorine solution for commercial use (Khanna and Naidu, 2000). Biofilm cells do appear to be less sensitive to chlorine than planktonic cells (Simpson and Sofos, 2009). A commercial product containing potassium peroxymonosulfate and sodium chloride (PP/SC) is available for use as a broad spectrum hard surface disinfectant in livestock production and veterinary facilities, but is not approved to sanitize food contact surfaces. In general, PP/SC is active against both gram-positive and gram-negative bacteria, and antimicrobial activity is markedly, but not entirely reduced in the presence of organic material or when mixed using a hard water source (http://www.biosecuritycenter.org/content/labelClaim/virkonS.pdf). The efficacy of PP/SC solutions against biofilms, and specifically *E. coli* O157:H7 biofilm cells, is unclear.

Frank et al. (2003) investigated the efficacy of multiple cleaning agents in combination with different sanitizers against *L. monocytogenes* biofilm cells on stainless steel soiled with a moderate degree of food processing residue (i.e., chicken serum albumin and rendered chicken fat). The researchers found that static cleaning of stainless steel and sanitizer application was effective at controlling the pathogen. Cleaning with an alkaline cleaner and sanitizing with ASC was more effective than other programs, although QACs

and a PAA/octanoic acid mixture were also effective when applied after a cleaning compound (Frank et al., 2003). In one study, 200 ppm sodium hypochlorite solutions successfully detached *E. coli* biofilms from the surface of polished stainless steel, but not from scratched stainless steel (Lomander et al., 2004). In another study, sodium hypochlorite was found to more effectively inactivate *E. coli* on stainless steel than peracetic solutions (Rossoni and Gaylarde, 2000).

Eighteen different sanitizers were evaluated for their ability to inactivate *E. coli* O157:H7 or *Pseudomonas aeruginosa* at 10 or 20°C when suspended in a broth system with or without bovine serum albumin to simulate dirty or clean conditions, respectively (Taylor et al., 1999). Sodium hypochlorite and PAA/hydrogen peroxide solutions reduced both pathogens by ≥ 5 log CFU/ml after 5 min, at both temperatures under clean or dirty conditions (Taylor et al., 1999). Overall, the QACs were effective against *E. coli* O157:H7 and reduced the pathogen by ≥ 5 log CFU/ml after 5 min, but failed to reduce *P. aeruginosa* by ≥ 5 log CFU/ml under dirty conditions at 20°C, or under clean or dirty conditions at 10°C (Taylor et al., 1999). Of the 18 solutions, sodium hypochlorite was the least affected by temperature.

Farrell et al. (1998) examined the efficacy of common procedures and chemicals used to clean and sanitize meat grinders after grinding beef contaminated with *E. coli* O157:H7 (2 to 6 log CFU/g). Stainless steel chips were secured to the inside of a grinder and then removed for microbiological analysis after grinding, cleaning and sanitizing were complete. Approximately 3 to 4 logs of *E. coli* O157:H7 were attached to the inside of

the grinder after grinding beef with lean:fat ratios of 75:25, 80:20 and 90:10. Level of attachment was not influenced by fat content; the grinder was then washed with detergent (with or without manual scrubbing), rinsed and treated with chlorine or PAA. In that study: (i) grinding samples with higher fat contents generally resulted in a greater number of chips which were positive for the pathogen after cleaning and sanitation, indicating that fat content did not influence attachment but did influence the efficacy of cleaning and sanitation; (ii) between 88 and 100% of the chips were positive by enrichment after cleaning, and manual scrubbing decreased the number of positive chips; and (iii) chlorine and PAA were equally effective against *E. coli* O157:H7 although neither consistently eliminated the pathogen on stainless steel chips, regardless of fat content or the addition of manual scrubbing during cleaning. The results of this study also indicated that ATP bioluminescence and enrichment were more accurate indicators of sanitation efficacy when compared to plate count methods (Farrell et al., 1998).

Lomander et al. (2004) found that 200 ppm sodium hypochlorite solutions effectively inactivated *E. coli* biofilm cells on polished stainless steel, but were ineffective against biofilm cells on scratched stainless steel. In another study, sodium hypochlorite was more effective than peracetic acid solutions against *E. coli* on stainless steel (Rossoni and Gaylarde, 2000). Ryu and Beuchat (2005) examined formation of *E. coli* O157:H7 biofilms on stainless steel and the efficacy of sodium hypochlorite sanitizing solutions against biofilm cells and their planktonic counterparts. Under the conditions examined, *E. coli* O157:H7 strains did form biofilms (7.5 to 10.0 log CFU/coupon) on stainless steel, and biofilm cells were not reduced to below the level of detection (2.5 log CFU/coupon)

even after being exposed to 200 ppm chlorine solutions for up to 5 min (Ryu and Beuchat, 2005). Planktonic cells were reduced to below the level of detection, although cells cultured at 12°C were inactivated more slowly than those cultured at 22°C (Ryu and Beauchat, 2005).

The extracellular matrix that surrounds biofilm cells acts as a protective barrier, and the results of one study indicated that only 20% of a chlorine solution diffused through the surrounding layer to the biofilm cells (DeBeer et al., 1994). Two minute exposures to QAC sanitizer (1:64 v/v dilution) reduced planktonic *E. coli* O157:H7 (7.5 log CFU/ml) by 3.0 to 7.0 log CFU/ml, but were ineffective against biofilm cells on glass coupons (Uhlich et al., 2006). As part of the same study, 10 min exposures to 5.0% hydrogen peroxide reduced biofilm and planktonic *E. coli* O157:H7 populations to below the level of detection or by 1 to 4 log CFU/ml, respectively (Uhlich et al., 2006). Stopforth et al. (2002) also found that *L. monocytogenes* biofilm cells were less susceptible to sanitizer treatments compared to their planktonic counterpart; it was also noted that planktonic cells and biofilm cells that were detached from stainless steel coupons by vortexing in buffer that contained glass beads exhibited similar sensitivities to the sanitizer treatments. Under the conditions examined, Stopforth et al. (2002) concluded that PAA treatments were also more effective against cells in biofilm than cells in suspension. The authors attributed such findings to the ability of PAA to penetrate glycocalyx and theorized that PAA compounds may also possess an increased affinity for the proteinaceous and/or polysaccharide molecules that comprise the outer peptidoglycan layer which surrounds bacterial cells (Baldry, 1983; Jolivet-Gougeon et al., 1996).

In one study, which evaluated the effects of daily production cycles associated with food processing facilities on the fate of *L. monocytogenes* biofilm cells on stainless steel and Teflon, biofilm cells were subjected to 24 h daily cycles, with three phases per cycle: (i) 60 sec exposure to peroxide-based sanitizer or saline (control), (ii) storage (15 h) in dry sterile tubes, and (iii), incubation (8 h) in diluted growth media (Pan et al., 2006). Daily cycles were repeated for up to three weeks; during the first week, biofilm populations treated with the peroxide-based sanitizer were reduced by approximately 1.8 log CFU/cm^2, but then increased by an additional 2.0 log CFU/cm^2 during the following 2 weeks of storage (22.5°C) (Pan et al., 2006). After 3 weeks of storage, surviving pathogen populations exhibited an increased resistance to the peroxide-based sanitizer, as well as chlorine sanitizers and QACs (Pan et al., 2006). Manual scrubbing was not evaluated as part of this study.

When Virkon® S (1%) was added to footbaths and footmats, total aerobic bacteria counts were 1.4 log CFU/ml lower than counts on untreated or water-treated boots (Dunowska et al., 2006). While a second study reported similar results (Morely et al., 2005), another found that 1% Virkon® S treatments were ineffective against total microbial populations on soiled boots (Amass et al., 2001). Different rates of application, exposure time, microbial populations, and type or amount of soil may have contributed to such disparity (Payne et al., 2005). Two of these groups (Morely et al., 2005; Payne et al., 2005) also found that 1% Virkon® S treatments were more effective than commercial QAC solutions against naturally occurring bacteria or *S.* Typhimurium populations. Virkon® S

is primarily used to decontaminate animal holding facilities and research regarding its use as a food contact-surface sanitizer is not available, as it is not currently approved for such applications.

2.4 *Listeria monocytogenes*

2.4.1 Introduction

L. monocytogenes is ubiquitously distributed throughout the environment, including soil, water, and foliage, and can be transferred to foods, such as fruits and vegetables, raw meats, and dairy products, via water, soil or fecal contamination (CDC, 2005). *L. monocytogenes* contamination is of most concern for raw food products and RTE foods which require post-lethality exposure to the environment (Altekruse et al., 1997; CFSAN/FSIS/CDC, 2003). Even though listeriosis represents a far less significant proportion of annual foodborne infections compared to other foodborne pathogens, such as *E. coli* O157:H7, approximately 20% of invasive listeriosis infections are fatal (CDC, 2002b; Mead et al., 1999).

2.4.2 Characteristics

Listeria are noncapsulated, non-spore forming, Gram-positive rods. Other genera within this classification include *Brochothrix, Lactobaccillus*, and *Erysipelothrix* (Seeliger and Jones, 1986; CDC, 2005; Jay et al., 2005). The genus *Listeria* includes non-pathogenic (*seelegeri, welshimeri, grayi*, and *innocua*) and pathogenic species (*ivanovii* and *monocytogenes*), and serovars within each pathogenic species exhibit various levels of virulence (Farber and Peterkin, 1991; Jay et al., 2005). Two new species of *Listeria*, *L. marthii* and *L. rocourtii,* have also been proposed (Ivanek et al., 2009; Monnier and

Leclercq, 2007). In 1924, *L. monocytogenes* was first isolated from laboratory rabbits and guinea pigs with septicemic infections (Murray et al., 1926). Pathogenic strains of *L. monocytogenes* are capable of listeriolysin O-mediated ß-hemolysis of erythrocytes within blood agar and production of lactic acid via fermentation of rhamnose (Jay et al., 2005); *L. ivanovii* and *seeligeri* are also capable of hemolysis (Khelef et al., 2005).

The most suitable conditions for growth of *L. monocytogenes* include moderate O_2 concentration, 30 to 37ºC, and neutral (7.0) environmental pH, with approximately 0.5% NaCl (Bacon and Sofos, 2003; McClure et al., 1991; Seeliger and Jones, 1986). Although these are the most optimal conditions for growth, *L. monocytogenes* are capable of growing, or at least surviving, in an extreme range of conditions. As a facultative anaerobe, *L. monocytogenes* can survive in both aerobic and anaerobic conditions, including open-air and vacuum-sealed environments, as well as in the human body (Bacon and Sofos, 2003; Premaratne et al., 1991). Replication of *L. monocytogenes* can occur at temperatures between 1 and 45ºC (Seeliger and Jones, 1986) and inactivation temperatures depend on numerous other factors including duration of exposure, food matrix and previous stress on the cell (Novak and Juneja, 2003; Poysky, 1997; Yoon et al., 2006). *L. monocytogenes* has also been shown to persist at a pH of 4.5 to 9.0, and in the presence of up to 24% NaCl (McClure et al., 1991; Stenberg and Hammainen, 1955). Metabolic requirements include a carbohydrate source (xylose, lactose, galactose, rhamnose, or mannitol), amino acids (cystine, valine, leucine, glutamine, and isoleucine), vitamins (biotin, riboflavin, thiamine, and thioctic acid), and minerals such as iron. Pathogen growth and the expression or action of virulent mechanisms may be inhibited

by aminoglycosides, tetracyclines, macrolides, chloramphenicols, and penicillins (CDC, 2005; Jones et al., 2002; Khelef et al., 2005).

2.4.3 Human Illness

The most common syndromes associated with invasive listeriosis include meningitis, meningoencephalitis, and encephalitis; swelling of the general and cervical lymphatic tissues (commonly misdiagnosed as mononucleosis) is also observed in a small number of cases (Jay et al., 2005). *L. monocytogenes* can be differentiated into 13 serovars, some of which also represent *L. innocua* and *L. seeligeri*; of these serovars, 1/2a, 1/2b and 4b have been associated with the most significant number of listeriosis cases (Farber and Peterkin, 1991; Kathariou, 2002). Serovar 4b is also more commonly associated with neonatal infection than with infection in non-pregnant persons (McLaughlin, 1990). Individual symptoms suffered during a listerial infection have not yet been linked to any specific serovar (Kathariou, 2002).

Those with compromised immune systems in infancy or before birth, or due to AIDS, alcoholism, type I diabetes, heart disease, renal transplant, and corticosteriod therapy are most at risk of contracting invasive listeriosis. Pregnant women may or may not exhibit signs of illness, and when present, symptoms usually include vomiting, fever, and diarrhea. During pregnancy, fetal exposure to the pathogen (perinatal listeriosis) can result in spontaneous abortion, premature delivery, neonatal illness, or death. Infants who contract the illness from their mothers typically exhibit symptoms associated with meningitis (CFSAN/FSIS/CDC, 2003; CDC, 2005; Jay et al., 2005). Non-pregnant

persons with normal immune function generally remain unaffected or suffer only mild and self-limiting symptoms including vomiting, fever, and diarrhea (CFSAN/FSIS/CDC, 2003; CDC, 2005; Jay et al., 2005; Rocourt, 1988). Invasive listeriosis infections are primarily treated using ampicillin or amoxicillin in combination with an aminoglycoside like gentamicin (the use of gentamicin is not recommended during pregnancy) (Charpentier and Courvalin, 1999; Hof, 2004). Trimethoprim plus a sulfonamide may be administered if the previously described treatments are ineffective (Charpentier and Courvalin, 1999). *L. monocytogenes* is innately resistant to third-generation cephalosporins and fosfomycin (Charpentier and Courvalin, 1999). In *L. monocytogenes*, antibiotic resistance genes are often acquired via conjugative plasmids or transposons (Teuber, 1999) and survival under unfavorable environmental conditions (i.e., low pH, low temperatures) may necessitate the acquisition of such resistance genes, as well as the genetic mutation or phenotypic alteration of sublethally-stressed cells (Simpson and Sofos, 2009). For example, exposure to the harsh conditions of the gastrointestinal tract, which in the presence of drug-resistant enterococci or streptococci, can result in the transfer of resistance genes to *L. monocytogenes* (Teuber, 1999). For these reasons, generic applications of broad spectrum antibiotic therapies, food antimicrobials and sanitizer treatments are not appropriate and may only compound long-term control efforts (Simpson and Sofos, 2009).

In 2007, 122 cases of listeriosis were reported in the U. S. (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5714a2.htm). A regression model was designed to estimate statistically significant changes in incidence of laboratory-

confirmed listeriosis infections in 2007 compared to previous years, while accounting for changes in the amount of surveillance and incidence of infections between surveillance sites each year. Using this model, there was a 42.0% decrease in the annual incidence of laboratory-confirmed listeriosis in 2007, compared to 1996-1998. While the 42% decrease was substantial, it did not meet the Healthy People 2010 goal for national disease prevention which called for the annual incidence of foodborne listeriosis to be reduced by at least 50.0% by the end of 2010 (CFSAN/FSIS/CDC, 2003). For these reasons, additional routes of contamination, foodborne vehicles, and control strategies must be investigated.

2.4.4 Mechanisms of Virulence

In *L. monocytogenes*, the presence of positive regulatory factor A (PrfA) is required for the expression of specific virulent mechanisms, including those involved in cellular attachment, nonphagocytic host cell invasion, pathogenesis, replication *in vivo*, lysis of phagocytes, and cell-to-cell translocation of the pathogen (Khelef et al., 2005; Todar, 2002). Transcription of the prfA coding sequence is mediated by the presence and concentration of substrates used in metabolic pathways, H_2O_2, and H^+ concentration and temperature, and, when certain metabolic needs are compromised, prfA transcription may be up-regulated (Khelef et al., 2005).

Gastric exposure should inactivate the majority of pathogenic cells (Kathariou, 2002); however, those cells that are not inactivated prior to entering the lower gastrointestinal tract may cause infection. Certain host mechanisms, such as induced phagocytosis, can

allow the pathogen to evade host immune response (Khelef et al., 2005). *L. monocytogenes* may also invade host epithelial cells via internalin protein (InlA-H)-mediated host cell attachment where the pathogen is slowly engulfed by host cells (Mengaud et al., 1996), which then serve as sites for replication of the pathogen (Gaillard et al., 1991; Mengaud et al., 1996; Nightingale et al., 2005; Portnoy et al., 1992). *In vivo*, a transcriptional protein product of the *actA* coding sequence found within the *L. monocytogenes* genome (ActA) initiates the recruitment of actin from the host cytoskelton (Todar, 2002; Khelef et al., 2005). The resulting actin filaments surround replicating pathogens while simultaneously driving them toward the host cell wall (Todar, 2002). Long finger-like projections containing *L. monocytogenes* then protrude into neighboring host cells, which subsequently take up the protrusion, allowing the pathogen to laterally infect additional host cells without triggering a host immune response (Cossart and Bierne, 2001; Khelef et al., 2005).

Listeriolysin O (LLO; 55 - 60 kDa) is a 504 amino acid hemolysin (Hly) produced during the exponential or logarithmic phase of growth. This protein belongs to a collection of sulfhydryl-activated pore-forming cytolysins, and is closely related to streptolysin O and pneumolysin (Farber and Peterkin, 1991; Jay, 2000; Portnoy et al., 1992). An absence of the gene necessary for Hly expression does not appear to affect host cell invasion, although Hly^- strains do not replicate following invasion and are generally eliminated from the host within 24 h (Kathariou et al., 1988). In one study, the introduction of the genetic coding sequence for LLO protein expression restored virulence capabilities in LLO^- strains (Cossart et al., 1989); and thus, it is widely accepted that LLO plays an

intricate role in the virulence of *L. monocytogenes*. Fully functioning LLO creates pores in the membranes of host vacuoles, thereby freeing previously engulfed *L. monocytogenes* cells (Todar, 2002). In the absence of LLO, engulfed cells remain in the vacuoles and cannot replicate (Portnoy et al., 1992). Strains producing truncated Hly or LLO may be capable of cellular attachment but incapable of hemolysis, and therefore, considered inactive (Farber and Peterkin, 1991). Expression of the proteins phosphatidylinostitol phospholipase C (PI-PLC-A) and phosphatidylcholine-specific phospholipase (PC-PLC) induces the reorganization of the lipid membrane of host cells and augments LLO-mediated degradation of host cell vacuoles (Todar, 2002; Marquis and Hager, 2000).

2.5 *Listeria monocytogenes* in Processed Meats

2.5.1 Introduction

L. monocytogenes emerged as a foodborne pathogen during the 1980s as outbreaks of related illnesses were documented in the U.S., Canada and Europe (CFSAN/FSIS/CDC, 2003). Given that *Listeria* species are commonly associated with soil, water, and foliage, the potential for pathogenic species to enter the food chain is highly likely (CFSAN/FSIS/CDC, 2003; CDC, 2005; Jay et al., 2005; Ryser and Marth, 2007). According to the CDC, causal agents in the vast majority of listeriosis cases are foodborne, although the actual food products are seldom identified (CFSAN/FSIS/CDC, 2003). A lack of known sources, in conjunction with an ever-increasing capacity to distribute food products nation- and world-wide, may increase the proportion of listeriosis infections that appear to be sporadic or isolated (CDC, 2005; Jay et al., 2005;

CFSAN/FSIS/CDC, 2003; Killalea et al., 1996). Although the precise food product associated with each case of listeriosis is not usually determined, high risk foods have been identified based on product processing, handling, and distribution, as well as the level of consumption by persons from different age groups (CFSAN/FSIS/CDC, 2003).

As many pathogens, including *Listeria,* are easily inactivated by cooking or pasteurization processes (CDC, 2005; Farber and Peterkin, 1991; Jay et al., 2005), unpasteurized and refrigerated RTE foods subject to post-lethality handling are viewed as most vulnerable to all types of pathogenic contamination. *L. monocytogenes* are an even greater threat to the safety of these foods compared to other pathogens, as these bacteria are capable of replication in the absence of air, and under cold storage (Ryser and Marth, 2007). Foods most commonly associated with listeriosis are luncheon and delicatessen meats, improperly re-heated hot dogs, refrigerated meat spreads and pates, uncooked or RTE fish and seafood (including raw, smoked, pickled, nova-style, kippered, dried, jerky, and lox), soft unpasteurized cheeses such as queso blanco and brie, unpasteurized milk and ice cream, deli-sliced cheeses, and inadequately washed raw or dried fruits and vegetables (CFSAN/FSIS/CDC, 2003; CDC, 2005; Farber and Peterkin, 2001; Henao et al., 2006; Lay et al., 2002; Rorvick and Yndestad, 1991; Ryser and Marth, 2007; Wang et al., 1992). Other vectors, although less commonly associated with disease, include raw meat and poultry, pasteurized milk and ice cream, and other high fat dairy products (CFSAN/FSIS/CDC, 2003; CDC, 2005; Farber and Peterkin, 1991; Fleming et al., 1985; Heisick et al., 1989; Jay et al., 2005; Kennedy et al., 2000; Lyytikainen et al., 2000; Ryser and Marth, 2007; Varma et al., 2005).

2.5.2 Outbreaks

In 1983, pasteurized milk was implicated as the source of listeriosis in 49 Massachusetts residents. All laboratory-confirmed cases were observed in adults with weakened immune systems or in the fetuses of pregnant women. Fourteen of these cases (29%) were fatal (Fleming et al., 1985). In 1992, smoked mussels were associated with an outbreak of perinatal and adult listeriosis in New Zealand (Brett et al., 1998). In 1994, nearly half (46%) of the guests at an Italian dinner party reported symptoms of listeriosis, and rice salad, among other food items, was implicated as a source of contamination (Salamina et al., 1996). Serovar 1/2b was recovered from clinical patients, food items and kitchen utensils present during the reception (Salamina et al., 1996). In Finland, serovar 3a was recovered from clinical human fecal samples (n = 25), as well as from dairy butter from which identical isolates had been recovered two years prior. All ill persons had consumed butter which was related to the butter that was found to be contaminated (Lyytikainen et al., 2000). Cold corn and tuna salad were associated with 1,566 clinical cases in patrons of the same catering agency (Aureli et al., 2000). Many additional outbreaks, involving food such as hot dogs, poultry delicatessen meat, have also been reported (CDC, 1998, 1999, 2000b, 2002b; Conly and Johnston, 2008).

2.5.3 Antimicrobial Ingredients in Processed Meat Formulations

Fresh meat is rich in nutrients, has a high water activity (a_w) and a near-neutral pH, all of which encourages growth of microorganisms involved in spoilage, and, if improperly refrigerated or inadequately cooked, in foodborne illness. For centuries, preservation methods have been utilized to modify the physical, chemical, and/or biological properties

of fresh meat in order to extend shelf life and improve safety. Physical preservation methods include heat, refrigeration, drying, smoking, packaging and irradiation. The U. S. Food and Drug Administration (FDA) defines chemical preservatives as "any chemical that, when added to food, tends to prevent or retard deterioration." Many common food preservatives, including sodium chloride, sugars, vinegar, spices and oil extracts of spices, naturally occurring components of wood smoke, and chemicals originally applied for insecticidal/herbicidal purposes, may have antimicrobial properties but are not considered chemical preservatives by the FDA (21 CFR 101.22). It should be noted, however, that almost all ingredients incorporated into processed meat and poultry product formulations exhibit some degree of antimicrobial activity.

In response to multistate outbreaks of listeriosis associated with RTE meat and poultry products (CDC, 1998, 2000b, 2002b), manufacturers of products which may be exposed to post-lethality *L. monocytogenes* contamination and are capable of supporting its growth must include measures to control the pathogen in their HACCP plans or prerequisite programs (USDA-FSIS, 2003b). Such control measures include the incorporation of antimicrobial ingredients in product formulations and other post-lethality treatments, comprehensive sanitation programs, and intensive microbiological testing.

Sodium/potassium lactate alone or in combination with sodium or potassium acetate and diacetate may be added to processed meat and poultry products to control *L. monocytogenes*. Sodium diacetate is also effective against pathogenic *E. coli*, *Pseudomonas fluorecens*, *Salmonella*, *Shewanella putrefaciens*, *Bacillus cereus*, and

some aerobic bacteria involved in spoilage (Ajjarapu and Shelef, 1999; Shelef and Addala, 1994). Microorganisms inhibited by lactates also include Gram-negative and gram-positive bacteria and fungi (Sofos et al., 1998). Currently available data indicate that approved levels of lactate and sodium diacetate sufficiently control *L. monocytogenes* in RTE meat products (Blom, et al., 1997; Glass et al., 1999; Schlyter et al., 1993; Seman et al., 2002), and that the pathogen is most effectively controlled by use of combinations of lactate and sodium diacetate (Barmpalia et al., 2005; Blom et al., 1997; Seman et al., 2002). In one study, 1.5% potassium lactate plus 0.05% sodium diacetate extended the lag phase (10 d) of *L. monocytogenes* (3.2 log CFU/cm^2) on frankfurters compared to controls (0 d) and inhibited growth throughout storage at 10ºC for 48 d (Geornaras et al., 2006). Combinations of potassium lactate and sodium diacetate also inhibited *L. monocytogenes* on commercial ham following two contamination scenarios (manufacturing- versus retail-level), and during three storage scenarios (processing, retail, home) involving various temperature (refrigerated storage at 4 or 7ºC, or intermittent temperature abuse at 25ºC for 90 min) and atmospheric conditions (vacuum- and aerobic packaging) (Lianou et al., 2007). Pathogen counts were generally lower on ham formulated with antimicrobials, and the level of inhibition depended on contamination scenario and packaging method, with the highest pathogen counts found on samples contaminated at the manufacturing-level and stored in aerobic packaging materials (Lianou et al., 2007).

In general, the efficacy of lactate is enhanced under modified atmosphere conditions, and in the presence of bacteriocins, acetates and diacetates, potassium sorbate, nisin, nitrites,

glucono-delta-lactone, or ethanol (Doores, 2005; Jordan et al., 1999; Malicki et al., 2004; Scannell et al., 1997). Calcium, potassium, and sodium lactates are limited to 100 mg/kg of body weight/d (FAO/WHO, 1966), and L- and DL-lactates are prohibited in infant formulas (Doores, 2003). Daily intake of calcium, potassium or sodium acetate are not limited for humans, regardless of age (FAO/WHO, 1966, 1974). Sodium diacetate is limited to15 mg/kg of body weight/d (FAO/WHO, 1974).

Directly adding antimicrobials to product formulations is an effective way to control microorganisms for extended periods, although many factors will affect the resulting level of microbial inactivation and/or inhibition. The characteristics of a given food product must be considered when selecting an antimicrobial ingredient, as the pH, water content and availability, fat, protein content, and other non-meat ingredients can significantly influence antimicrobial activity (Grau and Vanderlinde, 1992; Padilla-Zakour, 1998; Sofos et al., 1998). Other factors that influence the efficacy and longevity of an antimicrobial include extent and type of microbial contamination, as well as the processing, packaging and storage conditions associated with the product (Sofos, 2004).

2.5.4 Home Storage and Thawing of Processed Meats

The ability of *L. monocytogenes* to grow at refrigeration temperatures (4 to 7°C) is well-documented (Barmpalia et al., 2004, 2005; Novak and Juneja, 2003; Seeliger and Jones, 1986). In general, growth rates are higher at 7°C compared to 4°C, and under aerobic versus vacuum-packaged conditions (Lianou et al., 2007). *L. monocytogenes* can survive, but does not grow during frozen storage. While frozen storage at -18°C was more lethal

than storage at -5, -28, or -198°C, *L. monocytogenes* populations were generally only reduced by ≤ 1 log cycle during storage at -18°C (≤ 10 months) (Chou et al., 1999; Gianfranceschi and Aureli, 1996; Elkest and Marth, 1992). Fat, protein and glycerol content may increase survival of *L. monocytogenes* during frozen storage (Elkest and Marth, 1992; Gianfranceschi and Aureli, 1996) and degree of lethality during frozen storage may also be strain-dependent (Elkest and Marth, 1992; Gianfranceschi and Aureli, 1996). In one study, *L. monocytogenes* was inactivated less quickly on cut strawberries when stored at -20°C compared to 4 or 24°C, as pathogen populations on frozen berries were not subject to the antimicrobial compounds present in liquid phase strawberry juice (Flessa et al., 2005). While freezing did not increase susceptibility of *L. monocytogenes* to thermal inactivation (Novak and Juneja, 2003), a single freeze/thaw cycle did increase susceptibility to the antimicrobial agents lipase and lysozyme (Elkest and Marth, 1992). Freezing-stress also increased lag time and growth rate of *L. monocytogenes* on smoked salmon (Yoon et al., 2004). In general, the effects of freezing on lag time and growth were more apparent when products were stored at 4°C compared to 10°C after being thawed (Yoon et al., 2004). Whiting and Bagi (2002) reported similar effects on the lag phase of *L. monocytogenes* after freezing. In general, repeated freezing/thawing cycles appear to exacerbate the lethality of freezing, thermal treatments, antimicrobials, and other previously sublethal stresses (Elkest et al., 1991; Elkest and Marth, 1992; Novak and Juneja, 2003; Papageorgiou et al., 1997).

The USDA-FSIS recommends that consumers store opened packages of frankfurters for no longer than 7 d at 4°C. Unopened packages may be stored for up to 14 d at 4°C, and

both opened and sealed packages may be held for 1 to 2 months in home freezers (http://www.fsis.usda.gov/PDF/Basics_for_Safe_Food_Handling.pdf). Although recent surveys indicate that many consumers store unopened packages of frankfurters in home freezers (Porto et al., 2004), little information is available on the fate of *L. monocytogenes* on these products during and after thawing. Freezing recommendations are usually present on frankfurter package labels, while thawing instructions are generally absent. When available, instructions usually recommend thawing frozen products under refrigeration, discourage countertop thawing and ignore microwave defrosting options. However, thawing frozen foods under refrigeration, in cold water, or by microwave defrosting are considered basic good handling practices for consumers, as set forth by the USDA-FSIS (2005b). As part of such recommendations, products thawed under refrigeration should be safe for consumption if cooked and consumed within 3 to 5 d of initial thawing, while those thawed in cold water or by microwave defrosting should be cooked immediately after thawing, and before being consumed or re-frozen. Thawing products in cold water is often recommended, but does raise some concerns. Unless cold water is continually refreshed the temperature will likely reach room temperature in a short amount of time. Some resources suggest letting cold water flow continuously from a faucet into a container holding the frozen item(s) (http://www.ces.ncsu.edu/depts/foodsci/agentinfo/projects/fss4.pdf; http://www.nraef.org/foodsafetycenter/downloads/factsheets_activities/thawing_food.pdf); this however, is not an economical option. Furthermore, if the frozen food is not covered by packaging material, surface contaminants and purge will overflow into the sink area, increasing the potential for future cross-contamination events. Countertop

thawing at room temperature is discouraged by most, but not all groups. Snyder (1999) reviewed literature pertaining to countertop thawing and concluded that the practice did not pose more significant food safety risks than thawing under refrigeration. Jiménez et al. (2000) investigated the effect of different thawing methods on the growth of spoilage bacteria and *Salmonella* Hadar on the surface of thawed poultry carcasses. Frozen carcasses were thawed under refrigeration (3.5 to 7.2°C), under running water (21°C) or on a countertop at room temperature (22°C) until internal carcass temperature reached 4.4°C. Total aerobic bacteria, total pseudomonad and *S.* Hadar populations were not significantly different among treatments, indicating that under the conditions of this study and according to the authors, countertop thawing did not increase the food safety risk of these products (Jiménez et al., 2000). It should be noted, however, that thawing treatments were suspended as soon as internal carcass temperatures reached 4.4°C. If the treatments were extended, the outcome of countertop treatments may have differed dramatically. The likelihood that all consumers will closely monitor the internal temperature of thawing products and transfer them to refrigerators immediately after reaching 4.4°C is extremely unlikely. External carcass temperatures may also reach temperatures above 4.4°C before internal carcass temperatures and could reach up to 22°C for a significant amount of time. Lianou and Koutsoumanis (2009) examined the effect of different thawing methods on the inactivation of *Salmonella* Enteritidis and *L. monocytogenes* in ground beef and the development of heat resistance in surviving cells. Frozen ground beef samples were thawed under refrigeration (5°C, 15 h), on a countertop at room temperature (25ºC, 12 h) or in a microwave oven at a defrost setting. Thermal inactivation of *S.* Enteritidis was not significantly affected by different thawing

treatments and surviving cells were not increasingly heat tolerant after thawing treatment. In contrast, *L. monocytogenes* populations on samples thawed at room temperature were more heat tolerant than those recovered from samples thawed under refrigeration or defrosted in a microwave (Lianou and Koutsoumanis, 2009).

Industrial microwave applications include the tempering, preheating or vulcanization of commercial rubber products, the sintering and synthesis of ceramics, microwave-drying technologies used to process wood, plastics, and other textiles, and rapid thawing of frozen red blood cells and other intravenous solutions (Ausman et al., 1980; Beck et al., 2002; Osepchuk, 2002). Food applications include tempering, blanching, drying, dry frying, pasteurization, sterilization, precooking, and cooking processes applied to bacon, sausage, canned meats, potato chips, baked goods, sherry and beer, chewing gum, pasta and green tea (Industrial Microwave Systems, LLC., http://www.industrialmicrowave.com/foodprocessing.thm; Osepchuk, 2002). The popularity of home microwave ovens has increased dramatically over the last two decades, and according to a fairly recent estimate (Osepchuk, 2002), there are over 100 million such units currently in use by U. S. consumers. Microwave defrosting is much faster than other thawing methods, and, because most modern consumers now have microwaves installed in their homes, is a likely method of thawing frozen foods.

According to Chamchong and Datta (1999), proper microwave defrosting does not cook frozen products, because the power output is cyclical instead of continuous. By cycling power output, a reduced yet seemingly continuous level of power is generated and

applied to the frozen item. Cyclical applications may increase the uniformity of thawing (Taher and Farid, 2001); however, at such low power levels, microwaves only penetrate to just below the surface and thus, adequate defrosting requires significantly longer applications as the surface temperature increases much faster than the interior. As a rule, longer thawing programs typically result in decreased uniformity of microwave defrosted products (Chamchong and Datta, 1999). During thawing, microwaves are preferentially absorbed into the liquid phase of a food product, which leads to partial thawing, excessive water loss, chemical deterioration and decreased palatability (Rosenberg and Bogl, 1987; Taher and Farid, 2001; Virtanen et al., 1997). Although water loss and chemical reactions caused by excessive heating are irreparable, observing a two min dwell time after thawing will generally increase temperature uniformity throughout the product. Rate of thawing is dependent on microwave size, power, and presence of turntables or stirrers, as well as food product components, temperature, shape, size, density, water content, and packaging or container characteristics (Li and Sun, 2002; Osepchuk, 2002; Taher and Farid, 2001).

The effects of microwave defrosting on food product properties are well-understood; unfortunately, little is known about its effects on microbial contaminants during and after thawing. For this reason, the effect of different thawing methods on the fate of *L. monocytogenes* on frankfurters formulated with and without antimicrobial agents should be investigated. Thawing methods selected for investigation include thawing under refrigeration, thawing at ambient temperature on a countertop, and microwave defrosting. Fate of the pathogen on frankfurters immediately after thawing treatments and under

post-thawing home storage conditions (7°C, 14 d, aerobic packaging) has not been previously described, and should provide useful information for researchers, manufacturers and consumers.

2.6 Multiple Hurdle Technology

A systematic approach to the control of microbial contaminants in food is the most effective and economical way to minimize safety concerns and human health risks. Thus, the sequential application of individual antimicrobial agents or interventions, commonly referred to as "multiple hurdles", is a logical approach to pathogen management (Leistner and Gould, 2002). The combined effect of several inhibitory compounds and/or interventions should result in greater reductions in a broader range of microbial populations than if each factor were used alone. For this very reason, the use of various combinations of fresh meat decontamination treatments has been the topic of extensive investigation (Bacon et al., 2003; Graves-Delmore et al., 1998; Hardin et al., 1995; Pohlman et al., 2002; Sofos and Smith, 1998; Stopforth and Sofos, 2006). Inhibitory ingredient combinations may also exhibit synergistic activity when used in combination. Examples include nitrite and NaCl or lactate and sodium diacetate in RTE meat and poultry products (Barmpalia et al., 2005; Blom et al., 1997; Sofos, 1984; Sofos et al, 1979). Sequential applications of some compounds can induce microbial sensitivity to otherwise non-lethal agents. For example, gram-negative bacteria are sensitized to the antimicrobial mechanism of bacteriocins after being exposed to compounds that alter cell membrane permeability; most microorganisms also become increasingly sensitive to irradiation treatments in the presence of non-meat ingredients (Bricher, 2005; Delves-Broughton and Gasson, 1994). Antagonism, or the diminished activity of one or more

compounds, may also result when one or more chemical reactions take place between agents (Ahn et al., 2002). Concentration of antimicrobial compounds, level and type of microbial contaminants, and/or processing and storage conditions may influence antagonistic reactions (Simpson and Sofos, 2009).

Optimizing all parameters related to antimicrobial activity in complex food systems can be a difficult process. Antimicrobial activity is generally optimized in broth systems and in the absence of lipids (Ray and Miller, 2000; Smith, 2003; Sofos et al., 1998). The pH of a solution or food product can also influence antimicrobial activity and degree of hardness or presence of excess solute associated with water sources used to mix antimicrobial solutions can also limit the efficacy of some agents (Cords et al., 2005). Friction may denature some antimicrobial proteins, and effort should be made to minimize friction-induced denaturation when attempting to homogenously distribute antimicrobials in processed meat products (Romans et al., 2001). Emulsified products are the exception, and a fairly homogenous ingredient distribution is expected. Thermal processes and irradiation treatments may diminish activity or even destroy some antimicrobial compounds (Prakash, 2000). Some antimicrobials may also be depleted by surviving microorganisms during storage (Grau and Vanderlinde, 1992; Whiting and Masana, 1994).

Previous exposures to sublethal stress may alter the ability of a microbial cell to endure additional types or degrees of stress (Gahan et al., 1996; Koutsoumanis and Sofos, 2004; Maxcy et al., 1971; Samelis and Sofos, 2003; Whiting, 1993). Microbes subjected to

sublethal stress may recover and proliferate in food products or in food processing environments and/or become increasingly resistant to previously inhibitory or lethal treatments (Duffy et al., 2000; Davidson and Harrison, 2002; Samelis and Sofos, 2003; Sofos and Smith, 1998). Sublethally-stressed survivors may also be more likely to overcome the low pH conditions of the gastrointestinal tract (Rombouts et al., 2003). As an example, mildly acidic conditions (pH 5.0-6.0) may initiate survival mechanisms that enable microorganisms to overcome increasingly severe acid exposures (Koutsoumanis and Sofos, 2004). Acid adaptation may also result in increased resistance to thermal and osmotic stresses (Gahan et al., 1996). The number of published reports which indicate some level of pathogen resistance to antimicrobials in response to sublethal exposures has increased in recent years. There are three basic forms of antimicrobial resistance including innate, acquired, and apparent resistance (Davidson and Harrison, 2002). Defense mechanisms such as efflux pumps or the ability to degrade a compound are both examples of innate resistance. Acquired resistance is the result of lateral transfers of genetic material (e.g., plasmid acquisition), genetic mutations, or the deletion of a specific biochemical target (Souza et al., 2002). Apparent resistance is generally reversible, and depends on the conditions under which an antimicrobial is applied (Davidson and Harrison, 2002), reinforcing the need for strict and specific guidelines for the application of antimicrobial agents.

CHAPTER THREE

Fate of Natural Contamination and *Escherichia coli* O157:H7, Inoculated in Beef Slaughter Floor Runoff Fluids and Introduced in Beef Carcass Tissue Pockets, during Simulated Carcass Chilling and Post-Chilling Decontamination

3.1 Abstract

The outer fat layer of the surface of beef carcasses may be pulled and torn away from underlying lean tissue during mechanical hide removal creating pockets where bacteria and decontamination fluid or other liquids may collect. This study evaluated such pockets as potential reservoirs of *Escherichia coli* O157:H7 contamination. Hot (82°C) water carcass washings were collected as they ran off of beef carcasses and fresh beef brisket tissue samples were collected at a commercial beef packing facility. Brisket tissue samples were dissected to separate the fat from the lean tissue in order to create a pocket. Runoff fluid pH was adjusted to 3, 5 or 7 using lactic acid and then adjusted to 25, 45 or 65°C. Prepared runoff fluids were inoculated (4 log CFU/ml) with a six-strain rifampicin-resistant *E. coli* O157:H7 composite and poured (200 ml) into tissue pockets, which were then covered with aluminum foil and stored at 4°C for 48 h to simulate beef carcass chilling. At 0, 12, 24, 36 and 48 h of storage, fluid aliquots were removed from the pockets and analyzed for pH and microbial survivors. In addition, after 48 h at 4°C, fluid that had been adjusted to pH 3, 5 or 7 and to 25°C was removed from pockets and inoculated (1.5 log CFU/cm^2) onto fresh beef brisket fat. These inoculated fat samples were then sprayed (25°C, 20 psi/1.38 bar, 3 sec) with water (control), 5.0% lactic acid

(LA), 0.5% ceytlpyridinium chloride (CPC), or 0.02% peroxyacetic acid (PAA) to determine the efficacy of post-chilling decontamination treatments against cells that survived in the pockets during the simulated carcass chilling process. Microbial reductions, following exposure to runoff fluid, were pH and temperature dependent. Adjusting runoff fluid to pH 3 and to 65°C reduced initial total aerobic bacteria and *E. coli* O157:H7 from 5.5 and 4.0 log CFU/ml, respectively, to below the detection limit (0.0 log CFU/ml by spread plating). Adjusting runoff fluids (pH 5 or 7) to 45 or 65°C reduced initial total aerobic bacteria and pathogen counts by 1.1 to 3.2 and 0.0 to 1.3 log CFU/ml, respectively. Microbial populations associated with ambient temperature (25°C) runoff fluids adjusted to pH 5 or pH 7 did not change. Exposure to heated runoff fluids (45 or 65°C) resulted in immediate microbial reductions but did not have a lasting effect on survivors during storage (4°C, 48 h). Adjusting fluids to pH 3 did suppress growth of natural contamination during storage. Post-chilling decontamination treatments reduced *E. coli* O157:H7 on beef fat by 0.1 to 1.3 log CFU/cm^2, with overall reductions decreased in order of CPC > LA > PAA = water. Treatments were generally more effective against cells derived from pH 7 compared to pH 5 or 3 runoff fluids with pathogen reductions ranging from 0.4 to 1.3 compared to 0.1 to 0.9 log CFU/cm^2, respectively. These data indicate that *E. coli* O157:H7 entrapped in defects on carcass surfaces may evade slaughter-floor decontamination treatments and survive the carcass chilling process, with the potential for survivors to contaminate other carcasses or the surrounding environment during fabrication. These data also indicated that fabrication-level CPC, LA and PAA treatments were only moderately effective against *E. coli* O157:H7, and less effective against acid-stressed cells.

3.2 Introduction

Pathogen control measures have been established in response to multiple outbreaks of *E. coli* O157:H7 infections associated with consumption of contaminated ground beef and mechanically tenderized beef products (Bell et al., 1994; CDC, 1996, 2002a; Riley et al., 1983; USDA-FSIS, 2005a). Such control strategies include robust sampling and testing programs, feed supplements, antimicrobial hide and carcass rinses, steam pasteurization and irradiation treatments (Simpson Beauchamp and Sofos, 2009; Ransom et al., 2003; VanOverbeke, 2007), with the greatest amount of focus having been placed on slaughter-floor interventions. The implementation of such control efforts appeared to be successful when, the USDA-FSIS (2006) reported an 80% decrease in the prevalence of *E. coli* O157:H7-positive samples between 2001 and 2006 (< 1% of samples were positive in 2001) (http://www.beefusa.org/uDocs/ecolinumbersdecline.pdf). Unfortunately, this steady decline was reversed in 2007 and the annual number of *E. coli* O157:H7-positive samples has since continued to rise (http://www.fsis.usda.gov/Science/Ground_Beef_E.Coli_Testing_Results/index.asp). Small outbreaks and large recalls also continue to plague the beef industry (http://www.fsis.usda.gov/News_&_Events/Recall_034_2009_Expanded/index.asp; http://www.cdc.gov/ecoli/june2008outbreak/). For these reasons, additional potential sources of beef product contamination must be investigated and controlled.

In recent years, commercial beef packers have replaced labor-intensive manual hide removal with mechanical methods. Mechanical removal requires only a few principal manual cuts before the hide is pulled free from the carcass in a single motion. The

adoption of mechanical hide removal allows commercial packers to maintain faster chain speeds but can also create defects on the carcass surface (MLA, 2004). During mechanical removal the subcutaneous fat layer of beef carcasses may be pulled away from the underlying lean tissue, creating an area where fluid may collect, especially when carcasses are sprayed or rinsed with water or antimicrobial intervention solutions (Simpson et al., 2006). Bacterial contaminants on carcass surfaces may also be washed with slaughter-floor fluids into the resulting "pockets". Simpson et al. (2006) found that, although aerobic plate counts, total coliform counts, and *E. coli* Biotype I counts were below the level of detection, 0.5% (2/40) of such "pockets" were positive for *E. coli* O157:H7. Previous studies (Berry and Cutter, 2000; Brackett et al., 1994; Samelis et al., 2002; Stopforth et al., 2007) also found that various decontamination treatments were capable of significantly reducing levels of natural flora, but allowed acid-stressed *E. coli* O157:H7 to survive during cold storage. *E. coli* O157:H7 has been shown to survive for 13 d in 2% lactic acid run-off fluid (Berry and Cutter, 2000; Samelis et al., 2004). Another study also indicated that *E. coli* O157:H7 exposed to low, yet sublethal pH conditions during chilling (pH 4.89-5.22; 4°C), was increasingly tolerant to subsequent acid exposure (Stopforth et al., 2007).

In general, fluid-filled pockets on the surfaces of carcasses are manually removed during fabrication, along with the outer fat layer, and ultimately become incorporated into beef trimmings or fat (Simpson et al., 2006). In these instances, contaminated pockets could remain intact until grinding and potentially remain undetected even if robust trim sampling and pathogen detection methods were utilized (USDA-FSIS, 2009a).

Fortunately, these sites may be easily eliminated, and as observed in one plant, plastic film was tightly fastened over cuts and tears in the fat layer immediately after hide removal and prior to carcass decontamination (Simpson et al., 2006). In accordance with 9 CFR 416.1, each official establishment must be operated and maintained in ways that prevent creation of insanitary conditions and ensure that products are not adulterated. Due to the continued incidence of *E. coli* O157:H7 in ground beef, a large-scale investigation of the fate of *E. coli* O157:H7 in slaughter-floor fluids during carcass chilling is needed to eliminate the need for undue concern, or to highlight these "pockets" as reservoirs of pathogen contamination. Therefore, the objectives of this study were to: (i) determine the efficacy of different slaughter-floor decontamination treatments against natural contamination and *E. coli* O157:H7; (ii) determine the ability of naturally occurring contamination and inoculated *E. coli* O157:H7 to survive in such decontamination fluids during beef carcass chilling; and, (iii) determine the efficacy of post-chilling decontamination treatments against pathogen cells that survive the chilling process.

3.3 Materials and Methods

3.3.1 Culture Preparation and Inoculation

The *E. coli* O157:H7 inoculum consisted of six rifampicin-resistant strains including ATCC 43895, ATCC 43895/ISEH-GFP (Noah et al., 2005), ATCC 51657, ATCC 51658, and F284 and F469 (Carlson et al., 2006). Active cultures of individual strains were first prepared (35ºC, 24 h) in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) and then subcultured (0.1 ml) under the same conditions. Individual cultures were then harvested by centrifugation (4,629 x *g* at 4ºC for 15 min) (Eppendorf, 5810 R, Brinkman Instruments, Inc., Westbury, NY) followed by washing in 10 ml phosphate-buffered

saline (PBS, pH 7.4; 0.2 g KH_2PO_4, 1.5 g $Na_2HPO_4 \cdot 7H_2O$, 8.0 g NaCl, and 0.2 g KCl in 1 L distilled H_2O). Washed cells of each strain were resuspended in 10 ml of PBS and combined to generate a 6-strain composite inoculum which was serially diluted in PBS to reach a target inoculum level of 6 log CFU/ml.

Fluid was collected in sterile containers as it ran off of carcasses exiting a hot (82°C; 180°F) water spraying cabinet at a commercial beef packing facility. Fluid was cooled to 4°C, transported on ice to the laboratory and immediately prepared for inoculation. In order to determine the ability of the inoculum to survive decontamination treatments, the pH of the fluid was not adjusted (pH 7), or was adjusted with lactic acid (lactic acid 88% FCC, Birko Corporation, Henderson, CO) to target pH values of 3 or 5. Following pH adjustment, the temperature of fluids was adjusted to 25, 45 or 65°C using a 65°C water bath. One ml of the inoculum was then added to 200 ml of pH and temperature adjusted decontamination fluid and gently agitated for 10 sec (target inoculum level of 4.0 log CFU/ml).

3.3.2 Storage of Inoculated Runoff Fluid

Beef tissue (approximately 15 cm deep, 20 cm wide, 50 cm long) was excised from the brisket area of chilled (4°C) beef carcasses and then cut in half to yield two 15 x 20 x 25 cm pieces. An incision was then made to separate the fat from the lean tissue in order to create a "pocket", taking care not to disrupt the lean and cut along the membrane between the fat and lean tissue. Beef pieces were placed "pocket" side up into sterile plastic containers (20 cm dia. x 30 cm deep) and then warmed to 35°C in an incubator (35°C) to

more accurately represent beef carcass temperatures at slaughter. Immediately after being inoculated (described above), 200 ml of runoff fluid was poured into each beef tissue pocket (three per treatment, in each of two biologically independent experiments). Containers were then covered with sterile aluminum foil and inoculated pockets were systematically sampled and analyzed for microbial survivors and fluid pH values during storage (48 h) at 4°C.

3.3.3 Post-Chilling Decontamination Treatments

To simulate contamination events that may occur during carcass fabrication, runoff fluids remaining in tissue pockets after 48 h were inoculated onto strips of fresh beef fat. Inoculated fat strips were then sprayed with different antimicrobial solutions in order to determine the ability of fabrication-level decontamination treatments to control similar contamination events. The runoff fluids used to inoculate the beef fat had been adjusted to pH values of 3, 5 or 7 and stabilized at 25°C prior to inoculation, poured into tissue pockets, and stored for 48 h at 4°C. Strips (surface area of 10 cm^2, 5 mm thick) of fresh beef fat were excised from a single side of beef and then inoculated under a biosafety cabinet. Runoff fluid was evenly spread over one side of each strip using a sterile bent glass rod to achieve a target pathogen load of 1.5 log CFU/cm^2. A 15 min bacterial cell-to-fat surface attachment time was allotted between inoculation and decontamination spray treatments. Antimicrobial spray treatments were applied for 3 sec (25°C, 20 psi, 1.38 bar) using a portable hand-held single conical mist nozzle spray system positioned to allow 20 cm between the nozzle and the inoculated surface. Treatments included water (control), 5.0% lactic acid (LA), 0.5% cetylpyridinium chloride (CPC), and 0.02%

peroxyacetic acid (PAA); inoculated, untreated samples also served as a control. Inoculated strips were placed on a wire rack fitted over a sterile plastic container to collect residual treatment fluids, sprayed for the prescribed time, and then allowed to dwell for 20 sec. Strips were then transferred to filter WhirlPak bags (Whirl-Pak™ Bag, Nasco International, Fort Akinson, WI) containing 20 ml Dey/Engley neutralizing broth (Difco), pummeled (Masticator, IUL Instruments, Barcelona, Spain) for 2 min and analyzed microbiologically.

3.3.4 Microbiological and pH Analyses

Immediately after inoculation (0 h) and at 12, 24, 26 and 48 h of storage, fluid in tissue pockets was sampled and analyzed for total aerobic bacteria, rifampicin-resistant *E. coli* O157:H7, and pH. A sterile plastic pipette was used to extract 30 ml of fluid from each pocket at each sample collection interval and placed in a sterile sampling bag (Nasco). Decontamination fluid pH and the pH of antimicrobial spray treatments was measured using a digital pH meter fitted with a glass electrode (Denver Instruments, Arvada, CO). Samples were diluted in 0.1% sterile buffered peptone water (BPW; Difco) and plated onto trypic soy agar (TSA; Difco) for enumeration of total bacterial populations, and TSA supplemented with rifampicin (100 µl/L) (TSArif) for enumeration of pathogen populations. Plates were incubated at 25°C for 48 h (TSA) or 35°C for 24 h (TSArif) and colonies were enumerated.

All samples were also enriched for subsequent detection of *E. coli* O157:H7 in case no growth was observed on TSArif plates after 22 h of incubation. Enrichment and

detection was conducted using a modified version of the procedure of the Microbiology Laboratory Guidebook 5.04 (USDA-FSIS, 2008b). A 25 ml aliquot of fluid was taken from each sample bag and used for enrichment. Sample aliquots were suspended in 225 g tryptic soy broth (TSB, International BioProducts, Bothwell, WA), incubated for 22 h at 45 ^{o}C, and then pummeled for 2 min. A 1 ml aliquot of each sample was then added to a 1.5 ml microcentrifuge tube containing 20 µl of anti-*E. coli* O157 Dynalbeads (Dynal Laboratories, Lake Success, NY) (5x per sample), and incubated at room temperature on a rocker for 10-15 min. Tubes were removed from the rocker, placed on a magnetic strip and allowed to incubate at room temperature for 5 min. Supernatant was removed and beads were washed by adding 1 ml of E-buffer (filter sterilized [0.2 µm] 0.5 g bovine albumin, 50 µl Tween-20 [Fisher Scientific, Fair Lawn, NJ] in 100 ml BPW) to each tube. Tubes were capped, inverted several times to resuspend beads and then placed on a magnetic strip for 5 min at room temperature. This wash step was repeated three more times for a total of four wash cycles. Following the fourth wash cycle, tubes were placed on a magnetic rack for 5 min at room temperature, supernatant was removed and beads were resuspended in 100 µl E-buffer. A 50 µl portion of the suspended bead solution was spread onto TSArif and another 50 µl was spread on Rainbow-plus agar (Rainbow-Agar O157, Bilog Inc., Hayward, CA) containing 0.8 mg/L potassium tellurite (Sigma, St. Louis, MO) and 20 mg/L novobiocin (Sigma) and incubated for 18 h at 35^{o} C. Following incubation, at least one typical *E. coli* O157-like colony found on the TSArif (opaque white colonies), or Rainbow-agar (dark slightly blue colonies), was removed and screened using the latex agglutination assay of the RIM^{TM} *E. coli* O157:H7 Test Kit (Remel; Lenexa, KS), and each isolate was checked with a test reagent and control

reagent located on the test card. Isolates were also checked against known positive and negative strain agglutination test reactions.

3.3.5 Statistical Analysis

Three samples were analyzed per treatment in each of two biologically independent experiments and microbiological data were converted to log CFU/ml before statistical analysis with a complete factorial design using the analysis of variance in the mixed model procedure of SAS (version 9.1, SAS Institute, Cary, NC). Treatment effects for the first phase of the study which examined the fate of the pathogen when stored in runoff fluid in pockets of beef tissue during simulated beef carcass chilling were: runoff fluid pH (pH 3, 5, and 7) x runoff fluid temperature (25, 45 and 65°C) x time (0, 12, 24, 36 and 48 h). Treatment effects in the second phase of the study which examined the fate of post-chilling *E. coli* O157:H7 survivors during decontamination of inoculated beef fat were: origin of inoculum (25°C; pH 3, 5 or 7) x decontamination treatment (water control, 5.0% LA, 0.5% CPC or 0.02% PAA). Under the conditions of this study, the minimum detection level of cells in inoculated runoff fluid was 0.0 log CFU/ml and the minimum detection limit of cells on decontaminated fat pieces was 0.3 log CFU/cm^2; all samples with microbial populations below the level of detection were assigned a value of -0.01 CFU/ml or 0.29 log CFU/cm^2, respectively. All treatment effects were analyzed individually and interactively and a confidence limit of 95% was assigned to all significant treatment effects ($P = 0.05$).

3.4 Results and Discussion

3.4.1 Effect of Slaughter-Floor Decontamination Treatments

The original pH of the runoff fluid collected from the plant was 6.86 ± 0.02 (19°C). As indicated, fluid aliquots also were adjusted to target pH values of 3 and 5 using lactic acid, and actual pH values following pH adjustment were 3.08 ± 0.06 and 4.90 ± 0.01 (Fig. 3.1A). For ease of discussion, pH 6.89, 4.90 and 3.08 will hereafter be referred to as pH 7, 5 and 3, respectively. Treatment fluids (pH 7, 5 or 3) were then stabilized at 25, 45 or 65°C, while unadjusted decontamination fluid (pH 7; 4°C) served as a control. Initial populations of natural flora in runoff fluids before pH or temperature adjustment were 5.4 ± 0.42 log CFU/ml. Following pH adjustment to 5 and 3, total aerobic bacteria counts were 4.0 ± 0.31 and 2.1 ± 0.8 log CFU/ml, respectively.

In general, microbial reductions increased as the pH of treatment fluids decreased or temperature increased and a significant interaction ($P < 0.05$) between pH and temperature was observed. Adjusting runoff fluid to pH 3 and to 65°C reduced natural contamination levels from 5.4 log CFU/ml to below the level of detection (0.0 log CFU/ml), and reduced inoculated *E. coli* O157:H7 from 4.0 log CFU/ml to below the level of detection (0.0 log CFU/ml) (Table 3.1). Adjusting runoff fluid to pH 5 and to 65°C reduced natural contamination and inoculated *E. coli* O157:H7 populations by 3.2 and 1.3 log CFU/ml, respectively (Table 3.1). Adjusting runoff fluid to pH values of 5 or 7 and to 45°C resulted in less meaningful microbial reductions (Table 3.1); ambient temperature treatments were generally ineffective (Table 3.1). These results were

expected as the increased efficacy of heated (55°C) versus ambient temperature acid treatments has been well established (Hardin et al., 1995; Simpson and Sofos, 2009).

3.4.2 Microbial Fate during Chilled Storage in Runoff Fluids

Runoff fluid pH increased from 3 to 4.11 and 5 to 5.65, or decreased from 7 to 5.76 during the first 12 h of storage at 4°C (Fig. 3.1A). No significant pH changes were obseved after 12 h (Fig. 3.1A). Adjusting runoff fluid temperature did not have a lasting effect on microbial survivors during storage (Fig. 3.1B). Microbial growth was suppressed during storage in runoff fluid adjusted to pH 3 (Fig. 3.1B and 3.1C) as indicated by total aerobic bacteria populations which reached 3.5 ± 0.2 log CFU/ml at 12 h and remained similar throughout storage. Total aerobic bacteria counts in runoff fluids adjusted to pH values of 5 or 7 ranged from 4.3 to 5.5 log CFU/ml at 12 h and reached 6.2 ± 0.5 log CFU/ml at 48 h of storage at 4°C (Fig 3.1B). *E. coli* O157:H7 populations generally did not change during storage (Fig. 3.1C). This was expected as *E. coli* O157:H7 does not grow at 4°C (Simpson and Sofos, 2009). Pathogen populations in runoff fluid adjusted to pH 3 and 45°C decreased by 1 log cycle between 0 and 12 h of storage (Fig. 3.1C), indicating continued inactivation of cells after the first sample collection interval. As previously described, adjusting runoff fluids to pH 3 and heating to 65°C inactivated inoculated *E. coli* O157:H7 populations to below the level of detection and no survivors were recovered by direct plating during the 48 h storage period (Fig. 3.1C); thus, these samples were enriched. The pathogen was not detected in enriched samples collected at 0 and 12 h of storage at 4°C, but was detected in samples collected at 24, 36 and 48 h of storage (Table 3.2). The number of samples positive by enrichment increased as storage time increased and more samples were detected on

TSArif than on Rainbow Agar (Table 3.2). When present, typical colonies on both types of media were removed and screened for presence of the O157 somatic antigen and the H7 flagellar antigen using a latex agglutination assay (Table 3.2). It should be noted that all colonies recovered on TSArif were positive for both the somatic and the flagellar antigens, while those recovered from Rainbow Agar-O157 were positive for the somatic antigen but negative for the flagellar antigen (Table 3.2). These results were not expected as all of the *E. coli* O157 inoculum strains possessed both antigens. However, colonies recovered on Rainbow Agar should be transferred onto Blood Agar and colonies present on the Blood Agar should be used for agglutination assay (USDA-FSIS, 2008b). We did not confirm which of the six inoculum strains were represented in each of the colonies recovered with the selective media and additional research is needed to determine the identity of inoculum strains present in each sample type and on each media, and to substantiate the discrepancies between colonies recovered from TSArif versus Rainbow Agar-O157.

3.4.3 Post-Chilling Survival on Decontaminated Beef Fat

Microbial contamination present on beef carcasses at fabrication must survive slaughter-floor decontamination treatments, as well as the carcass chilling process which may involve extended exposure to sublethal pH values, lactic acid or other antimicrobial compounds. The potential for *E. coli* O157:H7 to survive for extended periods under low pH and temperature conditions has been established (Berry and Cutter, 2000; Brackett et al., 1994; Samelis et al., 2002, 2004; Stopforth et al., 2007), while extended exposure to sublethal conditions may support development of acid-tolerant survivors (Stopforth et al.,

2007). In order to determine the fate of such cells, following fabrication-level decontamination treatments, fluid from selected tissue pockets was removed after 48 h at 4°C and inoculated onto strips of chilled beef adipose tissue. Inoculated beef tissue was then sprayed with water (control), 5.0% LA, 0.02% PAA or 0.5% CPC (Fig. 3.2). *E. coli* O157:H7 counts on inoculated control (no treatment) samples were 1.6 ± 0.10 log CFU/cm^2. Decontamination treatment efficacy decreased in order of 0.5% CPC > 5% lactic acid > 0.02% PAA = water (Fig. 3.2) with reductions in *E. coli* O157:H7 populations ranging from 0.1 to 1.3 log CFU/cm^2 (Fig. 3.2). No treatment reduced *E. coli* O157:H7 to undetectable levels (Fig. 3.2). In general, decontamination treatments were less effective against inoculum cells that were habituated to a more acidic pH during previous storage at 4°C (Fig. 3.2). More specifically, 5.0% LA treatments were more effective ($P < 0.05$) against *E. coli* O157:H7 populations recovered from pH 7 runoff fluids compared to those stored in runoff fluids adjusted to pH 3 or 5 with lactic acid (Fig. 3.2).

To summarize, the first objective of this research was to determine the efficacy of different slaughter-floor decontamination treatments against natural contamination and *E. coli* O157:H7. The pH and/or temperature of carcass runoff fluid were adjusted before being inoculated with *E. coli* O157:H7, and when either factor was used alone, increasing temperature resulted in greater overall microbial reductions compared to lowering runoff fluid pH. However, when both factors were adjusted, microbial reductions increased as runoff fluid temperature increased or pH decreased and the greatest reductions were observed when runoff fluids were adjusted to pH 3 and 65°C. Thus, corresponding

efforts should be made to ensure that antimicrobial interventions are applied at the lowest pH values and highest temperatures recommended by the manufacturer and allowed under regulatory guidelines. Application temperature should also be measured at the carcass surface and not at the nozzle. Reusable and disposable temperature monitoring strips have been designed to monitor the temperature of a liquid as it contacts a surface, and are available for such verification purposes.

The second objective of this research was to determine whether natural contamination and inoculated *E. coli* O157:H7 survived in decontamination fluids under beef carcass chilling conditions. Both populations survived in decontamination fluids for up to 48 h at 4°C. The survival of *E. coli* O157:H7 during storage (as detected by direct plating or enrichment), even after being exposed to the extreme conditions (45-65°C, pH 3-5) associated with each decontamination treatment, reinforces the importance of limiting reservoirs for fluids and microbial contamination to collect during decontamination treatments (Samelis et al., 2001, 2002, 2004; Simpson et al., 2006; Stopforth et al., 2003a, 2003b, 2007). These data also suggest that validation of slaughter-floor interventions should encompass the fate of microbial populations directly after treatment (before chilling), and after chilling.

The third and final objective of this research was to determine the efficacy of post-chilling decontamination treatments against pathogen cells that survive the chilling process. If applied under the conditions described in this study, approved PAA solutions would be ineffective against *E. coli* O157:H7 contamination on chilled beef carcasses.

The inability of PAA treatments to inactivate *E. coli* O157:H7 on carcass tissue has also been described in other published reports (Ellebracht et al., 2005; FAO/WHO, 2004; Ransom et al., 2003; Stopforth et al., 2004). While the efficacy of lactic acid treatments (2 to 5%) against *E. coli* O157:H7 has been well established (Simpson and Sofos, 2009), lactic acid treatments applied during fabrication may be less effective against cells that survive previous sublethal acid exposures (Cheng et al., 2003). Multiple studies (Breen et al., 1995; Ransom et al., 2003; Stopforth et al., 2004) have described the benefits of using CPC to decontaminate beef carcass tissue and the results of this study also indicate that fabrication-level CPC applications are effective against *E. coli* O157:H7 contamination on beef carcasses. Solutions of CPC are currently approved for use as an antimicrobial for poultry but not for use to decontaminate red meat (USDA-FSIS, 2009b).

Multiple hurdle decontamination programs are designed to systematically challenge microbes on carcass surfaces. Bacteria washed into defects on the carcass surfaces may evade one or more of these hurdles and ultimately survive the decontamination process. These data indicate that *E. coli* O157:H7 can survive decontamination treatments applied at ≤ 65°C and/or ≥ pH 3 and survive during carcass chilling (4°C) for at least 48 h at pH values as low as 4.11. These data also indicate that acid-stressed survivors may be less susceptible to ensuing decontamination treatments. These data are important as cells which survive carcass decontamination and chilling processes may: (i) be displaced from these reservoirs and (re)contaminate carcass surfaces or the surrounding environment during carcass fabrication; or (ii) remain intact and eventually be incorporated into beef trimmings destined for grinding.

The continued incidence of *E. coli* O157:H7 contaminated beef products and negative consequences associated with manufacturing, purchasing and consuming such products should encourage processors to minimize the creation of such reservoirs by optimizing the hide removal process and covering remaining tears before decontamination treatments are applied. If these reservoirs are encountered during fabrication, employees should be trained to carefully remove the pockets, taking care not to disrupt the fluid inside, and then discard them as inedible. Carcasses or equipment that become contaminated with fluid originating from such pockets should immediately be trimmed or cleaned and then sanitized. And finally, antimicrobial interventions should be applied at the fabrication level, and when applied, should be scientifically validated to reduce *E. coli* O157:H7 under the same conditions being used at each facility and should differ from those applied during slaughter.

3.6 Acknowledgements

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Table 3.1. Surviving natural contamination (APC) and inoculated *Escherichia coli* O157:H7 (EC) (log CFU/ml ± standard deviation) recovered from runoff fluid adjusted to pH 3, 5 or 7 with lactic acid and stabilized at 25, 45 or 65°C. Natural contamination and inoculated *E. coli* O157:H7 levels in untreated control samples (pH 7, 4°C) were 5.5 and 4.0 log CFU/ml, respectively.

pH	25°C		45°C		65°C	
	APC	EC	APC	EC	APC	EC
3	3.8±0.14 bA	3.8±0.04 bA	3.2±0.39 bA	3.2±0.71 bA	0.00±0.0 aA	0.0±0.00 aA
5	5.0±1.09 bB	4.2±0.36 bA	4.4±0.54 bB	4.0±0.08 bA	2.3±0.12 aB	2.7±0.32 aB
7	5.5±0.25 bB	4.0±0.14 aA	4.1±0.05 aB	4.0±0.02 aA	3.4±0.98 aA	3.2±0.62 aB

Least squares mean values with different lower case letters in the same row are different ($P < 0.05$).
Least squares mean values with different upper case letters in the same column different ($P < 0.05$).

Table 3.2. Detection of *Escherichia coli* O157:H7 in enriched samples. Cells were exposed to runoff fluid (pH 3, 65°C) and then stored in the fluid for 48h (4C°). Following enrichment, colonies recovered on TSArif or Rainbow Agar-O157 were screened for somatic (O157) and flagellar (H7) antigens using latex agglutination.

Time (h)	TSArif (n/N)[a]			Rainbow Agar-O157 (n/N)		
	Growth	O157	H7	Growth	O157	H7
0	0/6	0/0	0/0	0/6	0/0	0/0
12	0/6	0/0	0/0	0/6	0/0	0/0
24	1/6	1/1	1/1	1/6	1/1	0/1
36	3/6	3/3	3/3	2/6	2/2	0/2
48	4/6	4/4	4/4	4/6	4/4	0/4

[a]n/N: number of positive samples/number of samples tested

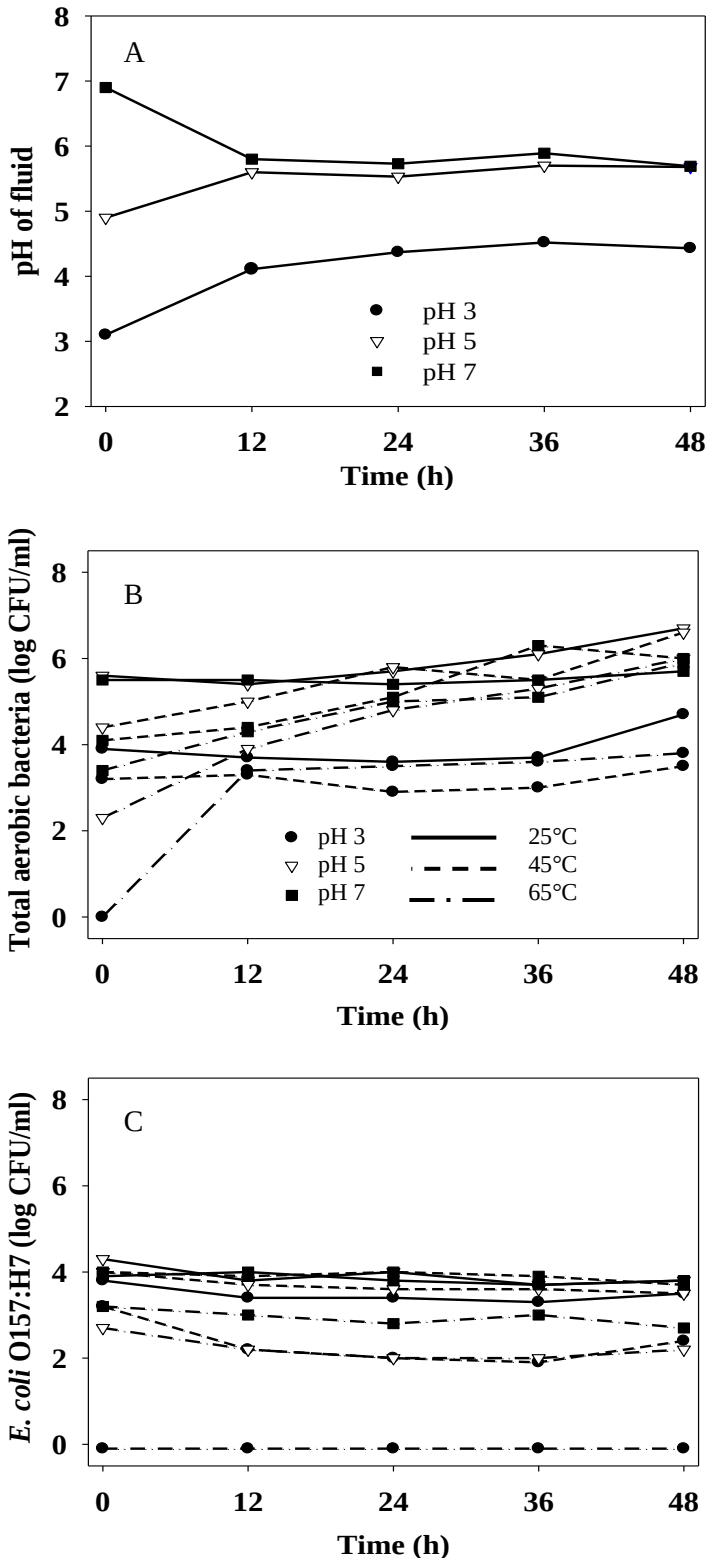

Fig. 3.1 (Data in Appendix Tables 1-3). Fluid pH (A) of runoff fluid, total aerobic bacteria (B), and *Escherichia coli* O157:H7 (C) populations (log CFU/ml) during storage (48 h, 4°C). Runoff fluid was adjusted to pH 3, 5 or 7 using lactic acid, adjusted to 25, 45 or 65°C, and then inoculated with *E. coli* O157:H7 (4.0 log CFU/cm^2)

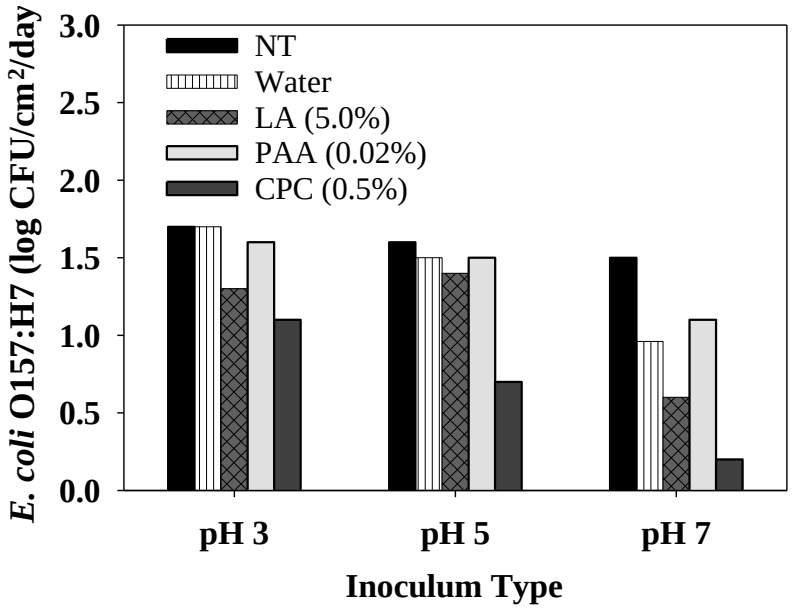

Fig. 3.2 (Data in Appendix Table 4). *Escherichia coli* O157:H7 (log CFU/cm^2) survivors recovered from decontaminated beef fat. Fluid (25°C) was adjusted to pH 3, 5 or 7 using lactic acid, inoculated with *E. coli* O157H7 and stored for 48 h (4°C). After 48 h, runoff fluid pH had increased from 3 to 4.11, 5 to 5.56, or decreased from 7 to 5.75 and runoff fluid was inoculated (1.5 log CFU/cm^2) onto fresh beef fat. Inoculated beef fat was then left untreated (NT) or sprayed (25°C, 20 psi, 1.38 bar, 3 sec) with water (control), 5.0% lactic acid (LA), 0.5% cetylpyridinium chloride (CPC) or 0.02% peroxyacetic acid (PAA).

CHAPTER FOUR

Optimization of Conditions for the Transfer, Attachment and Formation of Biofilms by *Escherichia coli* O157:H7 on Meat Contact Surface Materials

4.1 Abstract

Biofilms in food processing environments typically exhibit increased resistance to otherwise lethal interventions and can act as recurring sources of contamination. Studies examined the effects of fresh meat contact material types, inoculation substrate, surface pre-conditioning, presence or absence of fluid during incubation, presence of air at the liquid-surface interface, incubation substrate, and incubation time on the attachment of *Escherichia coli* O157:H7 cells and subsequent biofilm formation. The materials examined were selected following visual inspection of beef fabrication facilities and verbal communication with fabrication equipment manufacturers, sales personnel and purchasers which identified them as those most commonly used to construct hooks, knives, cutting tables, conveyor belts, storage containers and employee safety equipment. These materials included stainless steel (Type 304, #2b finish), acetal, polypropylene and high-density polyethylene (HDPE), and all surface materials were acquired and maintained in new, unblemished condition. A six-strain rifampicin-resistant *E. coli* O157:H7 composite was used to inoculate (6 log CFU/ml, g or cm^2) tryptic soy broth (TSB, pH 7.29), beef fat/lean homogenate (FLH, pH 5.66), fresh conveyor belt-runoff fluids containing dilute peroxyacetic/octanoic acid (CBRF, pH 4.6), fresh ground beef (pH 5.76), or fresh beef fat (pH 6.03). These substrates were then used to contaminate

dry, sterile coupons (2x5 cm) and coupons that were also pre-conditioned by submerging them in CBRF or beef fat homogenate (FH) for 45 min. Attachment of *E. coli* O157:H7 was surface material and substrate dependent, although beef fat appeared to negate differences among surface materials. Beef fat was the most effective ($P < 0.05$) inoculation substrate followed by ground beef, FLH and TSB. In general, pre-conditioning surfaces did not appear to influence attachment of *E. coli* O157:H7, regardless of inoculation substrate. As an exception, greater pathogen attachment was observed on pre-conditioned HDPE coupons compared to dry coupons when a liquid inoculum was used; no difference was observed when fat was used as the inoculation substrate. To determine the effect of production cycles on biofilm cells, inoculated coupons were incubated (15°C) in dry sterile tubes, in tubes containing 20 ml of diluted CBRF (dCBRF; pH 5.20), or in tubes containing 20 ml of dCBRF for 8 h and then in dry tubes for 16 h to simulate a normal beef processing facility production cycle. Coupons from each treatment were analyzed at 24, 48 and 96 h of incubation and at each sample collection interval, greater survival was observed on constantly hydrated coupons compared to coupons that were exposed to rotational hydration and drying. No *E. coli* O157:H7 survivors were observed on dry coupons after 96 h of incubation. To determine the effect of air at the incubation fluid-surface interface on biofilm formation, fat-inoculated coupons were placed in tubes containing either 20 ml (partially-submerged) or 30 ml (fully-submerged) of fresh CBRF (pH 3.07) or fresh beef fat homogenate (FH; 1:10 dilution in sterile distilled water, pH 4.21) for up to 16 d at 15°C. To determine the effect of incubation substrate on biofilm formation, fat-inoculated stainless steel and acetal coupons were partially-submerged (20 ml) in uninoculated TSB, FLH or CBRF for

up to 10 d at 15°C. Incubation in FLH allowed the pathogen to survive and grow on both surface materials, although TSB was the most optimal incubation substrate. Maximal biofilm formation was observed between 2 and 8 d of storage at 15°C, and growth was dependent on the presence of air and type of incubation media. These results indicate that the process of fabricating beef carcasses is conducive to the attachment of *E. coli* O157:H7 onto meat contact surfaces and the subsequent formation of biofilms. Furthermore, substrates generated, or otherwise encountered, during beef fabrication should be preferred in future studies designed to investigate *E. coli* O157:H7 biofilm development and control in these environments.

4.2 Introduction

Biofilms contain diverse communities of microorganisms and organic compounds from the surrounding environment encased by a protective layer of polysaccharides (Carpentier and Cerf, 1993), and represent the natural state of existence for most microbes (Terada et al., 2006). Medical implants and prostheses, contact lenses, ship-hulls, and fluid and food processing operations are examples of environments burdened by the formation and persistence of biofilms which can impede fluid flow in closed systems, interfere with heat transfer, corrode or otherwise damage metal or polymers, and lead to cross-contamination of the surrounding environment and/or human illness and infection (Chmielewski and Frank, 2003; Mittleman, 1998; O'Toole et al., 2000). Surface characteristics relevant to microbial cell attachment include charge, mass transport and hydrophobicity, and surface micro-topography as influenced by the age of a material and/or presence of a conditioning layer (Palmer et al., 2007). Brown et al. (1977) and Mittelman (1998)

attributed the initiation of biofilm formation to low nutrient availability or starvation conditions. Other environmental factors that may influence cell attachment and biofilm formation include temperature, pH, osmolarity, and the availability of oxygen or iron (O'Toole et al., 2000).

Biofilms have become one of the major concerns in the food industry related to food safety (Stopforth et al., 2002, 2007). The ability of *E. coli* O157:H7 to attach onto stainless steel (Wilks et al., 2005) and other meat contact surface materials has been documented and existing data suggest that beef fabrication equipment and other environmental locations may serve as potential reservoirs of *E. coli* O157:H7 contamination. *E. coli* O157:H7 has been recovered from fabrication-floor conveyor belt food contact surfaces during preoperational and mid-shift inspections (Chmielewski and Frank, 2003; Lindsay et al., 1996; Rivera-Betancourt et al., 2004). Gun et al. (2003) also recovered *E. coli* O157:H7 from processing room floors, cutting surfaces and conveyors, knives and hooks, aprons, saws, and the hands of abattoir employees. While interventions at the slaughter-level are critical for adequate pathogen control, their contribution may be nullified if contaminated food contact surfaces are encountered during fabrication, as the aforementioned studies indicate. Thus, pathogen control efforts should also address contamination encountered during beef carcass fabrication. Correspondingly, Kain et al. (1999) found that the overall frequency of pathogen contamination was greater on subprimals and steaks than on whole beef carcasses 24 h after the onset of chilling, indicating additional contamination events during fabrication handling, packaging and distribution. In another study, *E. coli* isolates recovered from animal hides, carcasses and

conveyors were genetically related to isolates recovered from ground beef samples (Aslam et al., 2004).

The impact of biofilms in food processing environments, and specifically during beef fabrication, is unclear due to the lack of research under conditions that simulate food systems and the food processing environment (Chmielewski and Frank, 2003). While it is apparent that *E. coli* O157:H7 biofilm formation occurs on food contact surface materials, little information is available regarding the types of surface materials and processing conditions and/or residues which retard or facilitate such formation (Bower and Daeschel, 1999; Brightwell et al., 2006). For example, the nutritional, atmospheric and protective properties associated with the aqueous, gaseous and solid phases of an environment should influence subsequent colonization by biofilm cells. While all of these phases are present during food processing applications, the majority of studies have only examined *E. coli* O157:H7 biofilm formation on surfaces which are entirely submerged in fluid. The positive influence of an air-liquid interface at the site of biofilm formation has been reported for *Salmonella*, *Pseudomonas* and *Bacillus* species (Giaouris and Nychas, 2006; Koza et al., 2009; Wijman et al., 2007). While Cabellos-Avelar et al. (2006) and Colón-Gonzalez et al. (2004) have described the general absence of *E. coli* biofilm formation under anaerobic conditions, the impact of an air-liquid interface on biofilm formation by *E. coli* O157:H7 is not known.

An adequate understanding of the ecology and characteristics of biofilms in food processing facilities are essential elements of effective pathogen control. Both proactive

and reactive control strategies may be developed following determination of the food processing conditions which inhibit or facilitate cell attachment and subsequent biofilm formation. Variables which optimize biofilm formation must first be identified in order to establish "worst-case" scenarios and identify adequate interventions for biofilm control. For these reasons, the ability of *E. coli* O157:H7 cells to attach onto, and form biofilms on the materials most commonly used to construct beef carcass fabrication equipment was examined, as well as the influence of different fabrication-floor residues and fluids on such attachment and biofilm formation. The materials examined were selected following visual inspection of beef fabrication facilities and verbal communication with fabrication equipment manufacturers, sales personnel and purchasers who identified them as those most commonly used to construct hooks, knives, cutting tables, conveyor belts, storage containers and employee safety equipment. These materials included stainless steel, acetal, polypropylene, and high density polyethylene (HDPE) and all surface materials were acquired and maintained in new, unblemished condition. The influence of an air-liquid interface at the site of *E. coli* O157:H7 biofilm formation was also examined.

4.3 Materials and Methods

4.3.1 Culture and Inoculum Preparation

The *E. coli* O157:H7 inoculum was comprised of six rifampicin-resistant derivatives of strains ATCC 51658, ATCC 51657 (not included in the air-liquid interface portion of the study), ATCC 43895, ATCC 43895/ISEH-GFP (Noah et al., 2005), and two strains (F284, F469) isolated from beef cattle feces (Carlson et al., 2006). Active cultures of

individual strains were first prepared (35ºC, 24 h) in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) and then subcultured (0.1 ml) under the same conditions. Individual cultures were then harvested by centrifugation (4,629 x *g* at 4ºC for 15 min) (Eppendorf, 5810 R; Brinkman Instruments, Inc., Westbury, NY) followed by washing in 10 ml phosphate-buffered saline (PBS, pH 7.4; 0.2 g KH_2PO_4, 1.5 g $Na_2HPO_4 \cdot 7H_2O$, 8.0 g NaCl, and 0.2 g KCl in 1 L distilled H_2O). Washed cells of each strain were resuspended in 10 ml PBS and combined to generate a 6-strain composite.

4.3.2 Inoculation Substrates

Fresh conveyor belt runoff fluid (CBRF) was obtained from the collection trays beneath fabrication floor conveyor belts used to transport beef subprimals between final trimming and vacuum-packaging stations at a commercial beef packing facility. Spraying bars were installed above the conveyor belts and were used to spray an antimicrobial solution (0.015-0.020%; Inspexx™, active ingredients include acetic acid, peracetic acid, octanoic acid, hydrogen peroxide, octaneperoxoic acid; Ecolab, St. Paul, MN) onto beef products as they passed underneath. Residual spraying solution and small pieces of beef tissue rinsed from subprimals during treatment traveled through the slotted plastic conveyor belts and into the fluid collection trays installed underneath, where it was then collected in sterile containers for use in the studies. Fresh beef fat, lean tissue and ground beef also were collected from a commercial beef processing facility. All items were transported (4°C) to the laboratory and immediately prepared for inoculation. The prepared inoculum was used to inoculate (6 log CFU/ml, g or cm^2) tryptic soy broth (TSB, pH 7.29), beef fat/lean tissue homogenate (1:10 dilution of fresh 50/50 beef fat/lean tissue

homogenized in sterile water; FLH, pH 5.66), fresh CBRF (pH 4.6), fresh ground beef (pH 5.76), or fresh beef fat (pH 6.03). Beef fat was inoculated and then stored at 4°C for 24 h before being used to simulate beef carcass chilling.

4.3.3 Inoculation of Surface Materials

The materials most commonly used to construct meat fabrication equipment were identified as stainless steel (Type 304, #2b finish), acetal, polypropylene, and high-density polyethylene (HDPE). New coupons (2x5 cm) of each material were washed with dish soap and water to remove any processing residue, rinsed with water and then soaked in 70% ethyl alcohol. Coupons were allowed to dry and then sterilized at 121°C for 18 min. Sterile/dry coupons of each material were submerged (4°C, 30 min) in inoculated fluids and ground beef or placed between two pieces of inoculated beef fat and pressure (20 kg) was applied. Using a plastic pipette, inoculated coupons were rinsed with 20 ml of sterile distilled water, and prepared for microbiological analysis or subsequent incubation as described below.

4.3.4 Fluid Level during Storage of Inoculated Coupons

To determine the effect of air at the liquid-solid interface on subsequent biofilm formation, inoculated coupons were placed in sterile polypropylene tubes (50 ml, Fisher Scientific, Fair Lawn, NJ) containing either 20 ml (partially-submerged) or 30 ml (fully-submerged) of CBRF (pH 3.07) or fresh beef fat homogenate (1:10 dilution of fresh beef fat homogenized in sterile water FH; pH 4.21). Tubes were incubated under static conditions for up to 16 d at 15°C. At 0, 1, 2, 3, 8, 12 and 16 d of storage, coupons were

removed from tubes, rinsed with 20 ml sterile distilled water, and then prepared for microbiological analysis.

4.3.5 Storage of Fat-Inoculated Coupons

The effect of incubation substrate on *E. coli* O157:H7 biofilm formation also was examined. Stainless steel and acetal coupons were exposed to inoculated beef fat, as described above, and then rinsed with 20 ml of sterile distilled water, using a plastic pipette, to remove unattached or loosely attached cells. Rinsed coupons were then placed in sterile polypropylene tubes (50 ml, Fisher Scientific) containing 20 ml of uninoculated TSB, FLH or CBRF and incubated statically for up to 10 d at 15°C. At 0, 1, 3, 7 and 10 d of storage, coupons were removed from tubes, rinsed with 20 ml sterile distilled water, and prepared for microbiological analysis.

4.3.6 Microbiological and pH Analyses

To enumerate microbial populations on coupon surfaces after inoculation and during storage, rinsed coupons (three per treatment in each of two biologically independent experiments) were placed in tubes containing 40 ml maximum recovery diluent (0.85% NaCl [Fisher] and 0.1% peptone [Bacto^{TM} Proteose Peptone, Difco]) and 10 glass beads, and vortexed for 2 min (3200 rpm) to remove attached cells. Sample aliquots were diluted in 0.1% sterile buffered peptone water (BPW; Difco) and plated onto trypic soy agar (TSA; Difco) for enumeration of total bacterial populations, and TSA supplemented with rifampicin (100 µl/L) (TSArif) for enumeration of pathogen populations. Plates were incubated at 25°C for 48 h (TSA) or 35°C for 24 h (TSArif) and colonies were

enumerated. Samples collected during the experiment designed to examine biofilm formation on partially- and fully-submerged coupons were, in addition, spread plated onto sorbitol MacConkey agar (SMAC; Difco) supplemented with cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L) (SMACct), incubated at 35°C for 24 h, and colonies were enumerated. SMACct was used for the recovery of both the inoculum population and naturally occurring *E. coli* O157:H7 in the FH and CBRF. SMACct was also used to indicate the presence of injured inoculum populations as it contains selective compounds that can inhibit the growth of injured cells (Hara-Kudo et al., 2000). Because both media were appropriate for the recovery of inoculum cells, fewer colonies on SMACct compared to TSArif plates should effectively indicate the presence of injured cells (Hara-Kudo et al., 2000; Taormina et al., 1998). Following incubation, at least three morphologically typical *E. coli* O157-like colonies found on the SMACct plates (colorless with or without a dark center) were removed and screened with the latex agglutination assay of the DrySpot™ *E. coli* O157:H7 Test Kit (Oxoid; Ogdensburg, UK), and each isolate was checked with a test reagent and control reagent located on the test card. Isolates also were checked against known positive and negative strain agglutination test reactions. The pH of substrates was measured at inoculation and at each sampling interval throughout storage using a digital pH meter fitted with a glass electrode (Denver Instruments, Arvada, CO).

4.3.7 Statistical Analysis

Three samples were analyzed per treatment in each of two biologically independent experiments and microbiological data were converted to log CFU/cm^2 and least squares

means were calculated using the analysis of variance in the general linear model procedure of SAS (version 9.1, SAS Institute, Cary, NC). Under the conditions of this study, the minimum detection level was 0.3 log CFU/cm^2. All samples with microbial populations below the level of detection were assigned a value of 0.29 log CFU/cm^2. For the first study, which examined cell attachment/transfer onto coupons, treatment effects included surface material (stainless steel, acetal, polypropylene or HDPE) and inoculation substrate (TSB, FLH, CBRF, ground beef or beef fat). For the second study, which examined the effect of air at the liquid-solid interface on biofilm formation, treatment effects included surface material (stainless steel, acetal, polypropylene, or HDPE), incubation fluid (CBRF or FH), fluid level (partially- or fully-submerged), and length of storage at 15°C (0, 1, 2, 5, 8, 12 or 16 d). For the third study, which examined biofilm formation by attached cells, treatment effects included surface material (stainless steel or acetal), incubation fluid (TSB, FLH or CBRF), and length of storage at 15°C (0, 1, 3, 7 or 10 d). All treatment effects were analyzed individually and interactively and a confidence limit of 95% was assigned to all significant treatment effects (P = 0.05).

4.4 Results and Discussion

4.4.1 Cell Attachment onto Meat Contact Surfaces

Although the process is not completely understood, initial cell attachment onto a surface is the result of interactions among cell-mediated properties, characteristics of the attachment surface, and the surrounding environment (O'Toole et al., 2000). This study investigated the attachment of *E. coli* O157:H7 cells onto different materials commonly encountered during beef carcass fabrication, and the influence of different substrates also

encountered during beef fabrication on such attachment.

In order to determine the effect of a conditioning layer of beef fabrication residues on initial attachment of *E. coli* O157:H7 onto stainless steel, acetal, polypropylene and HDPE, coupons of each material were submerged (25°C, 40 min) in CBRF or beef fat homogenate (FH; 1:10 dilution in sterile distilled water) to create a conditioning layer of fluid meat processing particles and natural flora on coupon surfaces. Both dry and conditioned coupons were exposed (15°C, 30 min) to inoculated (5 log CFU/ml or cm^2) laboratory buffer (PBS) or beef fat, and the effect of conditioning on cell attachment and/or transfer from each inoculation substrate onto coupons was examined. When inoculated with PBS, attachment of *E. coli* O157:H7 ranged from 1.2 to 2.2 log CFU/cm^2, with the greatest degree of attachment onto stainless steel, and the least attachment onto acetal coupons. The presence of a conditioning layer did appear to enhance attachment of *E. coli* O157:H7 onto HDPE (1.2 and 2.1 log CFU/cm^2 of attached cells onto dry or conditioned coupons, respectively), but did not influence attachment onto stainless steel, acetal or polypropylene (Table 4.1). When using beef fat as the inoculation substrate, *E. coli* O157:H7 cell attachment or transfer onto coupons was similar (2.4 ± 0.3 log CFU/cm^2) on all surface materials, and regardless of presence/absence of a conditioning layer (Table 4.1). Because attachment or transfer of *E. coli* O157:H7 onto coupons was generally unaffected by the presence/absence of a conditioning layer, the process of conditioning coupons was eliminated in susequent studies.

Under the conditions of this study, when dry, sterile coupons were exposed to inoculated TSB, CBRF, FLH, ground beef or beef fat, affinity of total aerobic bacteria (Fig. 4.1) and *E. coli* O157:H7 cells (Fig. 4.2) for each of the surface materials varied. When coupons were submerged in inoculated TSB, greater ($P < 0.05$) attachment of *E. coli* O157:H7 was observed on polypropylene and HDPE versus stainless steel or acetal (Fig. 4.1). When submerged in inoculated FLH or beef fat, greater ($P < 0.05$) attachment of *E. coli* O157:H7 was observed on stainless steel compared to plastic coupons (Fig. 4.1). When submerged in inoculated ground beef, greater ($P < 0.05$) attachment of the pathogen was observed on stainless steel and HDPE compared to acetal or polypropylene coupons (Fig. 4.1). Finally, when exposed to inoculated beef fat, attachment of the pathogen was not significantly different between any of the surface materials (Fig. 4.1).

It is imperative to study microbial attachment in the presence of substrates commonly encountered during food processing (Terada et al., 2006). Inoculated beef fat transferred the greatest ($P < 0.05$) amount of microbial contamination onto dry, sterile stainless steel, acetal, polypropylene and HDPE coupons, followed by ground beef, beef FLH, and TSB (Fig. 4.1). The greater level of contamination on surfaces inoculated with fat was probably due to a combination of more attached cells and cells fixed within the fat and to the surfaces by fat residues. No cells were recovered from the CBRF immediately after inoculation or from CBRF-inoculated coupons (Fig. 4.1). The absence of surviving cells was most likely due to the low pH (4.6) and other compounds in the CBRF (organic acids and peroxygens) which are known to be lethal against *E. coli* O157:H7 cells (Simpson and Sofos, 2009).

As indicated by these results, inherent differences between surface materials (i.e., hydrophobic, hydrophilic) may be negated when exposed to beef fat and/or lean tissues and tissue homogenates. Furthermore, beef fat and ground beef appeared to aid in initial physical transfer of bacteria, and specifically *E. coli* O157:H7, onto contact surfaces and then act as a physical protective barrier against the antimicrobial components found in the CBRF (Fig. 4.2 and 4.3). Therefore, studies which use laboratory media to investigate the attachment and growth of natural flora and *E. coli* O157:H7 on meat contact surfaces may not provide realistic representations of the events involved in physical transfer of cells onto surfaces and/or biofilm formation and maturation, in beef fabrication environments. Substrates found in beef fabrication settings should be used to determine the ability of *E. coli* O157:H7 to attach and grow on surface materials used to construct knives and hooks, saws, cutting boards, conveyor belts and storage containers.

4.4.2 Biofilm Formation on Meat Contact Surfaces

Cells first attach reversibly to a surface via van der Waals forces and electrostatic and hydrophobic cell-surface interactions (Chmielewski and Frank, 2003). Contaminated organic material such as ground beef or beef fat may also physically fix cells onto a surface. Reversible attachment requires as little as 5 to 30 sec, and can be reversed by applying very little force (Chmielewski and Frank, 2003; Mittleman, 1998). "Irreversible" adhesion is mediated by cell anchoring proteins and the production of protective polysaccharides (Terada et al., 2006). Remove of cells adhered to surfaces by proteins and polysaccharides requires manual scrubbing, which physically removes

protective layers and tears or breaks chemical bonds within appendages anchoring cells to the surface (Chmielewski and Frank, 2003). Once established, biofilms generally exhibit increased resistance to antimicrobials and/or other previously lethal interventions and create the potential for reoccurring contamination events as outer layers of the biofilm slough off into the surrounding environment (Chmielewski and Frank, 2003).

The effect of post-contamination hydration (dry, constant hydration or rotational hydration) on biofilm formation and survival of *E. coli* O157:H7 on stainless steel, acetal, polypropylene and HDPE also was examined (Table 4.2). Briefly, inoculated coupons were either incubated (15ºC) in empty centrifuge tubes (dry), or in centrifuge tubes containing 20 ml of diluted (1:4 in sterile distilled water) CBRF (dCBRF, pH 5.20). The effect of rotational versus constant hydration was observed by removing fluid from half of fluid-filled tubes after 8 h; fluid was replaced 16 h later (fluid in constantly-hydrated tubes also was replaced at this time for consistency among treatments). The rotational hydration schedule (8 h wet, 16 h dry; repeat) was maintained for 96 h, as were constant hydration and dry treatments. At 96 h of incubation, *E. coli* O157:H7 survivors were only recovered from coupons that were incubated under constant or rotational hydration (Table 4.2). Based on these data, coupons in subsequent studies were constantly hydrated during incubation in order to optimize biofilm development.

The influence of air on biofilm formation by *E. coli* O157:H7 at the liquid-solid interface of meat contact surfaces was determined using stainless steel, acetal, polypropylene and HDPE coupons which were contaminated using inoculated beef fat and then partially- or

fully-submerged in fresh beef FH or CBRF during incubation (15°C, 16 d). During incubation, the pH of the FH increased from 4.21 ± 0.11 on d-0 to 5.01 ± 0.36 and 5.90 ± 0.22 at d-1 and d-2 of storage at 15°C (Table 4.3). For the remainder of the storage period, pH values were approximately 6.00 (Table 4.3). The pH of CBRF increased from 3.07 ± 0.16 on d-0 to 4.11 ± 0.08 at d-1 of storage, and exhibited a similar pH for the duration of storage. The pH of the fluid was not different ($P \geq 0.05$) between partially- or fully-submerged samples. Correspondingly, incubation in FH promoted microbial growth, while CBRF resulted in growth inhibition or death (Fig. 4.2).

There was a three-way interaction between incubation media, fluid level and length of storage. Total aerobic bacteria and *E. coli* O157:H7 populations on coupons submerged in FH were not affected by fluid level during the first 48 h of storage (Fig. 4.2). Populations on fully-submerged coupons were static after 48 h of storage in FH while partially-submerged populations increased by an additional 1.2 log CFU/cm^2 before reaching stationary phase on d-8 of incubation (Fig. 4.2). No major growth was observed on coupons submerged in CBRF, regardless of fluid level, although partially-submerged conditions were more conducive ($P < 0.05$) to the survival of total aerobic populations stored in CBRF (Fig. 4.2). Rifampicin-resistant *E. coli* O157:H7 counts were reduced to below the detection limit immediately after being introduced into the CBRF and remained at or below the detection limit throughout storage (Fig. 4.2). The presence of air at the solid-liquid interface of coupons did not affect ($P \geq 0.05$) the survival of *E. coli* O157:H7 inoculum cells in CBRF (Fig. 4.2), but did appear to influence the survival of other naturally occurring sorbitol-negative *E. coli* O157:H7 (Fig. 4.2) that were present

on the beef fat used to inoculate coupons and/or in the CBRF. Counts recovered on SMACct should represent both surviving rifampicin-resistant inoculum cells, as well as naturally occurring sorbitol-negative *E. coli* O157:H7 (as determined by latex agglutination) associated with the beef fat used to inoculate coupons, or the incubation fluids, while those recovered on TSArif should only represent surviving inoculum cells. *E. coli* O157:H7 populations recovered on SMACct were equal to or greater than those recovered on TSArif (Fig. 4.2 and 4.2) and the divergence between *E. coli* O157:H7 counts recovered on SMACct and TSArif after 48 h in CBRF may be indicative of increased adaptation and survival of naturally occurring strains compared to laboratory strains. The acid-tolerant nature of *E. coli* O157:H7 has been established (Simpson and Sofos, 2009) and survival at pH values $\geq$ 4.0 has been previously reported (Stopforth et al., 2003a, 2003b). Another possible explanation for the disparity between the two selective media is that more inoculum cells were recovered on SMACct compared to TSArif, although TSArif should be a more favorable media for recovery of injured and uninjured rifampicin-resistant *E. coli* O157:H7 compared to SMACct (Simpson Beauchamp and Sofos, 2009).

Published data indicate, that for some species of bacteria, biofilm formation is enhanced under aerobic conditions with preferential colonization of biofilm cells at the air-liquid interface (Cabellos-Avelar et al., 2006; Wijman et al., 2007). Colón-Gonzalez et al. (2004) and Cabellos-Avelar et al. (2006) found that 90 to 100% of *E. coli* laboratory strains did not form biofilms in the absence of oxygen. While it is unclear whether biofilm formation occurred on the fully-submerged coupons in this study, continued

growth of the pathogen on partially-submerged coupons indicates that biofilms were formed on coupons when air was present at the liquid-solid interface. Additional research designed to identify the presence or absence of biofilm proteins and polysaccharides on such coupons is needed to determine whether biofilm formation occurred, and if so, which strains of *E. coli* O157:H7 were represented in such biofilms. In any respect, the increased growth of the pathogen on contaminated surfaces in the presence of air is significant and should be considered when designing future biofilm studies.

In order to optimize cell attachment or transfer and biofilm formation, all coupons were inoculated by exposure to inoculated beef fat and then partially submerged in fabrication floor fluids during incubation to allow air at the fluid-surface interface. In order to determine whether surface material influenced biofilm formation, stainless steel and acetal coupons were selected because these materials exhibited the highest and lowest level of *E. coli* O157:H7 attachment during the previously described attachment studies. The incubation fluids used included TSB, FLH and CBRF (Fig. 4.3). Initial pH values of TSB, FLH and CBRF were 7.22 ± 0.08, 5.56 ± 0.14 and 4.1 ± 0.22, respectively (Table 4.4). During incubation, TSB pH decreased to 6.13 ± 0.18 and 5.66 ± 0.14 at d-1 and d-3 of storage at 15°C, respectively, and was 5.71 ± 0.24 for the remainder of the storage period. The pH of FLH was similar (5.52 ± 0.18) at each sample collection interval during the 10 d storage period. CBRF pH increased from 4.1 ± 0.09 on d-0 to 5.11 ± 0.28 at d-1 of storage, and exhibited a similar pH for the duration of storage (Table 4.4).

Type of surface material did not influence ($P \geq 0.05$) biofilm formation during incubation for 10 d at 15ºC (Fig. 4.3). After 3 d of incubation, initial pathogen populations (4.0 log CFU/cm^2) on fat-inoculated surfaces in FLH and TSB increased by 1.5 or 3.0 log CFU/cm^2, respectively, or decreased by 2.0 log CFU/cm^2 in CBRF, and then remained constant for the remainder of storage (Fig. 4.3). Total aerobic bacterial populations on coupons stored in TSB, FLH, and CBRF responded similarly to *E. coli* O157:H7 populations (Fig. 4.3). These data indicate that at 15°C (the average temperature of commercial beef fabrication facilities non-production hours), FLH provided sufficient nutrients for the growth of microbial contaminants, and specifically *E. coli* O157:H7 on meat contact surface materials. Thus, in the absence of proper cleaning and sanitation, beef fabrication residues may support formation and persistence of *E. coli* O157:H7 biofilms in beef processing environments.

In contrast, exposing soiled coupons to the CBRF immediately reduced total aerobic bacteria by 3.2 ± 0.2 log CFU/cm^2, and *E. coli* O157:H7 populations to below the level of detection (0.3 log CFU/cm^2) (Fig. 4.2). Microbial inactivation and inhibitory effects were most likely due to the low pH and antimicrobial compounds in the CBRF (peroxyacetic and octanoic acid derivatives) (Simpson and Sofos, 2009). Cells adhered to coupons with beef fat may have been protected from the lethal properties of the CBRF as indicated by the presence of surviving inoculum cells on soiled coupons during extended storage in the fluid (Fig. 4.2 and 4.3). This highlights the importance of thoroughly cleaning soiled surfaces to remove all remnants of beef fat or other organic

material which may harbor or protect microbial contaminants during otherwise lethal antimicrobial interventions.

To summarize, under the conditions of this study, *E. coli* O157:H7 cells exhibited the ability to attach onto stainless steel, acetal, polypropylene and HDPE. The greatest level of attachment occurred when beef fat was used to transfer cells onto the surface materials, followed by ground beef, FLH and then TSB. When coupons were exposed to inoculated fluids (FLH or TSB), attachment of *E. coli* O157:H7 was dependent ($P < 0.05$) on type of surface material and inoculation substrate. Surface material did not significantly influence the level of microbial attachment when inoculated beef fat was used to transfer microbial contamination onto coupons, and attachment of *E. coli* O157:H7 was similar across all surface materials. In general, preconditioning coupons did not significantly influence attachment of *E. coli* O157:H7. As an exception, attachment of *E. coli* O157:H7 was enhanced on preconditioned HDPE when using a liquid inoculum. Under the conditions of these studies, maximal biofilm formation was observed between 2 and 8 d of storage at 15°C. Degree of biofilm formation also was dependent on the presence or absence of air at the coupon surface during incubation and the type of incubation media. Biofilm formation was retarded on inoculated coupons stored under dry conditions or under rotationally hydrated conditions (8 h hydrated, 16 h dry), and biofilm populations persisted longer on coupons which were maintained under constantly hydrated conditions. Incubation in beef lean tissue homogenate allowed the pathogen to survive and grow on both stainless steel and plastic coupons during storage,

although laboratory medium (TSB) was the most optimal incubation substrate. Surface material did not have a significant impact on biofilm formation.

These findings are significant since the outer fat layer of beef carcasses is the least hygienic area of the carcass. Because of the repeated contact between the outer fat layer of beef carcasses and carcass cutting and breaking surfaces during fabrication, the potential for cross-contamination is high. The ability of ground beef to transfer microbial contaminants onto contact surfaces is also important due to the serious implications associated with pathogens in ground products and the establishment of *E. coli* O157:H7 biofilms in grinding facilities. Beef fat also appeared to protect cells adhered to surfaces from the antimicrobial properties of peroxyacetic and octanoic acids found in the incubation substrate. The importance of adequate cleaning protocols that incorporate scrubbing to remove physical contamination has been established (Farrell et al., 1998; Taylor et al., 1999). Physical removal of fat residues prior to the application of chemical sanitizers is imperative for effective cleaning and sanitation, and scrubbing contact surfaces even when visible contamination is not present is required to remove biofilm cells. Care should also be taken to manually scrub the inside of grinders and storage containers, and where scrubbing is not possible, well designed clean-in-place (CIP) systems should be used. Furthermore, water and/or processing fluids should not be allowed to stand on cooler floors or in drip pans, floor drains or storage containers for long periods of time. These data also indicate that spraying antimicrobial solutions, such as peroxyacetic acid and/or octanoic acid on conveyor belts during fabrication, may

reduce planktonic contamination levels and mitigate initial attachment and subsequent growth of *E. coli* O157:H7 on meat contact surfaces.

4.5 Acknowledgements

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Table 4.1. Total aerobic and *Escherichia coli* O157:H7 populations (mean ± standard deviation; log CFU/cm^2), recovered with tryptic soy agar (TSA) without or with rifampicin (100 μg/ml; TSArif), respectively, attached to stainless steel (SS), acetal, polypropylene (PP), or high-density polyethylene (HDPE) coupons (2×5 cm) immediately after two inoculation scenarios. Coupons were either clean and dry, or preconditioned by being submerged for 45 min in conveyor belt runoff fluids (CBRF) or beef fat homogenate (FH) and then: (i) submerged in liquid inoculum (cells suspended in PBS; 5 log CFU/ml) for 30 min; or, (ii) placed between two pieces of inoculated beef fat.

Surface material	Surface condition	Liquid inoculum		Inoculated fat	
		TSA	TSArif	TSA	TSArif
SS	Dry	2.2±0.3 aA	2.2±0.1 aA	2.8±0.3 bBCD	2.4±0.2 aBC
	Preconditioned in FH	[a] -	-	2.6±0.2 deCD	2.1±0.1 D
	Preconditioned in CBRF	2.2±0.3 Aa	2.1±0.2 aA	2.5±0.5 aD	2.1±0.6 aD
Acetal	Dry	1.4±0.2 aB	1.4±0.4 aBC	3.3±0.3 bA	2.6±0.3 bAB
	Preconditioned in FH	-	-	2.9±0.6 BC	2.4±0.3 BC
	Preconditioned in CBRF	1.5±0.2 aB	1.4±0.4 aBC	2.8±0.2 bBCD	2.4±0.3 bBC
PP	Dry	1.5±0.4 aB	1.6±0.0 aB	2.9±0.2 bBC	2.5±0.3 bABC
	Preconditioned in FH	-	-	3.2±0.3 AB	2.7±0.3 A
	Preconditioned in CBRF	1.9±0.2 aA	1.9±0.3 aA	2.8±0.6 bBCD	2.3±0.4 bCD
HDPE	Dry	1.3±0.4 aB	1.2±0.6 aC	3.0±0.2 bB	2.5±0.1 bABC
	Preconditioned in FH	-	-	2.0±0.1 E	2.4±0.2 BC
	Preconditioned in CBRF	2.1±0.2 aA	2.1±0.0 aA	2.8±0.2 bBCD	2.3±0.4 aCD

[a] - Samples were not collected

Mean values with different lower case letters, in the same row for each recovery medium are different ($P < 0.05$).

Mean values with different upper case letters in the same column are different ($P < 0.05$).

Table 4.2. *Escherichia coli* O157:H7 populations (mean ± standard deviation; log CFU/cm^2), recovered with tryptic soy agar plus rifampicin (100 µg/ml), attached to stainless steel (SS), acetal, polypropylene (PP), or high density polyethylene (HDPE) coupons (2×5 cm) that were dry/sterile or preconditioned (PC) in diluted conveyor belt runoff fluid (dCBRF, pH 5.2) prior to being submerged in a liquid inoculum (5 log CFU/ml, 30 min), and then incubated in dCBRF (15ºC) under rotational hydration (Rotation; 8 h hydrated, 16 h dry), constant hydration (Wet), or constant dry (Dry) conditions.

Surface material	Surface condition	Initial population (0 h)	Incubation conditions	Incubation period (h)			
				8	24	48	96
SS	Dry	2.0±0.1 aA	Rotation	1.9±0.1 a	0.4±0.5 b	<0.3 b	<0.3 b
			Wet	[a] -	1.3±0.4 b	<0.3 c	0.3±0.6 c
			Dry	-	0.4±0.5 b	<0.3 b	<0.3 b
	PC	1.9±0.5 aA	Rotation	1.7±0.2 a	0.3±0.3 b	<0.3 b	<0.3 b
			Wet	-	0.9±0.9 b	<0.3 c	0.5±0.2 bc
			Dry	-	<0.3c	<0.3 c	<0.3 c
Acetal	Dry	1.2±0.6 aB	Rotation	<0.3 b	<0.3 b	<0.3 b	<0.3 b
			Wet	-	0.4±0.5 b	<0.3 b	<0.3 b
			Dry	-	<0.3 b	<0.3 b	<0.3 b
	PC	2.0±0.1 aA	Rotation	1.0±0.3 b	0.4±0.5 c	<0.3 c	<0.3 c
			Wet	-	0.5±0.2 b	0.5±0.8 b	<0.3 b
			Dry	-	0.4±0.5 b	<0.3 b	<0.3 b
PP	Dry	1.4±0.2 aB	Rotation	1.0±0.2 a	<0.3 b	<0.3 b	<0.3 b
			Wet	-	1.0±0.2 b	0.5±0.2 c	<0.3 c
			Dry	-	0.7±1.0 b	<0.3 b	<0.3 b
	PC	2.0±0.2 aA	Rotation	0.5±0.3 b	0.4±0.5 b	<0.3 b	<0.3 b
			Wet	-	0.7±0.1 b	0.4±0.5 b	<0.3 b
			Dry	-	0.7±0.6 b	0.5±0.3 b	<0.3 b
HDPE	Dry	1.3±0.1 aB	Rotation	1.0±0.1 a	<0.3 b	<0.3 b	<0.3 b
			Wet	-	0.5±0.8 b	0.5±0.8 b	<0.3 b
			Dry	-	0.6±0.6 b	<0.3 b	<0.3 b
	PC	1.8±0.2 aA	Rotation	1.4±0.1 a	0.4±0.5b	<0.3 b	0.4±0.5 b
			Wet	-	0.8±0.6 b	<0.3 c	<0.3 c
			Dry	-	1.3±0.2 b	1.2±0.1 b	<0.3 c

<0.3 log CFU/cm^2 = detection limit

[a] - Samples not collected

Mean values with different lower case letters in the same row are different ($P < 0.05$).

Mean values with different upper case letters in the same column and within each surface material are different ($P < 0.05$)

Table 4.3. The pH values (mean ± standard deviation) of beef fat homogenate (FH; 1:10 in sterile distilled water) or fresh conveyor belt runoff fluid (CBRF; dilute peroxyacetic/octanoic acid, pH 4.60) during incubation (16 days, 15°C) of inoculated stainless steel (SS), acetal, polypropylene (PP), and high density polyethylene (HDPE) coupons (2×5 cm).

Substrate	Partially or Fully Submerged	Food contact surface	Storage days[a]					
			1	2	5	8	12	16
FH	Partially-submerged	SS	4.64±0.27 bA	6.10±0.20 bB	6.02±0.08 bB	6.08±0.02 bB	6.06±0.03 bB	6.06±0.07 bB
		acetal	4.97±0.14 bA	5.85±0.11 bB	5.95±0.02 bB	6.04±0.02 bB	6.01±0.02 bB	6.01±0.02 bB
		PP	5.06±0.05 bA	5.83±0.03 bB	6.03±0.04 bB	6.04±0.02 bB	5.99±0.05 bB	5.99±0.04 bB
		HDPE	5.00±0.08 bA	5.68±0.06 bB	6.11±0.07 bC	6.03±0.05 bC	6.43±0.48 bD	6.43±0.00 bD
	Fully-submerged	SS	5.03±0.53 bA	5.98±0.20 bB	5.95±0.01 bB	5.88±0.05 bB	5.87±0.01 bB	5.87±0.06 bB
		acetal	4.95±0.35 bA	5.88±0.04 bB	5.94±0.02 bB	5.92±0.03 bB	5.87±0.02 bB	5.87±0.09 bB
		PP	5.21±0.38 bA	5.89±0.33 bB	6.10±0.37 bB	5.89±0.07 bB	5.90±0.04 bB	5.90±0.08 bB
		HDPE	5.18±0.22 bA	6.00±0.20 bB	5.83±0.12 bB	5.89±0.07 bB	5.88±0.06 bB	5.88±0.03 bB
CBRF	Partially-submerged	SS	4.03±0.01 aA	4.20±0.04 aA	3.92±0.03 aA	4.06±0.11 aA	4.13±0.01 aA	4.13±0.02 aA
		acetal	4.12±0.04 aA	4.30±0.07 aA	4.03±0.02 aA	4.06±0.02 aA	4.13±0.01 aA	4.13±0.00 aA
		PP	4.09±0.01 aA	4.29±0.05 aA	3.99±0.02 aA	4.18±0.02 aA	4.15±0.03 aA	4.15±0.01 aA
		HDPE	4.09±0.05 aA	4.25±0.07 aA	3.98±0.01 aA	4.04±0.19 aA	4.13±0.01 aA	4.13±0.02 aA
	Fully-submerged	SS	4.10±0.04 aAB	4.27±0.05 aB	3.97±0.07 aA	4.04±0.00 aA	4.13±0.02 aAB	4.13±0.01 aAB
		acetal	4.12±0.03 aA	4.34±0.02 aB	3.98±0.03 aA	4.06±0.01 aA	4.12±0.01 aA	4.12±0.00 aA
		PP	4.13±0.05 aA	4.30±0.05 aB	3.97±0.03 aA	4.06±0.01 aA	4.11±0.00 aA	4.06±0.00 aA
		HDPE	4.16±0.01 aA	4.31±0.04 aB	3.97±0.03 aA	4.06±0.01 aA	4.12±0.03 aA	4.12±0.02 aA

[a] Initial (day-0) pH values of FH and CBRF were 4.21±0.37 and 3.07±0.01, respectively.

Least squares mean values with different lower case letters in the same row are different ($P < 0.05$).

Least squares mean values with different upper case letters in the same column are different ($P < 0.05$).

Table 4.4. The pH values (mean ± standard deviation) of tryptic soy agar (TSB) beef fat homogenate (FLH; 1:10 in sterile distilled water) or fresh conveyor belt runoff fluid (CBRF; dilute peroxyacetic/octanoic acid) during incubation (10 days, 15°C) of inoculated stainless steel (SS) or acetal coupons (2×5 cm).

Substrate	Storage days				
	0	1	3	7	10
TSB	7.22±0.08 cC	6.13±0.18 bB	5.66±0.14 bB	5.87±0.31 bB	5.37±0.08 aA
FLH	5.56±0.14 aB	5.51±0.13 aA	5.46±0.11 aAB	5.56±0.18 aB	5.50±0.32 aA
CBRF	4.10±0.22 aA	5.11±0.28 bA	5.16±0.36 bA	5.07±0.18 bA	5.11±0.30 bA

Mean values with different lower case letters in the same row are different ($P < 0.05$)
Mean values with different upper case letters in the same column are different ($P < 0.05$)

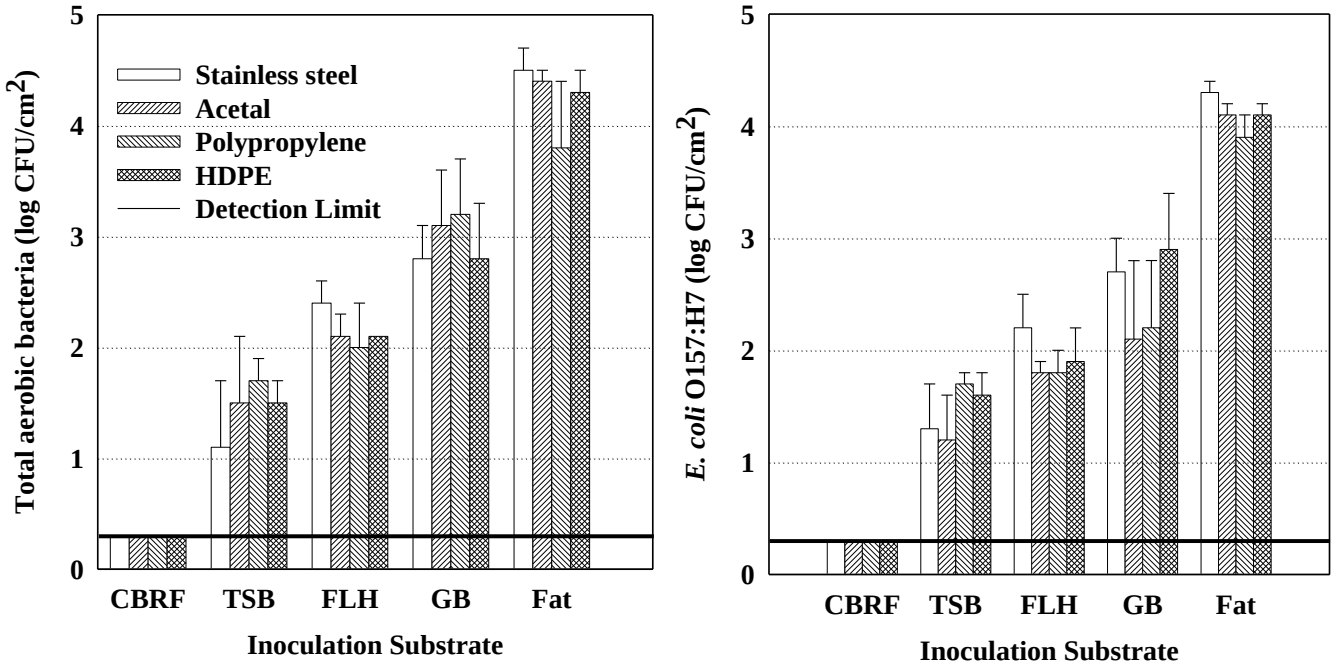

Fig. 4.1 (Data in Appendix Tables 5-6). Attachment (log CFU/cm^2) of aerobic bacteria and *Escherichia coli* O157:H7 to meat contact surface materials after 30 min exposure to inoculated conveyor belt runoff fluid (CBRF; pH 4.60), tryptic soy broth (TSB; pH 7.29), beef fat/lean tissue homogenate (FLH; pH 5.66), fresh ground beef (GB; pH 5.76), or fresh beef fat (pH 6.00).

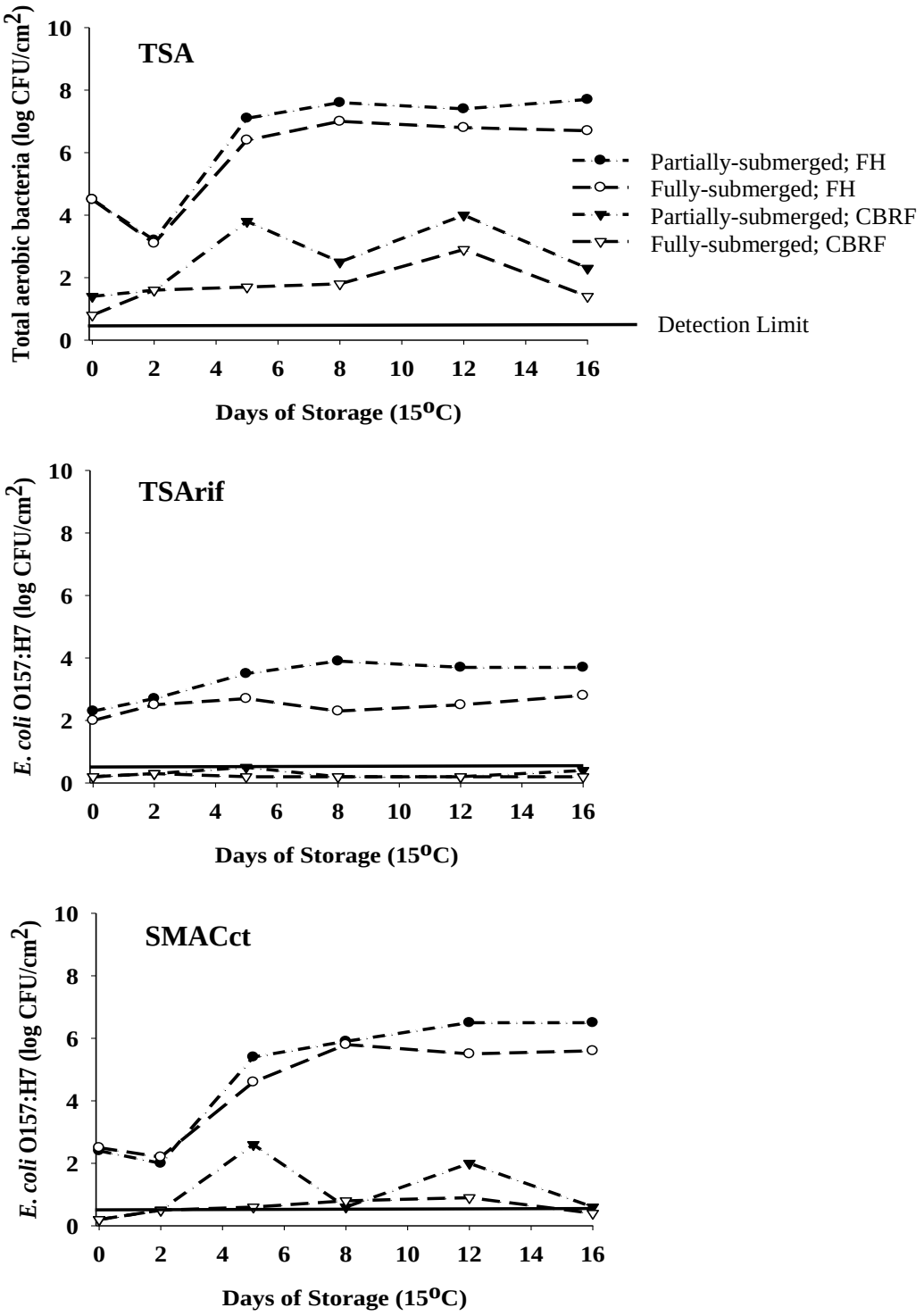

Fig. 4.2 (Data in Appendix Tables 7-9). Growth (log CFU/cm^2) of total aerobic bacteria recovered with TSA and *Escherichia coli* O157:H7 populations recovered with TSArif or SMACct, attached to meat contact surface materials which were incubated in 20 ml (partially-submerged) or 30 ml (fully-submerged) of beef fat homogenate (FH) or conveyor-belt runoff fluid (CBRF). Partial versus full submersion of inoculated coupons was used to examine the influence of air at the liquid-solid interface on biofilm formation by natural flora and *E. coli* O157:H7.

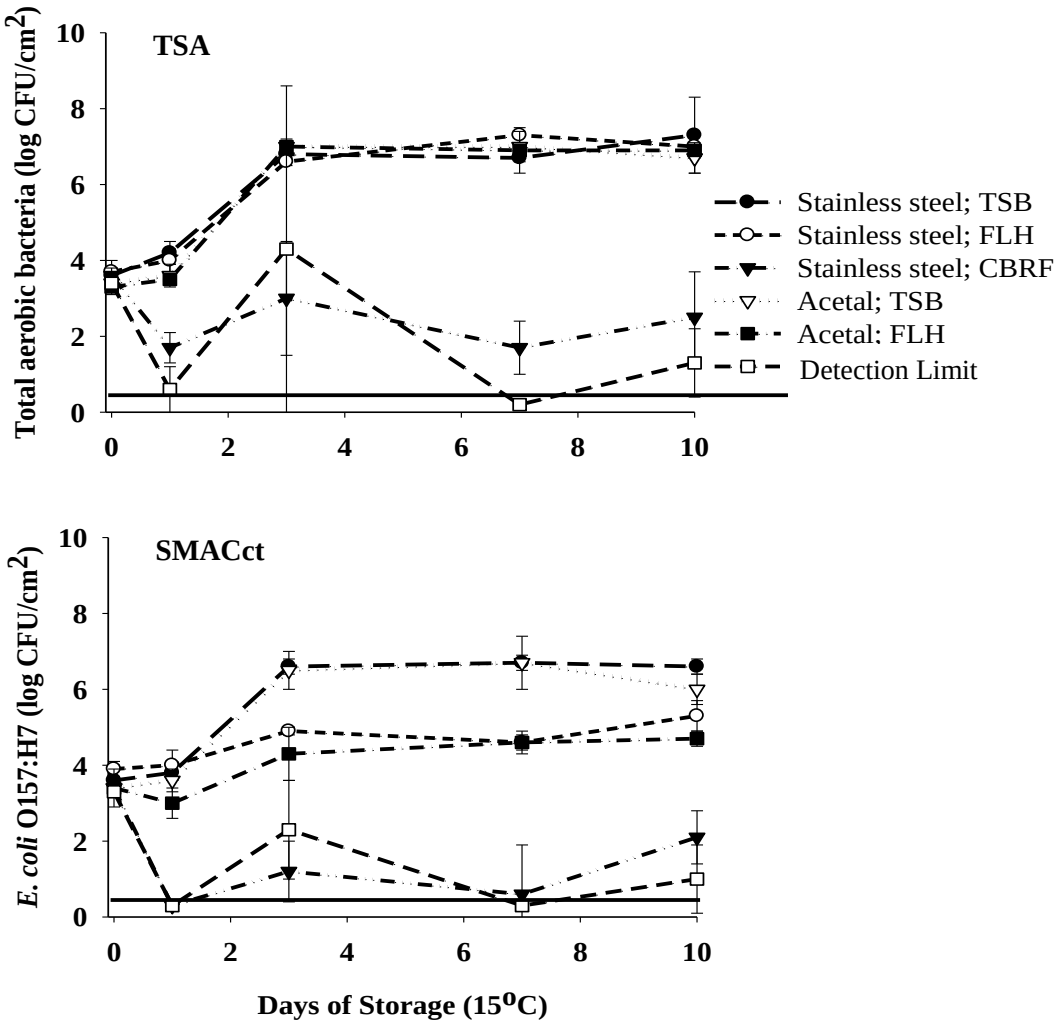

Fig. 4.3 (Data in Appedix Tables 11-12). Growth (log CFU/cm^2) of total aerobic bacteria recovered with TSA or *Escherichia coli* O157:H7 recovered with SMACct, attached to meat contact surface materials which were exposed (30 min) to inoculated beef fat and then incubated in tryptic soy broth (TSB), beef fat/lean tissue homogenate (FLH), or fresh conveyor belt runoff fluid (CBRF).

CHAPTER FIVE

Sanitizer Efficacy against *Escherichia coli* O157:H7 Biofilms As Influenced by Surface Material, Sanitizer Concentration and Exposure Time

5.1 Abstract

Negative consequences associated with pathogens on inadequately cleaned surfaces necessitate the identification of sanitizers which are effective on both clean and soiled surfaces. This study evaluated the efficacy of approved food contact surface sanitizers and sanitizers which are unapproved for use on food contact surfaces, against *Escherichia coli* O157:H7 cells in biofilms formed on surface materials used in beef fabrication facilities. Sanitizers applied at manufacturers' minimum and maximum recommended concentrations for use on food contact surfaces included acidified sodium chlorite (ASC), sodium hypochlorite (SH), potassium peroxymonosulfate/sodium chloride (PP/SC), two commercial quaternary ammonium compounds containing a mixture of ammonium chloride compounds (QUAT-A) or cetylpyridinium chloride (QUAT-B), peroxyacetic acid (PAA), and a peroxyacetic acid/octanoic acid mixture (PA/OA). Coupons (2x5 cm) of stainless steel, acetal, and high-density polyethylene were inoculated with a 6-strain rifampicin-resistant *E. coli* O157:H7 composite (3-4 log CFU/cm^2) and incubated (15°C) in fresh, unsterilized beef lean/fat tissue homogenate (pH 5.66). At d-3 of incubation, attached cells were challenged by submerging coupons in minimum and maximum recommended concentrations of each of the seven sanitizing solutions or water (control) for 1 or 10 min. Sanitizer treatments reduced *E. coli* O157:H7 on coupons by 0.0 to 2.2

log CFU/cm^2 and treatment efficacy decreased in order of ASC > PAA > PP/SC = PA/OA > QUAT-B > QUAT-A = SH. Maximum reductions and extent of inactivation generally increased as concentration and exposure time increased. Three sanitizers (SH, QUAT-A and PA/OA) were also applied on 0- and 7-d old biofilm cells at minimum and maximum recommended concentrations for 1 and 10 min. On d-0, 10 min exposures to maximum concentrations of PA/OA or QUAT-A reduced *E. coli* O157:H7 (3.6 log CFU/cm^2) by 1.5 or 0.8 log CFU/cm^2, respectively. On d-3 and -7, 10 min exposure to the maximum recommended concentration of PA/OA reduced *E. coli* O157:H7 (3.7-4.3 log CFU/cm^2) by only $\leq$ 0.9 log CFU/cm^2. In general, SH treatments were ineffective against *E. coli* O157:H7 biofilm cells. Surface material did not ($P \geq 0.05$) influence the fate of biofilm cells during sanitizing treatments. These data indicate that, while no sanitizer consistently reduced pathogen populations by more than 1 log cycle on soiled surfaces, approved concentrations of ASC and peroxyacetic acid-based sanitizers may be more effective against *E. coli* O157:H7 on inadequately cleaned surfaces than other sanitizers. Increased resistance of cells in older biofilms also emphasizes the importance of thorough cleaning before sanitation and applying sanitizers at the highest allowable concentrations for extended exposure times.

5.2 Introduction

Pathogens, including *E. coli* O157:H7, have been recovered from beef fabrication-floor conveyor belts during both pre-operational and mid-shift inspections (Rivera-Betancourt et al., 2004). Other research groups have recovered viable bacteria from conveyor belts and carcass breaking/cutting surfaces, and inadequately sanitized food contact surfaces

have been implicated in multiple outbreaks of foodborne illness (Chmielewski and Frank, 2003; Gill and Landers, 2004; Lindsay et al., 1996). *E. coli* O157:H7 was also shown to attach to stainless steel surfaces (Rivas et al., 2007; Ryu et al., 2004a, 2004b). Wilks et al. (2005) found that *E. coli* O157 survived for 28 d at 4°C on stainless steel, while copper and copper alloys were bactericidal. Even so, the functionality of stainless steel has resulted in its extensive use as a building material for food processing equipment and facilities. Other materials commonly used to construct food processing equipment include acetal, polypropylene and high density polyethylene (HDPE) (Chapter 4). While all aspects of biofilm formation and persistence are not clear, it is apparent that: (i) biofilms can form on meat contact surface materials, including those used to manufacture knives, cutting tables and conveyor belts; (ii) cells within biofilms generally exhibit an increased resistance to cleaning and sanitization programs; and, (iii) biofilms which are present on food contact surfaces can act as repeated sources of contamination (Bower and Daeschel, 1999; Brightwell et al., 2006; Chmielewski and Frank, 2003). Further research is needed to determine what, if any, type of meat contact surface material(s) is/are most optimal for use when constructing beef fabrication equipment and how to most effectively remove *E. coli* O157:H7 biofilms from such materials.

Sanitizers, as defined by the Environmental Protection Agency, are "intended to disinfect or sanitize, reducing or mitigating growth or development of microbiological organisms…on inanimate surfaces in the household, institutional, and/or commercial environment" (40 CFR 455.10). Sanitizers are designed to inactivate microorganisms which survive and/or are translocated during cleaning processes. Several chlorine

compounds, including sodium hypochlorite and acidified sodium chlorite, are approved for use as food contact sanitizers (21 CFR 178.1010) and are generally effective against both gram-positive and gram-negative bacteria, although biofilm cells tend to be less susceptible than planktonic cells (Simpson and Sofos, 2009). Sodium hypochlorite a widely used commercial sanitizer and efficacy increases when applied at pH values between 6.5 and 7.5 (Takeuchi and Frank, 2001). Decreased activity is observed in the presence of excess organic matter or when applied using hard water (Simpson and Sofos, 2009). Chlorous acid is the active compound produced when sodium chlorite is combined with a generally recognized as safe (GRAS) weak acid (i.e., citric acid), and as a sanitizer, exhibits an antimicrobial activity and spectrum similar to that of chlorine. Efficacy of chlorous acid is optimized at pH $\leq$ 2.5 and in the absence of organic material (EFSA, 2008). A commercial solution of potassium peroxymonosulfate and sodium chloride (PP/SC) is available for use as a broad spectrum hard surface disinfectant in livestock production and veterinary facilities, but is not approved as a sanitizer for food contact surfaces. In general, PP/SC is active against both gram-positive and gram-negative bacteria, and antimicrobial activity is markedly, but not entirely, reduced in the presence of excessive organic material or when mixed using hard water (http://www.biosecuritycenter.org/content/labelClaim/virkonS.pdf).

Peroxyacetic acid (PAA) is a powerful oxidizing agent approved for use on food contact surfaces, although gram-negative bacteria are more susceptible than gram-positive types (Simpson and Sofos, 2009). Optimal antimicrobial activity is observed at above ambient temperature, under acidic pH and in the absence of organic material. The antimicrobial

activity of PAA is not affected by the presence of metallic ions (hard water source) but is slightly diminished in cold temperature applications (Simpson and Sofos, 2009). The U.S. Food and Drug Administration (FDA) also approved a sanitizing solution mixture (i.e., Vortexx) which contains a mixture of peroxyacetic acid, acetic acid, octanoic acid, and hydrogen peroxide (21 CFR 178.1010) and is marketed by Ecolab, Inc. (http://www.ecolab.com/Initiatives/FoodSafety/AST/Vortexx.asp).

Quaternary ammonium compounds (QAC) are surface active agents (surfactants) approved for use on food contact surfaces (21 CFR 178.1010) although gram-negative bacteria are also less susceptible than gram-positive types (Simpson and Sofos, 2009). QAC are more effective when applied at higher temperatures (> 22°C, < 55°C) and at an acidic pH (IPCS, n.d.). Activity is generally reduced at lower then ambient temperature and in the presence of excessive organic material, metallic ions, anionic surfactants/detergents or soaps (Simpson and Sofos, 2009). Cetylpyridinium chloride (CPC) is a QAC commonly used in oral hygiene products for its ability to impede attachment of plaque-forming bacteria to tooth enamel (Simpson and Sofos, 2009). CPC is approved for use as a poultry carcass decontamination fluid (USDA-FSIS, 2009b) but is not approved for use on food contact surfaces.

Sanitizers are designed to inactivate microbial contaminants present on clean surfaces, in the absence of cleaning agents and organic material. Even so, the large size of commercial beef fabrication facilities, in conjunction with limited employee training and/or inadequate guidance during clean-up undoubtedly contributes to improperly

cleaned facilities and equipment. Therefore, while cleaning should always precede sanitation, it would be useful to identify a sanitizer which effectively reduces microbial contamination on both clean and soiled surfaces. For these reasons, this study was designed to investigate the effectiveness of seven different sanitizing solutions against *E. coli* O157:H7 biofilm cells on soiled food contact surfaces, and the effect of surface material and biofilm age on sanitizer efficacy.

5.3 Materials and Methods

5.3.1 Culture and Inoculum Preparation

The *E. coli* O157:H7 inoculum was comprised of six rifampicin-resistant derivatives of strains ATCC 51657, ATCC 51658, ATCC 43895, ATCC 43895/ISEH-GFP (Noah et al., 2005) and two strains recovered from beef cattle feces (F284, F469) (Carlson et al., 2006). Active cultures of individual strains were first prepared (35ºC, 24 h) in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) and then subcultured (0.1 ml) under the same conditions. Individual cultures were then harvested by centrifugation (4,629 x *g* at 4ºC for 15 min) (Eppendorf, 5810 R; Brinkman Instruments, Inc., Westbury, NY) followed by washing in 10 ml phosphate-buffered saline (PBS, pH 7.4; 0.2 g KH_2PO_4, 1.5 g $Na_2HPO_4 \cdot 7H_2O$, 8.0 g NaCl, and 0.2 g KCl in 1 L distilled H_2O). Washed cells of each strain were resuspended in 10 ml PBS and combined to generate a six-strain composite inoculum. Fresh beef fat and lean tissue were purchased from a commercial supplier and used within 48 h of receipt. Beef fat was prepared for inoculation by trimming pieces to uniform thickness and laying them side by side on sterile foil-covered trays (external fat side up) to create a solid sheet of beef fat. The prepared inoculum was

uniformly spread onto the beef fat (pH 6.03) using a sterile bent glass rod (target inoculum level of 6 log CFU/cm^2), trays of inoculated beef fat were then covered and stored at 4°C for 24 h to simulate beef carcass chilling.

5.3.2 Inoculation and Storage of Surface Materials

The materials most commonly used to construct meat fabrication equipment were identified as stainless steel (Type 304, #2b finish), acetal and high-density polyethylene (HDPE) (Chapter 4). Coupons (2x5 cm) of each material were washed with dish soap and water to remove any existing residue, rinsed with water and then soaked in 70% ethyl alcohol. Coupons were allowed to dry and then sterilized at 121°C for 18 min. Dry, sterile coupons of each material were placed between two pieces of inoculated beef fat (4°C, 30 min), and held under pressure (20 kg). Inoculated coupons were rinsed with 20 ml sterile distilled water and then partially submerged (Chapter 4) in sterile polypropylene tubes (50 ml, Fisher Scientific, Fair Lawn, NJ) containing 20 ml of fresh beef fat/lean tissue homogenate (FLH; 1:10 w/w of 50/50 fat and lean tissue in sterile distilled water, pH 5.60). Tubes were then incubated under static conditions for up to 7 d at 15°C (Chapter 4).

5.3.3 Sanitizer Treatment

At 0 (12 h), 3 and 7 d of incubation, coupons of each material (three per treatment in each of two biologically independent experiments) were removed from the incubation substrate, rinsed with 20 ml of sterile distilled water using a sterile pipette and then placed in 40 ml of sterile distilled water or sanitizing solution (Table 5.1) for 1 or 10 min.

Seven sanitizers (Table 5.1) were included in the study; acidified sodium chlorite (ASC), a commercial sodium hypochlorite solution (SH; XY-12, Ecolab, St. Paul, MN), a commercial potassium peroxymonosulfate/sodium chlorite solution (PP/SC; Virkon® S, Dupont, Wilmington, DE), a commercial mixture of four quaternary ammonium chloride compounds (QUAT-A; Oasis™ , Ecolab), a commercial quaternary ammounium formulation containing the active ingredient cetylpyridinium chloride solution (QUAT-B; Cecure® Safe Food Corporation, North Little Rock, AR), a commercial peroxyacetic acid solution (PAA; Oxonia Active™, Ecolab), and a commercial peroxyacetic acid/octanoic acid mixture (PA/OA; Vortexx®, Ecolab). All seven sanitizers were applied to 3-d old biofilm cells at the minimum and maximum concentrations recommended by the manufacturer (Table 5.1), and three of the seven sanitizers (SH, QUAT-A, PA/OA) were also applied at the same concentrations to d-0 and d-7 biofilm cells.

5.3.4 Microbiological and pH Analyses

Following inoculation, initial populations on coupon surfaces were determined by rinsing the surface of each coupon with 20 ml sterile distilled water to remove unattached and loosely attached cells, and then placing coupons in tubes containing 40 ml maximum recovery diluent (MRD; 0.85% NaCl [Fisher] and 0.1% peptone [Bacto™ Proteose Peptone, Difco]) and 10 glass beads. In order to enumerate microbial populations on coupon surfaces before and after sanitizer treatments, coupons (three per treatment in each of two biologically independent experiments) were rinsed with 20 ml sterile distilled water and then placed in tubes containing 40 ml Dey/Engley neutralizing broth (Difco)

and 10 glass beads. All samples were then vortexed for 2 min (3200 rpm), serially diluted in 0.1% sterile buffered peptone water (BPW; Difco) and plated onto trypic soy agar (TSA; Difco) for enumeration of total bacterial populations and TSA supplemented with rifampicin (100 µl/L) (TSArif) for enumeration of inoculum populations. Plates were incubated at 25°C for 48 h (TSA) or 35°C for 24 h (TSArif) and colonies were enumerated. The pH of inoculation and incubation substrates, as well as all sanitizer treatments was measured at each sampling interval using a digital pH meter fitted with a glass electrode (Denver Instruments, Arvada, CO).

5.3.5 Statistical Analysis

Three samples were analyzed per treatment in each of two biologically independent experiments and microbiological data were converted to log CFU/cm^2 and least squares means were calculated using the analysis of variance in the general linear model procedure of SAS (version 9.1, SAS Institute, Cary, NC). Under the conditions of this study, the minimum detection level was 0.3 log CFU/cm^2. All samples with microbial populations below the level of detection were assigned a value of 0.29 log CFU/cm^2. Treatment effects included surface material (stainless steel, acetal or HDPE), sanitizer (ASC, SH, PP/SC, QUAT-A, QUAT-B, PAA, or PA/OA), sanitizer concentration (minimum or maximum recommended), length of sanitizer treatment (1 or 10 min) and incubation period or age of biofilm (0, 3 or 7 d). All treatment effects were analyzed individually and interactively and a confidence limit of 95% was assigned to all significant treatment effects ($P = 0.05$).

5.4 Results and Discussion

5.4.1 Initial Cell Attachment and Biofilm Formation

The inoculation and incubation procedures used in this study were selected based on the results of previous studies conducted (Chapter 4). As described in Chapter 4, exposure to inoculated beef fat was a successful and realistic means of transferring microbial contamination onto meat contact surface materials. The outer surface of a beef carcass is comprised almost entirely of subcutaneous fat which serves as the primary site of microbial contamination encountered during carcass dressing. Thus, contaminated subcutaneous fat is the most logical vehicle for transferring contamination onto carcass cutting and breaking surfaces and utensils (Chapter 4). Once cells were attached and/or transferred onto coupons, incubation in beef fat and lean tissue homogenate allowed the pathogen to survive and grow on both stainless steel and plastic coupons during storage, and the presence of air at the liquid-surface interface during incubation allowed pathogen populations to grow whereas the absence of air inhibited growth on coupons that were fully submerged in fluid during incubation (Chapter 4). Under these conditions, maximal biofilm formation was observed between 2 and 8 d of storage at 15°C (the average temperature of commercial beef processing facilities during non-production hours), and was dependent on the presence/absence of air and characteristics of the incubation media (Chapter 4). Incubation under these conditions was designed to determine the degree of biofilm formation by *E. coli* O157:H7 cells under beef processing conditions: (i) during the limited time (12 h) between successful daily sanitation cycles and, (ii) during extended periods of time on inadequately cleaned and/or sanitized food contact surfaces. For these reasons, stainless steel, acetal and HDPE coupons were exposed to inoculated

beef fat, partially submerged in FLH and stored for up to 7 d at 15°C. The pH of the FLH was 5.60 at d-0 and was similar (5.49 ± 0.28) throughout storage (Table 5.2). At d-0, 3 and 7 of storage, total aerobic bacteria counts recovered from coupon surfaces were 5.0 ± 0.2, 6.4 ± 0.7 and 7.7 ± 0.3 log CFU/cm^2, respectively. At d-0, 3 and 7 of storage, *E. coli* O157:H7 counts recovered from coupon surfaces were 3.6 ± 0.4, 4.3 ± 0.6 and 3.7 ± 0.5 log CFU/cm^2, respectively.

5.4.2 Effect of Surface Material on Sanitizer-Induced Inactivation of Biofilm Cells

There were no significant ($P \geq 0.05$) sanitizer-induced differences in microbial reductions among the three surface materials at 0, 3 or 7 d of incubation (Fig. 5.1 and 5.2). When differing levels of reduction were observed among pathogen population on the different surface materials, larger reductions were generally observed on acetal coupons, followed by HDPE and then SS coupons (Fig. 5.1 and 5.2).

5.4.3 Effect of Sanitizer Type, Concentration and Length of Exposure on Inactivation of Biofilm Cells

All sanitizers were applied to 3-d old biofilm cells at the minimum and maximum recommended concentrations (Table 5.1) for 1 or 10 min exposure times. There was a three-way interaction between sanitizer type, sanitizer concentration and length of sanitizer treatment. Sanitizer treatments reduced initial total aerobic populations on coupon surfaces (6.4 ± 0.7 log CFU/cm^2) by 0.6 to 3.7 log CFU/cm^2 and sanitizer efficacy decreased in order of ASC > PAA > PP/SC > PA/OA > QUAT-B > QUAT-A = SH (Fig. 5.1). Efficacy against *E. coli* O157:H7 populations (4.3 ± 0.6 log CFU/cm^2)

attached to coupon surfaces decreased in the order ASC > PAA > PP/SC = PA/OA > QUAT-B > QUAT-A = SH, with sanitizer-induced reductions ranging from 0.0 to 2.3 log CFU/cm^2 (Fig. 5.2). Microbial reductions generally increased as concentration and exposure time increased, although ASC treatments were more effective against 3-d old *E. coli* O157:H7 biofilm cells than any other sanitizing solution, regardless of concentration/exposure time combination ($P < 0.05$) (Fig. 5.2). The differences between SH- and ASC-induced microbial reductions were the most obvious as the active agents in both sanitizers were chlorine derivatives. Chlorine, the active compound in SH solutions, is easily inactivated in the presence of organic material and, as an oxidizing agent, must interact with cell surfaces to be effective (Simpson and Sofos, 2009). Furthermore, the 100 and 200 ppm SH solutions used in this study were mixed according to the manufacturers' recommendations and had pH values of 8.91 and 9.42, respectively. The antimicrobial activity of SH solutions is optimized at a pH of 6.5 to 7.5 (Takeuchi and Frank, 2001), and greater activity against *E. coli* O157:H7 may have been observed if the pH of the SH solutions had been adjusted to a pH of ≤ 7.5. Chlorous acid, the primary active agent in ASC solutions, has a similar antimicrobial action to chlorine, and while chlorous acid shares a similar sensitivity to organic material, its oxidizing potential does exceed that of chlorine (EFSA, 2008). Thus, it's greater oxidizing potential, in combination with a very low solution pH (pH 2.5), may explain the increased efficacy of ASC as compared to SH.

5.4.4 Sanitizer-Induced Inactivation of *E. coli* O157:H7 as Affected by Age of Biofilm Cells

Three specific sanitizers, SH, QUAT-A and PA/OA (Table 5.1) were identified via personnal communication with chemical sales representatives and sanitation professionals as chemicals commonly used to sanitize food processing evironments. Therefore, in addition to the treatment at d-3, these sanitizers were also applied to 0- and 7-d old biofilms. The influence of biofilm age on sanitizer efficacy was determined by applying each of the three sanitizers at the minimum and maximum recommended concentrations (Table 5.1) for 1 and 10 min to coupons of each surface material at 0, 3, and 7 d of incubation. Thee three-way interaction between sanitizer type, sanitizer concentration and length of sanitizer treatment was modified by the addition of the fourth factor, or age of biofilm cells, indicating a four-way interaction between these factors. Initial total aerobic bacteria populations on d 0, 3 and 7 (5.0, 6.4 and 7.7 log CFU/cm^2, respectively) (Fig. 5.3) were reduced by 0.6 to 1.8, 0.7 to 1.9 and 0.6 to 3.2 log CFU/cm^2 (Fig. 5.3), respectively. Correspondingly, initial *E. coli* O157:H7 populations on d 0, 3 and 7 (3.6, 4.3 and 3.7 log CFU/cm^2, respectively) (Fig. 5.4) were reduced by 0.1 to 1.4, 0.1 to 0.8 and 0.0 to 0.9 log CFU/cm^2 (Fig. 5.4), respectively. Reductions generally increased as sanitizer concentration and exposure time increased (Fig. 5.3 and 5.4). PA/OA treatments were more effective ($P < 0.05$) against total aerobic bacteria (Fig. 5.3) and *E. coli* O157:H7 (Fig. 5.4) than QUAT-A or SH treatments, regardless of biofilm age. QUAT-A treatments were effective against newly established biofilms, but ineffective against older biofilm cells (Fig. 5.4). SH treatments were effective against total aerobic bacteria (Fig. 5.3) but ineffective against *E. coli* O157:H7 biofilm cells, regardless of biofilm age, sanitizer concentration or length of exposure (Fig. 5.4).

Quaternary ammonium compounds and sodium hypochlorite are among the most commonly used commercial sanitizers and are generally effective when applied to clean soil-free surfaces (Cords et al., 2005; Holah et al., 2002). In one study, food contact surface approved concentrations (200 ppm) of sodium hypochlorite effectively detached *E. coli* biofilms from the surface of polished stainless steel, but not from scratched stainless steel (Lomander et al., 2004). In another study, sodium hypochlorite was found more effective than peracetic solutions in inactivating *E. coli* on stainless steel surfaces (Rossoni and Gaylarde, 2000). The aforementioned studies evaluated sanitizer efficacy against cells on clean surfaces, whereas this study evaluated efficacy in the presence of organic soil. The limitations of sodium hypochlorite solutions as sanitizers include sensitivity to organic compounds (Cords et al., 2005) and the inability to inactivate the pathogen under the conditions of this study was not unexpected. Quaternary ammonium compounds are also sensitive to organic material, but to a lesser degree than chlorine, and also possess surfactant properties (Cords et al., 2005). Uhlich et al. (2006) found that 1 or 2 min exposures to quaternary ammonium sanitizer (1:64 v/v dilution) were ineffective against 48 h-old *E. coli* O157:H7 biofilms (7.5 log CFU/ml) on glass, Teflon or stainless steel coupons, while 10 min exposures to 5.0% hydrogen peroxide reduced biofilm populations by 1 to 4 log CFU/ml. The PA/OA solution used in this study was comprised of multiple antimicrobial compounds including hydrogen peroxide, acetic and peroxyacetic acid, and octanoic and peroxyoctanoic acid, which may, in part, explain the increased activity of PA/OA solutions against *E. coli* O157:H7 biofilms cells when compared to QUAT-A or SH solutions.

Overall, susceptibility of natural flora biofilm cells to sanitizer treatments did not change as age of biofilm increased (Fig. 5.3). In contrast, *E. coli* O157:H7 biofilm cells became less sensitive to most sanitizer treatments as age of the biofilm increased (Fig. 5.4). In general, 1 min exposures to sanitizing solutions were ineffective against *E. coli* O157:H7 biofilm cells, regardless of biofilm age, type of sanitizer or sanitizer concentration (Fig. 5.4). Efficacy of 10 min exposures against *E. coli* O157:H7 biofilm cells decreased ($p \geq 0.05$) as the age of the biofilm increased, and with the exception of maximum recommended concentrations of PA/OA for 10 min exposures, no concentration/exposure time combination of SH, QUAT-A or PA/OA was capable of reducing *E. coli* O157:H7 populations in 7 d-old biofilms by more than 0.5 log CFU/cm^2 (Fig. 5.4). While exposure to maximum concentration sanitizer treatments typically resulted in equivalent or greater microbial reductions than minimum concentration treatments, differences in reductions between minimum versus maximum recommended concentration treatments were not consistently significant, regardless of biofilm age (Fig. 5.3 and 5.4).

The increased resistance of biofilm versus planktonic cells to sanitizing treatments has been established (Bower and Daeshel, 1999; Chmielewski and Frank, 2003; Stopforth et al., 2002). While all aspects of increased resistance are not fully understood, it is generally recognized that cells attached to surfaces have less available surface area than planktonic cells for antimicrobial-cell interaction and that the glycoproteins, exopolysaccharides and other compounds (glycocalyx) surrounding biofilm cells also act to physically protect cells from surface-active agents (Uhlich et al., 2006). For these same reasons, mature biofilms with thicker glycocalyx layers should be more resistant to

sanitizing treatments than newly established biofilms (Pan et al., 2006; Ryu and Beuchat, 2005).

Commercial food processing facilities are usually large and house multiple types of equipment which require various degrees of disassembly and attention during cleaning and sanitation processes. At the end of a production shift, these surfaces are typically covered by a heavy layer of beef fat and lean tissue. Cleaning protocols which employ both physical and chemical removal of organic material from these surfaces are critical to the success of ensuing sanitizer treatments. Even so, the complete removal of all organic material from the entirety of every surface in such a large facility is unlikely even after intensive cleaning efforts. It is likely that small amounts of organic material are left behind on a daily basis.

Five of the seven sanitizers used in this study were approved for use as food contact surface sanitizers, and therefore must be capable of reducing 5 log CFU/cm^2 of *E. coli* O157:H7 (25°C) on clean surfaces (EPA, 1979); however, even at the highest recommended concentrations, no sanitizing solution was capable of reducing *E. coli* O157:H7 biofilm cells on soiled food contact surfaces by $\geq$ 2.3 log CFU/cm^2. Under the conditions of this study, concentrations of acidified sodium chlorite and peroxyacetic acid-based sanitizers that are approved for use on food contact surfaces were the most effective against total aerobic bacteria and *E. coli* O157:H7 attached onto soiled surfaces. Potassium peroxymonosulfate/sodium chlorite treatments (1.0%) also reduced total aerobic bacteria and *E. coli* O157:H7 on soiled surfaces; additional research is needed to

determine whether its use on food contact surfaces is appropriate. Concentrations of quaternary ammonium compounds and sodium hypochlorite solutions approved for use on food contact surfaces were the least effective sanitizers against *E. coli* 157:H7 biofilm cells, regardless of biofilm age, sanitizer concentration or exposure time. These results are important as quaternary ammonium compounds and sodium hypochlorite are two sanitizers used extensively in commercial food processing sanitation programs.

In general, type of surface material did not influence the efficacy of sanitizer treatments. It is important to note that all surface materials used in this study were in good condition and free of visible surface damage. It is generally accepted that even microscopic nicks and cuts on surfaces can harbor microbial contaminants. Therefore, while new surface material did not appear to directly influence sanitizer efficacy, care should be taken to select materials that can withstand daily wear and tear associated with manually boning beef and do not chip or feather easily, as rough or scratched surfaces are increasingly difficult to successfully clean and sanitize (Cliver, 2006; Lomander et al., 2004).

In summary, sanitizer-induced pathogen reductions on soiled surfaces were small (≤ 2.3 log CFU/cm^2), increased as sanitizer concentration and exposure time increased, and decreased as biofilm cells aged. It is well known that sanitizers alone cannot compensate for inadequate cleaning protocols and the removal of organic material from surfaces is essential for effective sanitation. It is also apparent that care should be taken to apply sanitizers at the highest allowable concentration for extended dwell times. While no sanitizer treatment investigated in this study was capable of reducing *E. coli* O157:H7

biofilm populations on soiled surfaces by more than 2.3 log CFU/cm^2, concentrations of acidified sodium chlorite and peroxyacetic acid-based sanitizers which are approved for use on food contact surfaces were more effective than other sanitizers against biofilm cells on soiled surfaces and their use may be considered. Furthermore, processors may consider weekly applications of sanitizing solutions that exceed approved levels for food contact surface sanitation, although food contact surfaces treated with such solutions would have to be rinsed with water before operations are allowed to resume. Such treatments would require an additional step but could help negate the establishment of microbial biofilms.

5.5 Acknowledgements

This work was funded in part by the Beef Checkoff, the United States Department of Agriculture Cooperative State Research, Education, and Extension Service (USDA-CSREES), Commission of the European Committees, specific RTD Program, IP Project No. 036241-ProSafeBeef, and the Agricultural Experiment Station of Colorado State University.

Table 5.1. Sanitizers used in biofilm sanitizer challenges. Sanitizers used were commercially available products or were manufactured in a laboratory setting to simulate other commercially available products. Sanitizing solutions included those most commonly used in the food industry as well as those not currently approved for use as food contact surface sanitizers. All sanitizing solutions were mixed and stored as directed by the manufacturer and were applied to contaminated coupons at the minimum and maximum concentrations recommended by the manufacturer for use on food contact surfaces.

Sanitizer	Commercial name (Manufacturer)	Active ingredient(s)	Manufacturers' recommended concentrations for use on food contact surfaces	Sanitizer treatments	
				Minimum concentration (pH)	Maximum concentration (pH)
Acidified sodium chlorite (ASC)		sodium chlorite	0.05-0.12% acidified to pH 2.5 with 20% hydrochloric acid solution	0.05% (2.50)	0.12% (2.50)
Sodium hypochlorite (SH)	XY-12 (Ecolab)	8.5% sodium hypochlorite	0.01-0.02% or 1-2oz/6.5gal	0.01% (8.91±0.33)	0.02% (9.42±0.23)
Potassium peroxymonosulfate (PP/SC)	Virkon S (Antec International)	20.4% potassium peroxymonosulfate 1.5% sodium chloride	Not an approved food contact sanitizer	0.5% (2.76±0.09)	1.0% (2.79±0.11)
Quaternary ammonium compound (QUAT-A)	Oasis (Ecolab)	3.0% alkyl dimethyl benzyl ammonium chloride 2.25% octyl decyl dimethyl ammonium chloride 1.35% didecyl dimethyl ammonium chloride 0.90% dioctyl dimethyl ammonium chloride	0.015-0.04% or 1.25-2.67oz/4gal (based on water hardness)	0.02% (6.77±0.88)	0.04% (6.81±0.89)
Quaternary ammonium compound (QUAT-B)	Cecure (Safe Foods Corp.)	40% Cetylpyridinium chloride	Not an approved food contact sanitizer	0.5% (8.02±0.33)	1.0% (7.13±0.61)
Peroxyacetic acid (PAA)	Oxonia Active (Ecolab)	5.8% peroxyacetic acid 27.5% hydrogen peroxide	0.20-0.28% v/v	0.20% (4.33±0.44)	0.28% (4.04±0.45)
Peroxyacetic acid/octanoic acid mixture (PA/OA)	Vortexx (Ecolab)	6.9% hydrogen peroxide 4.4% peroxyacetic acid 3.3% octanoic acid	0.13-0.26%	0.13% (4.43±0.37)	0.26% (3.98±0.30)

Table 5.2. The pH values (mean ± standard deviation) of beef fat lean homogenate (FLH; 1:10 in sterile distilled water) during incubation (7 days, 15°C) of inoculated stainless steel (SS), acetal and high density polyethylene (HDPE) coupons (2×5 cm).

Food contact surface	Storage days		
	0	3	7
SS	5.67±0.08 aA	5.64±0.09 aA	5.49±0.11 aA
Acetal	5.61±0.14 aA	5.68±0.11 aA	5.52±0.19 aA
HDPE	5.52±0.05 aA	5.77±0.12 aA	5.63±0.07 aA

Least squares mean values with different lower case letters in the same row are different ($P < 0.05$).
Least squares mean values with different upper case letters in the same column are different ($P < 0.05$).

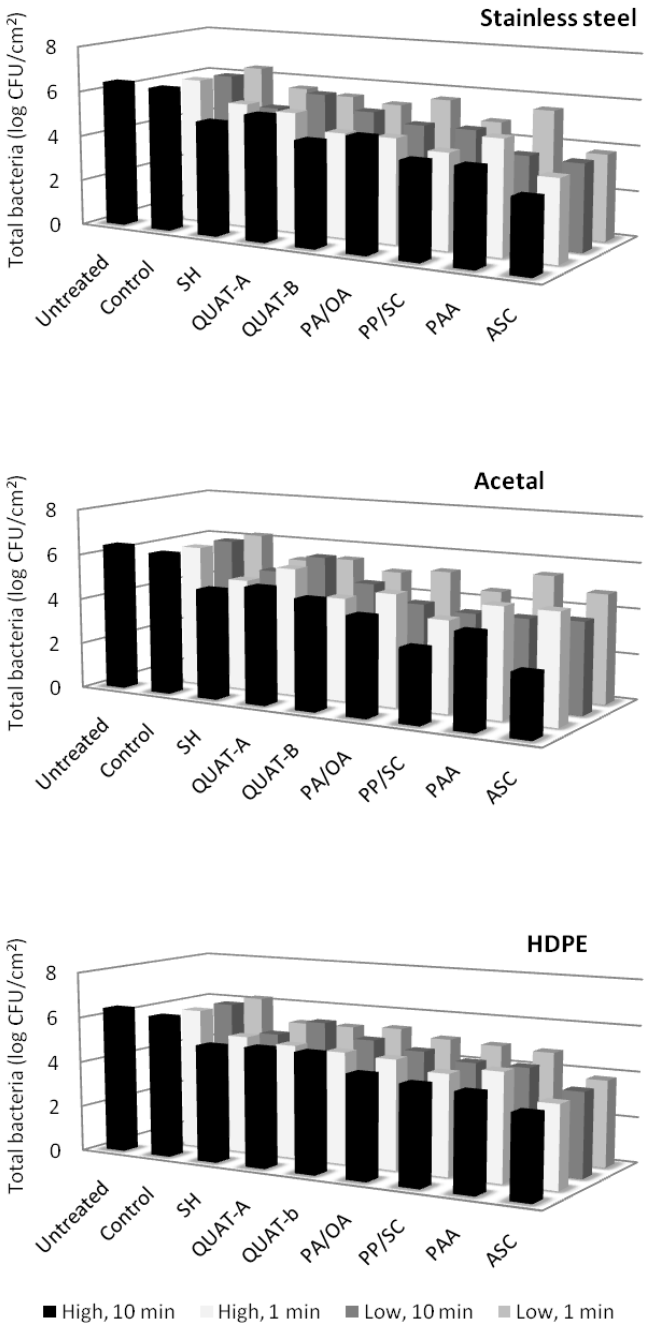

Fig. 5.1 (Data in Appendix Table 12). Surviving total aerobic bacteria (log CFU/cm^2) recovered on tryptic soy agar, attached to stainless steel, acetal or high-density polyethylene (HDPE) coupons (2x5 cm) after sanitizer treatment. Fat-inoculated coupons were placed in test tubes containing fresh beef fat-lean tissue homogenate (1:10 in sterile distilled water) and incubated for 3 d at 15°C. Coupons were then placed in the minimum or maximum recommended concentrations of sanitizing solution or in sterile distilled water (control) for 1 or 10 min. Sanitizers included acidified sodium chlorite (ASC), sodium hypochlorite (SH), two quaternary ammonium compounds (QUAT-A, QUAT-B), peroxyacetic acid (PAA), a peroxyacetic acid/octanoic acid mixture (PA/OA) and potassium peroxymonosulfate/sodium chloride (PP/SC).

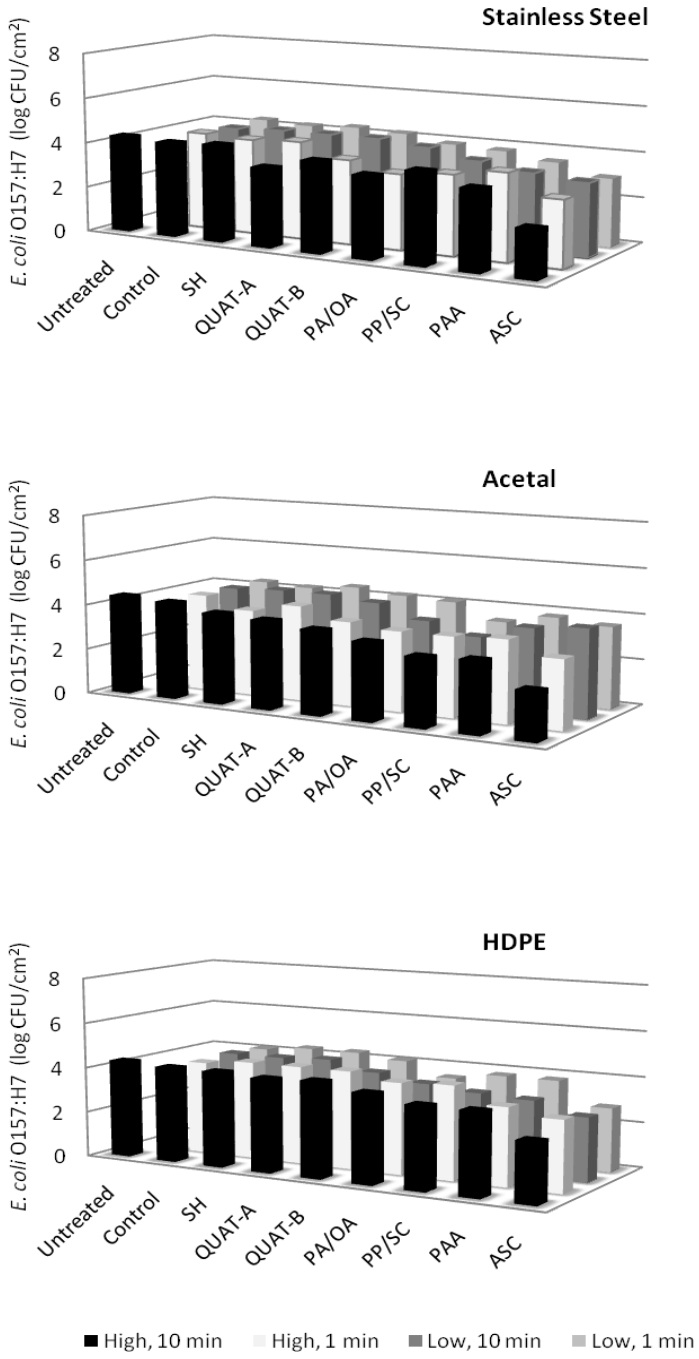

Fig. 5.2 (Data in Appendix Table 13). Surviving *Escherichia coli* O157:H7 (log CFU/cm^2) recovered with tryptic soy agar (TSA) plus rifampicin (100 µg/ml), attached to stainless steel, acetal or high-density polyethylene (HDPE) coupons (2x5 cm) after sanitizer treatment. Fat-inoculated coupons were placed in test tubes containing fresh beef fat-lean tissue homogenate (1:10 in sterile distilled water) and incubated for 3 d at 15°C. Coupons were then placed in the minimum or maximum recommended concentrations of sanitizing solution or in sterile distilled water (control) for 1 or 10 min. Sanitizers included acidified sodium chlorite (ASC), sodium hypochlorite (SH), two quaternary ammonium compounds (QUAT-A, QUAT-B), peroxyacetic acid (PAA), a commercial peroxyacetic acid/octanoic acid mixture (PA/OA) and potassium peroxymonosulfate/sodium chloride (PP/SC).

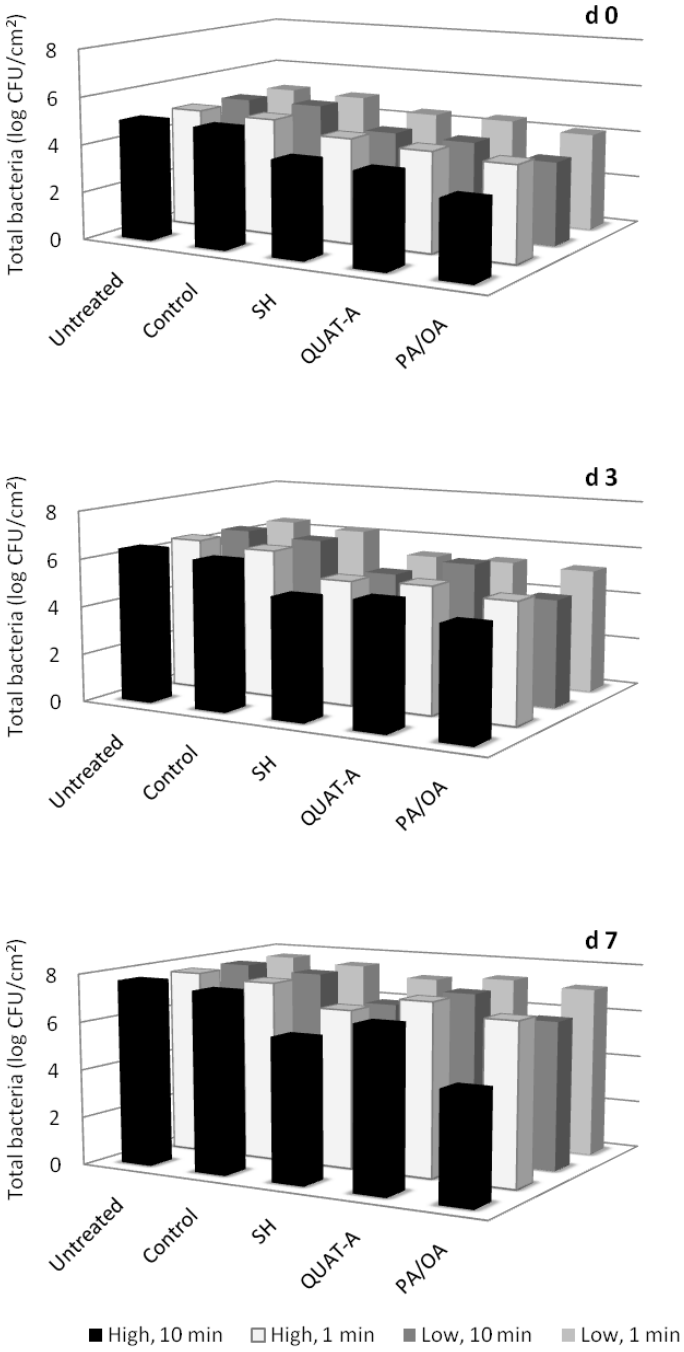

Fig. 5.3 (Data in Appendix Table 14). Surviving total aerobic bacteria (log CFU/cm^2) attached to coupons (2x5 cm), recovered with tryptic soy agar (TSA) following sanitizer treatment. Fat-inoculated coupons were incubated for 0, 3 or 7 d (15°C) in fresh beef fat-lean tissue homogenate and then placed in the minimum or maximum recommended concentrations of sanitizing solution or in sterile distilled water (control) for 1 or 10 min. Sanitizers included sodium hypochlorite (SH), quaternary ammonium compound (QUAT-A) and a peroxyacetic acid/octanoic acid mixture (PA/OA).

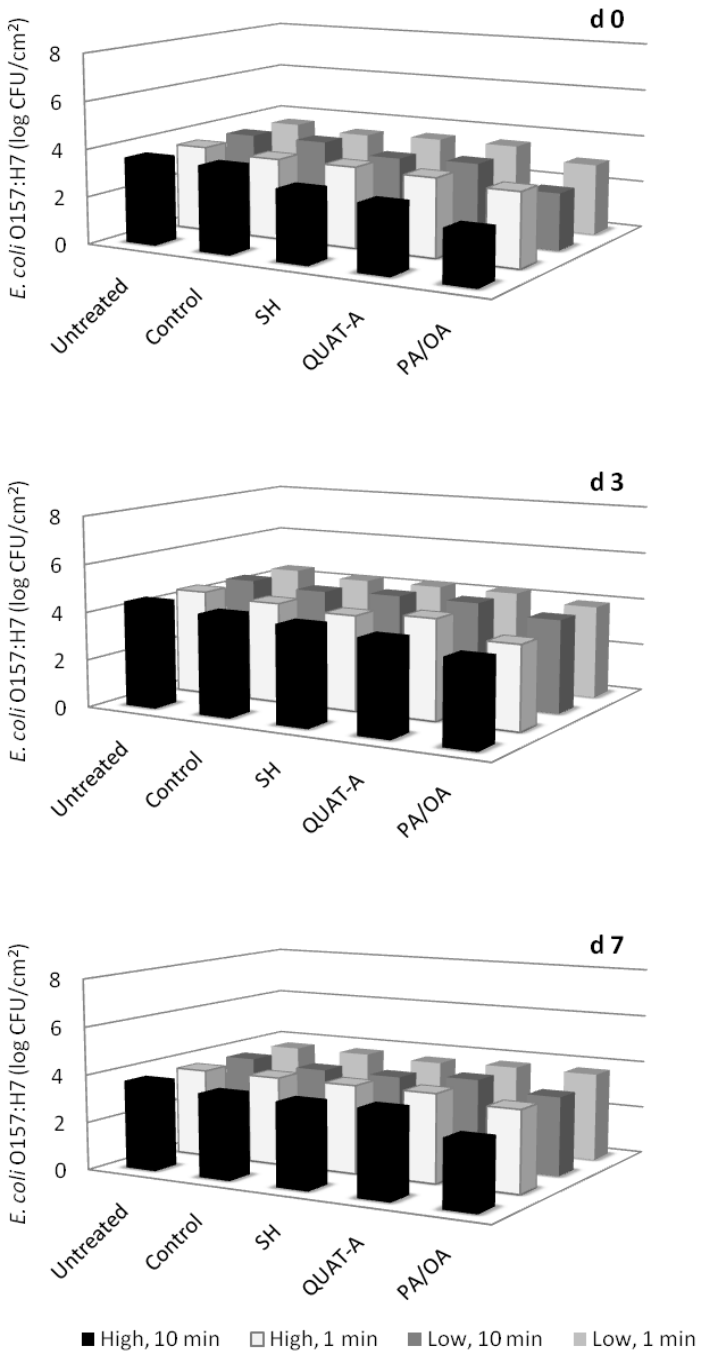

Fig. 5.4 (Data in Appendix Table 15). Surviving *Escherichia coli* O157:H7 (log CFU/cm^2) attached to coupons (2x5 cm), recovered with tryptic soy agar (TSA) plus rifampicin (100 µg/ml) following sanitizer treatment. Fat-inoculated coupons were incubated for 0, 3 or 7 d (15°C) in fresh beef fat-lean tissue homogenate and then placed in minimum or maximum recommended concentrations of sanitizing solution or in sterile distilled water (control) for 1 or 10 min. Sanitizers included sodium hypochlorite (SH), quaternary ammonium compound (QUAT-A) and a peroxyacetic acid/octanoic acid mixture (PA/OA).

CHAPTER SIX

Fate of *Listeria monocytogenes* during Freezing, Thawing and Home Storage of Frankfurters

6.1 Abstract

Little information is available regarding the fate of *Listeria monocytogenes* during freezing, thawing and home storage of frankfurters even though recent surveys show that consumers regularly store unopened packages in home freezers. This study examined the effects of antimicrobials, refrigerated storage, freezing, thawing method, and post-thawing storage (7°C) on *L. monocytogenes* on frankfurters. Inoculated (2.1 log CFU/cm^2) frankfurters formulated without (control) or with antimicrobials (1.5% potassium lactate plus 0.1% sodium diacetate) were vacuum-packaged, stored at 4ºC for 6 or 30 d and then frozen (-15ºC) for 10, 30, or 50 d. Packages were thawed under refrigeration (7ºC, 24 h), on a countertop (23 ± 2ºC, 8 h), or in a microwave oven (2450 MHz, 1100 watts, 220 s followed by 120 s holding), and then stored aerobically (7ºC) for 14 d. Bacterial populations were enumerated on PALCAM agar and tryptic soy agar plus 0.6% yeast extract. Antimicrobials completely inhibited ($P < 0.05$) growth of *L. monocytogenes* at 4ºC for 30 d under vacuum-packaged conditions, and during post-thawing aerobic storage at 7ºC for 14 d. Different intervals between inoculation and freezing (6 or 30 d) resulted in different pathogen levels on control frankfurters (2.1 or 3.9 log CFU/cm^2, respectively), while freezing reduced counts by <1.0 log CFU/cm^2.

Thawing treatments had little effect on *L. monocytogenes* populations (<0.5 log CFU/cm^2), and post-thawing fate of *L. monocytogenes* was not influenced by freezing or by thawing method. Pathogen counts on control samples increased by 1.5 log CFU/cm^2 at d-7 of aerobic storage, and reached 5.6 log CFU/cm^2 at d-14. As indicated by these results, consumers should freeze frankfurters immediately after purchase, and discard frankfurters without inhibitory agents within 3 d of thawing and/or opening.

6.2 Introduction

Frankfurters are in continuous demand by U.S. consumers, and retail sales exceeded $1.6 billion in 2008 (NHDSC, 2009). This is particularly important as inadequately reheated frankfurters are included among the food products of most concern regarding human listeriosis (CFSAN/FSIS/CDC, 2003). The USDA-FSIS currently recommends that consumers set home refrigerator temperatures at < 5ºC and store opened or unopened packages of frankfurters for no longer than 7 or 14 d, respectively (http://www.fsis.usda.gov/PDF/Basics_for_Safe_Food_Handling.pdf). Freezing may be used as a method of *Listeria monocytogenes* control in ready-to-eat (RTE) meat products that support its growth as per the USDA-FSIS Interim Final Rule on control of the pathogen (USDA-FSIS, 2003b). In addition, freezing is recommended to preserve quality and to minimize food safety concerns associated with frankfurters, and recent surveys indicate that unopened packages of frankfurters are regularly stored in home freezers (Porto et al., 2004). Freezing these products can dramatically increase shelf-life, and both opened and sealed packages may be held for 1 to 2 months in home freezers

(http://www.fsis.usda.gov/PDF/Basics_for_Safe_Food_Handling.pdf). Even so, scientifically validated thawing recommendations are not available and thawing instructions are consistently absent from frankfurter package labels.

Methods for thawing frozen foods in the refrigerator, immersed in cold water, or by microwave defrosting are outlined as part of basic good handling practices for consumers set forth by the USDA-FSIS (USDA-FSIS, 2005b). According to current recommendations, product thawed under refrigeration should be safe for consumption if cooked and consumed within 3 to 5 d of initial thawing, while those thawed in cold water or by microwave defrosting should be cooked immediately after thawing, and before consumption or re-freezing (USDA-FSIS, 2005b). Thawing products under cold running water is often recommended (SafeFood News, 2003), but unless the product is covered by packaging material, surface contaminants and purge will overflow into the sink area, increasing the potential for future cross-contamination events. The incidence of foodborne illnesses may be reduced by simply engaging consumers and equipping them with the information needed to avoid improper handling of food products.

The popularity of home microwave applications has increased dramatically over the last two decades, and according to a fairly recent estimate (Osepchuk, 2002), there were over 100 million microwave ovens currently in use by U.S. consumers. Microwave defrosting is much faster than other thawing methods, and, as most modern consumers have microwave ovens installed in their homes, is a likely method of thawing frozen food.

The effects of microwave defrosting on food product properties are well-understood (Chamchong and Datta, 1999; Osepchuk, 2002); unfortunately, little is known about its effects on microbial contaminants during and after thawing. The fate of *L. monocytogenes* on frankfurters immediately after thawing treatments and under post-thawing home storage conditions (7°C, 14 d, aerobic packaging) has not been previously described, and should provide useful information for researchers, manufacturers and consumers. For this reason, the effect of different thawing methods on the fate of *L. monocytogenes* on frankfurters formulated with and without antimicrobial agents was investigated. Selected thawing methods included thawing under refrigeration, thawing at ambient temperature on a countertop, and defrosting in a consumer-type microwave oven.

6.3 Materials and Methods

6.3.1 Preparation of Inocula

The *L. monocytogenes* inoculum was comprised of 10 strains of food, human clinical and environmental origin, including 558 (serotype 1/2, pork product isolate), N-7150 (serotype 3a, meat product isolate), NA-1 (serotype 3b, pork sausage isolate), R2-500, R2-764, and N1-227 (serotype 4b, epidemic-related food isolates), R2-501, R2-763, and N1-225 (serotype 4b, epidemic-related human clinical isolates), and R2-765 (serotype 4b, environmental source isolate). Strains R2-500, R2-501, R2-763, R2-764, R2-765, N1-225 and N1-227 (Fugett et al., 2006) were kindly provided by Dr. Martin Wiedmann (Cornell University, Ithaca, NY). A mixture of *L. monocytogenes* strains was used for

inoculation of the frankfurters to better simulate a contamination event occurring in a processing facility, and furthermore, to account for possible differences in survival and/or growth abilities among strains (NACMCF, 2005; Scott et al., 2005).

Active cultures of individual strains were first prepared (30ºC, 24 h) in tryptic soy broth (Difco, Becton Dickinson, Sparks, MD) + 0.6% yeast extract (Acumedia, Lansing, MI) and then subcultured (0.1 ml) under the same conditions. Individual cultures were then harvested by centrifugation (4,629 x *g* at 4ºC for 15 min) (Eppendorf, 5810 R; Brinkman Instruments, Inc., Westbury, NY) followed by washing in 10 ml phosphate-buffered saline (pH 7.4; 0.2 g KH_2PO_4, 1.5 g $Na_2HPO_4 \cdot 7H_2O$, 8.0 g NaCl, and 0.2 g KCl in 1 L distilled H_2O). Washed cells of each strain were then resuspended in 10 ml of autoclave-sterilized frankfurter extract (10% [w/w] in distilled water; pH 6.10), prepared as described by Lianou et al. (2007), and then habituated at 7ºC for 72 h before use, to acclimate the cells to a low-temperature food environment. Just before product inoculation, cultures of individually habituated strains were combined and serially diluted in fresh sterile frankfurter extract for a target concentration of 4.5 log CFU/ml.

6.3.2 Product Inoculation

Frankfurters (60% pork, 40% beef) were prepared as described by Byelashov et al. (2008) and formulated without or with 1.5% potassium lactate plus 0.1% sodium diacetate. Product was inoculated (2.1 log CFU/cm^2) under a biosafety cabinet by applying 0.2 ml of the 10-strain composite onto individual frankfurters. A sterile bent

glass rod was used to spread the inoculum over the surface of each frankfurter. Frankfurters were covered and held for 15 min at 4°C between inoculation and vacuum-packaging to allow inoculum cells to attach onto the product surface.

6.3.3 Storage and Thawing Treatments

Frankfurters were packaged (8 per pack) in re-sealable vacuum pouches (15 x 22 cm, 3 mil standard barrier, nylon/polyethylene vacuum pouch, water vapor and oxygen transmission rates of 8.0 $g/m^2/24$ h [37.8°C, 90% RH] and 52.0 $cm^3/m^2/24$ h [23ºC, 0% RH], respectively, Hollymatic Corp., Countryside, IL) and placed at 4°C to simulate storage of product before being purchased by the consumer. After 6 and 30 d at 4ºC, packages were transferred to frozen storage (-15ºC) to simulate storage conditions in a home freezer. Packages of frozen frankfurters were systematically removed from frozen storage after 10, 30 and 50 d and thawed using one of three methods: (i) holding packages under refrigeration (7ºC, 24 h); (ii) placing packages directly on a countertop (23 ± 2ºC, 8 h); or, (iii) microwave defrosting (Amana 1.4 cu. countertop microwave; 2450 MHz, 1100 watts for 220 s followed by 120 s holding). The length of each thawing treatment was determined by the amount of time required to completely thaw (measured by the absence of ice in or between frankfurters) whole packages of frozen frankfurters under the conditions of each method. Microbial populations on frozen frankfurters were used to determine the effect of freezing on *L. monocytogenes* populations, and also served as controls for the thawing portion of the study. Immediately after thawing, two frankfurters were collected from each vacuum-package for microbiological and pH

analyses. Packages were then re-sealed using the zip-closure feature of the pouch, and stored at 7ºC to simulate aerobic post-thawing conditions in a home refrigerator. At 3, 7 and 14 d, two frankfurters per pouch were removed for microbiological and pH analyses.

6.3.4 Microbiological, Chemical and Physical Analyses

To enumerate microbial populations during storage and after thawing, samples (two frankfurters per sample) were aseptically transferred from vacuum pouches to sterile WhirlPak bags (710 ml, 4 mil; Nasco, Modesto, CA) containing 50 ml of maximum recovery diluent (0.85% NaCl and 0.1% peptone [Bacto™ Proteose Peptone, Difco]), vigorously shaken 30 times in a 1 ft vertical arc (Samelis et al., 2002), and serially diluted in 0.1% buffered peptone water (Difco). Aliquots of appropriate dilutions were spread-plated, in duplicate, onto both PALCAM agar (Difco; for enumeration of *L. monocytogenes* populations) and tryptic soy agar (Difco) + 0.6% yeast extract (TSAYE; for enumeration of total aerobic bacterial populations), incubated (30ºC for 48 h, and 24 ± 2ºC for 72 h, respectively), and bacterial colonies were enumerated.

On the day of product inoculation (d-0), pH and a_w values were determined on samples from all treatments; pH measurements were also conducted on samples analyzed microbiologically throughout storage. The pH values of sample homogenates (Lianou et al., 2007) were determined using a pH meter with a glass electrode (Denver Instruments, Arvada, CO). The a_w was determined by placing small pieces ($\leq$ 0.5 cm) of frankfurters

into a plastic cup, and analyzing with an AquaLab a_w meter (Series 3; Decagon Devices, Inc., Pullman, WA).

6.3.5 Statistical Analysis

Three samples per treatment in each of two biologically independent experiments were analyzed at each sampling time. Microbiological data were converted to log CFU/cm^2 before statistical analysis with a complete factorial design ([product formulation; with or without antimicrobial ingredients] x [length of storage at 4ºC; 6 or 30 d] x [length of frozen storage at -15ºC; 10, 30 or 50 d] x [thawing method; under refrigeration, on a countertop or microwave defrosting] x [length of aerobic storage at 7ºC; 3, 7 or 14 d]) using the analysis of variance in the mixed model procedure of SAS (version 9.1, SAS Institute, Cary, NC). A confidence limit of 95% was assigned to all significant treatment effects (P = 0.05).

6.4 Results and Discussion

6.4.1 Physicochemical Characteristics of Frankfurters

On d-0, the pH of inoculated frankfurters formulated without or with 1.5% potassium lactate plus 0.1% sodium diacetate was 6.07 ± 0.03 and 6.00 ± 0.05, respectively (Table 6.1 and 6.2), and a_w values were 0.973 ± 0.007 and 0.970 ± 0.010, respectively. The pH values of frankfurters containing antimicrobial ingredients did not change (6.00 ± 0.11) during vacuum-packaged storage at 4°C (30 d), frozen storage (-15°C, 50 d), or post-thawing aerobic storage (7°C, 14 d) (Table 6.2). Thawing treatments did not affect ($P \geq$

0.05) product pH, regardless of product formulation. The pH values of control frankfurters (i.e., formulated without antimicrobials) remained static (6.07 ± 0.05) during vacuum-packaged storage at 4°C and during frozen storage, but were less consistent and declined during post-thawing aerobic storage (Table 6.1). For control frankfurters that were frozen after being stored for 6 d at 4°C, pH values (5.91 to 6.22) did not differ ($P \geq 0.05$) from initial product pH until d-14 of aerobic storage (7°C) when the pH ranged from 5.26 to 6.10 (Table 6.1). The pH values of control frankfurters that were frozen after 30 d at 4°C were significantly ($P < 0.05$) different from initial product pH at d-3 of aerobic storage (pH 5.38 to 5.91) and ranged from 5.14 to 5.76 at d-14 (Table 6.1). Changes in pH values during storage were most likely due to microbial growth.

6.4.2 Post-Inoculation Refrigerated and Frozen Storage

The ability of *L. monocytogenes* to grow at refrigeration temperatures is well-documented (Ryser and Marth, 2007). In general, growth rates are higher at 7°C compared to 4ºC, and under aerobic compared to vacuum-packaged conditions (Lianou et al., 2007). *L. monocytogenes* can survive, but does not grow during frozen storage (Ryser and Marth, 2007). According to the most recent U.S. Cold Temperature Evaluation Study conducted by the U.S. Food and Drug Administration (Audits International/FDA, 1999), the average temperature of retail backrooms is < 4ºC, and the average temperature of home freezers is approximately -15ºC. In accordance with the previous data, and recommendations by the National Advisory Committee on Microbiological Criteria for Foods (NACMCF, 2005),

the following storage temperatures were selected: finished product storage, 4ºC; (ii) frozen home-storage, -15ºC.

L. monocytogenes did not grow on control (without antimicrobials) frankfurters during the first 6 d of vacuum-packaged storage at 4ºC, but increased to 3.9 ± 0.4 log CFU/cm^2 at d-30 (Fig. 6.1). Total aerobic bacteria also did not grow on control frankfurters during the first 6 d at 4°C, but increased to 4.7 ± 0.7 log CFU/cm^2 at d-30 (Appendix Table 18). As indicated, after 6 and 30 d at 4°C, frankfurters were transferred to frozen storage (-15ºC) for 10, 30 and 50 d. Freezing did not affect ($P \geq 0.05$) *L. monocytogenes* populations on frankfurters formulated with antimicrobials or those on control frankfurters that were frozen after only 6 d at 4°C (Fig. 6.2). Freezing and frozen storage, however, reduced pathogen populations by < 1 log CFU/cm^2 on control frankfurters that were stored at 4°C for 30 d before freezing (Fig. 6.2). Total aerobic bacterial populations were not reduced during frozen storage of frankfurters, regardless of product formulation, storage interval between inoculation and freezing or length of frozen storage (Appendix Tables 16-19).

Potassium lactate (1.5%) plus sodium diacetate (0.1%) completely inhibited growth of *L. monocytogenes* on frankfurters during vacuum-packaged storage at 4°C for 6 and 30 d (Fig. 6.1), and during aerobic storage (7ºC, 14 d) after frozen samples were thawed, regardless of thawing method (Appendix Table 16). This outcome was expected, as published data collectively indicate that the levels of lactate and sodium diacetate

approved for use in RTE meat products are effective inhibitors of *L. monocytogenes* during product storage (Barmpalia et al., 2005; Geornaras et al., 2006; Glass et al., 1999; Lianou et al., 2007). Antimicrobials also inhibited growth of natural flora during vacuum-packaged storage at 4°C (30 d), and during post-thawing aerobic storage (7°C, 14 d) of frankfurters that were stored (4°C) for only 6 d between inoculation and freezing. Modest growth of total aerobic bacterial populations (counts were $\leq$ 3 log CFU/cm^2 and $\leq$ 5 log CFU/cm^2 at d-7 and -14 of aerobic storage, respectively) was observed on frankfurters that were stored longer (30 d, 4°C) before being frozen (Appendix Table 19).

6.4.3 Thawing Method

As indicated, the length of each thawing treatment was determined by the amount of time required to completely thaw whole packages of frozen frankfurters under the conditions of each method; consequently, the duration of individual thawing treatments differed (refrigeration, 24 h; countertop, 8 h; microwave defrosting, 220 s followed by 120 s holding). Overall, thawing method did not affect ($P \geq 0.05$) the fate of *L. monocytogenes* immediately after thawing or during subsequent aerobic storage (14 d, 7ºC). Pathogen counts following countertop and refrigerated thawing were similar to those of frozen samples, while counts on microwave defrosted samples were slightly ($P \geq 0.05$) lower in comparison (Fig. 6.3). Differences between thawing treatments (Fig. 6.3) were more apparent when higher levels of contamination were present on control frankfurters (2.0 versus 3.4 log CFU/cm^2 for vacuum-packaged frankfurters stored for 6 or 30 at 4ºC, respectively).

Proper microwave defrosting programs do not cook frozen products as the power output is cyclical instead of continuous (Chamchong and Datta, 1999). By cycling power output, a reduced yet seemingly continuous level of power is generated and applied to a frozen food item. Cyclical applications may increase the uniformity of thawing (Taher and Farid, 2001); however, at such low power levels, microwaves only penetrate to just below the product surface. For this reason, significantly longer applications are required to increase the internal temperature of frozen items. Longer programs also decrease temperature uniformity during defrosting (Chamchong and Datta, 1999). As a result, microwave defrosting applications are known for generating "hot-spots" in thinner areas and/or around the edges of partially frozen/thawed food products (Osepchuk, 2002; Taher and Farid, 2001). The development of such hot-spots (> 60ºC) was most likely responsible for the slightly lower pathogen counts associated with samples that were microwave defrosted, in this study.

6.4.4 Post-Thawing Aerobic Storage

In a recent survey related to the meat handling and home storage practices of Irish consumers, the average temperature of 100 home refrigerators was between 6 and 7ºC (Kennedy et al., 2005). Similar findings have been reported as a result of other studies conducted throughout Europe (Evans et al., 1991; Flynn et al., 1992; Laguerre et al., 2002; Taoukis et al., 2005). While Audits International found that only 10% of refrigerators in U.S. homes were set at or above 7°C (Audits International/FDA, 1999),

the NACMCF recommends that 7°C be used to represent refrigerated storage at the consumer level (NACMCF, 2005). In this study, thawed packages of frankfurters were stored aerobically for up to 14 d at 7°C to simulate post-thawing home storage conditions. *L. monocytogenes* grew on control frankfurters after 3 d of storage (7ºC) and continued to grow throughout the 14 d storage period (Fig. 6.4). While pathogen counts on product thawed under refrigeration tended to be higher than other treatments during aerobic storage and those exposed to microwave defrosting tended to be lower, such differences were not consistently significant (Fig. 6.4). Pathogen populations on control frankfurters increased by 1.5 ± 0.2 log CFU/cm^2 at d-7, and reached 5.6 ± 0.4 log CFU/cm^2 at d-14. Total aerobic bacteria counts increased by 2 log cycles at d-7 and by an additional 2-3 log cycles at d-14 (Fig. 6.4). Extent of growth was not affected by previous thawing treatment.

Under the conditions of this study, 1.5% potassium lactate plus 0.1% sodium diacetate in frankfurter formulations completely inhibited growth of *L. monocytogenes* on frankfurters throughout storage (4ºC, 30 d) in vacuum packages, and subsequent aerobic storage (7ºC, 14 d). In the absence of antilisterial agents, *L. monocytogenes* populations increased by 2.0 log cycles during vacuum-packaged storage (4ºC, 30 d) and by 1.5 log cycles during the recommended 7 d shelf-life of opened packages of frankfurters (7ºC) (http://www.fsis.usda.gov/PDF/Basics_for_Safe_Food_Handling.pdf). Freezing had little effect on *L. monocytogenes*, regardless of product formulation; only when present in high numbers (3.9 log CFU/cm^2) did freezing result in noticeable (≤ 1 log CFU/cm^2), but not

significant ($P \geq 0.05$) reductions. Overall, the thawing treatments evaluated in this study did not have a significant ($P \geq 0.05$) effect on *L. monocytogenes* populations immediately after thawing and during subsequent aerobic storage at 7ºC (14 d); however, microwave defrosting resulted in slightly ($P \geq 0.05$) lower pathogen populations compared to the other thawing treatments or controls. This was most likely due to the characteristic "hot-spots" that tend to develop during microwave defrosting cycles (Osepchuk, 2002; Taher and Farid, 2001).

After purchasing frankfurters devoid of inhibitory agents, consumers should freeze unopened packages immediately and discard uneaten frankfurters within 3 d of thawing and/or opening. These data also validate the effectiveness of using microwave defrosting programs to thaw frozen/packaged RTE meat products, and indicate that, when used appropriately, microwave defrosting is a quick yet safe alternative to more time consuming thawing methods. Given the high rate of frankfurter consumption and microwave use among U.S. consumers, manufacturers may consider including both freezing recommendations and microwave defrosting instructions on frankfurter package labels.

6.5 Acknowledgements

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Service (agreements 2004-51110-02160 and 2005-51110-03278), and by the Colorado State University Agricultural Experiment Station.

Table 6.1. pH of frankfurters formulated without antimicrobials during storage. Frankfurters (n = 8 per package) were inoculated, stored for 6 or 30 d at 4ºC to generate two inoculum levels (approximately 2.2 log or 4.2 log CFU/cm^2, respectively), and then frozen (-15ºC) for 10, 30, or 50 d. Frozen packages were thawed by microwave defrosting (220 sec), in a refrigerator (7ºC, 24 h), or on a countertop (23ºC, 8 h); immediately following thawing treatments and throughout aerobic storage (7ºC) frankfurters (n = 2) were systematically removed from packages for microbiological analysis.

Length of storage at 4ºC (d)	Length of frozen storage (d)	Storage day	Thawing method		
			Microwave	Refrigerator	Countertop
6	10	0	6.08±0.01 Aa	5.92±0.30 Aa	6.04±0.02 Aa
		3	6.14±0.01 Aa	6.14±0.02 Aa	6.15±0.03 Aa
		7	6.09±0.01 Aa	6.14±0.01 Aa	6.04±0.08 Aa
		14	6.13±0.05 Aa	6.12±0.03 Aa	6.05±0.08 Aa
	30	0	6.20±0.04 Aa	6.10±0.04 Ba	6.17±0.04 Ba
		3	6.07±0.02 Aa	6.07±0.01 Ba	6.09±0.04 Ba
		7	6.12±0.04 Aa	6.09±0.04 Ba	6.09±0.01 Ba
		14	5.62±0.31 Aa	5.22±0.07 Aa	5.29±0.21 Aa
	50	0	6.10±0.01 Aa	6.11±0.06 Aa	6.10±0.01 Aa
		3	6.16±0.02 Aa	6.10±0.04 Aa	6.23±0.04 Aa
		7	6.14±0.04 Aa	5.98±0.19 Aa	6.16±0.01 Aa
		14	6.14±0.14 Aa	5.79±0.19 Aa	6.13±0.07 Aa
30	10	0	5.99±0.10 Aa	6.01±0.01 Aa	5.99±0.06 Aa
		3	5.93±0.15 Aa	5.95±0.12 Aa	5.86±0.10 Aa
		7	5.85±0.16 Aa	5.84±0.03 Aa	5.79±0.14 Aa
		14	5.78±0.13 Aa	5.77±0.07 Aa	5.73±0.11 Aa
	30	0	6.07±0.01 Ba	6.09±0.05 Ba	6.16±0.05 Ba
		3	6.16±0.04 Ba	6.15±0.08 Ba	6.13±0.04 Ba
		7	6.07±0.06 Ba	6.07±0.06 Ba	6.13±0.08 Ba
		14	4.97±0.10 Aa	5.29±0.41 Aa	5.13±0.04 Aa
	50	0	6.24±0.01 Aa	6.20±0.07 Ba	6.16±0.05 Ca
		3	5.34±0.18 Aa	5.45±0.15 Aa	5.34±0.17 Aa
		7	6.02±0.14 Aa	5.95±0.27 Ba	5.79±0.02 Ba
		14	5.50±0.54 Aa	5.64±0.40 ABa	5.29±0.12 Aa

Least squares mean values with different lower case letters in the same row are different ($P < 0.05$). Least squares mean values with different upper case letters in the same column and within the same treatment are different ($P < 0.05$).

Table 6.2. pH values of frankfurters formulated with antimicrobials during storage. Frankfurters (n = 8 per package) were inoculated, stored for 6 or 30 d at 4ºC to generate two inoculum levels (approximately 2.2 log or 4.2 log CFU/cm^2, respectively), and then frozen (-15ºC) for 10, 30, or 50 d. Frozen packages were thawed by microwave defrosting (220 sec), in a refrigerator (7ºC, 24 h), or on a countertop (23ºC, 8 h); immediately following thawing treatments and throughout aerobic storage (7ºC) frankfurters (n = 2) were systematically removed from packages for microbiological analysis.

Length of storage at 4ºC (d)	Length of frozen storage (d)	Storage day	Thawing method		
			Microwave	Refrigerator	Countertop
6	10	0	5.91±0.02 Aa	5.89±0.03 Aa	5.94±0.02 Aa
		3	6.04±0.03 Aa	5.95±0.04 Aa	6.33±0.01 Aa
		7	5.99±0.03 Aa	5.94±0.03 Aa	6.01±0.03 Aa
		14	5.97±0.06 Aa	6.01±0.04 Aa	6.03±0.03 Aa
	30	0	5.98±0.06 Aa	6.06±0.01 Aa	6.07±0.04 Aa
		3	5.94±0.01 Aa	6.00±0.01 Aa	5.98±0.01 Aa
		7	5.90±0.11 Aa	5.84±0.08 Aa	5.90±0.05 Aa
		14	5.88±0.08 Aa	5.60±0.39 Aa	5.99±0.09 Aa
	50	0	6.02±0.02 Aa	6.02±0.08 Aa	6.04±0.03 Aa
		3	6.07±0.05 Aa	6.01±0.05 Aa	6.06±0.01 Aa
		7	6.04±0.03 Aa	6.02±0.04 Aa	6.04±0.05 Aa
		14	6.14±0.05 Aa	6.07±0.03 Aa	6.15±0.04 Aa
30	10	0	5.98±0.02 Aa	6.00±0.04 Aa	5.94±0.02 Aa
		3	6.02±0.04 Aa	6.00±0.10 Aa	6.03±0.04 Aa
		7	6.01±0.02 Aa	5.98±0.02 Aa	5.98±0.02 Aa
		14	5.98±0.05 Aa	5.98±0.05 Aa	5.97±0.04 Aa
	30	0	6.05±0.03 Aa	6.04±0.02 Aa	6.08±0.01 Aa
		3	6.07±0.03 Aa	6.07±0.03 Aa	6.07±0.03 Aa
		7	5.97±0.02 Aa	6.08±0.05 Aa	6.06±0.03 Aa
		14	5.82±0.26 Aa	5.96±0.12 Aa	5.84±0.24 Aa
	50	0	6.07±0.05 Aa	6.13±0.05 Aa	6.08±0.02 Aa
		3	5.91±0.10 Aa	6.01±0.06 Aa	5.98±0.11 Aa
		7	6.03±0.01 Aa	6.03±0.02 Aa	6.03±0.04 Aa
		14	6.07±0.03 Aa	5.92±0.04 Aa	5.92±0.10 Aa

Least squares mean values with different lower case letters in the same row are different ($P < 0.05$). Least squares mean values with different upper case letters in the same column and within the same treatment are different ($P < 0.05$).

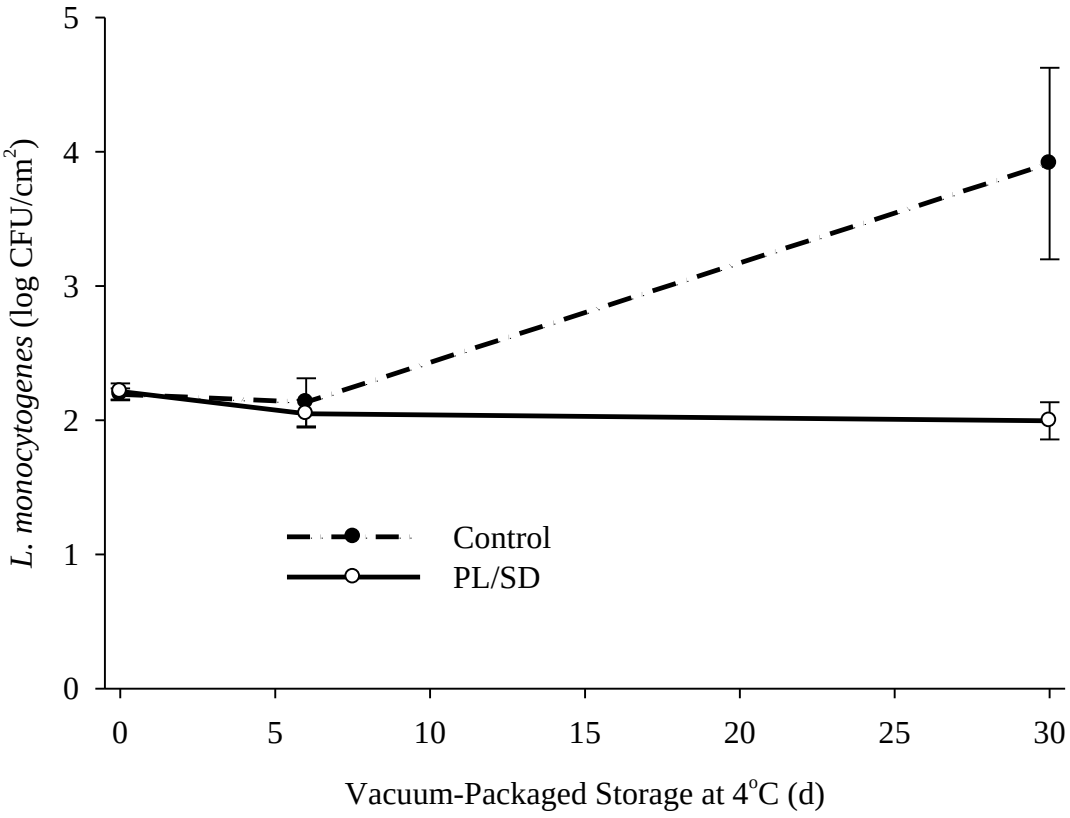

Fig. 6.1. (Data in Appendix Tables 16-19). *Listeria monocytogenes* (log CFU/cm^2) recovered from vacuum-packaged frankfurters formulated without (control) or with 1.5% potassium lactate plus 0.1% sodium diacetate (PL/SD) that were stored at 4ºC for up to 30 d.

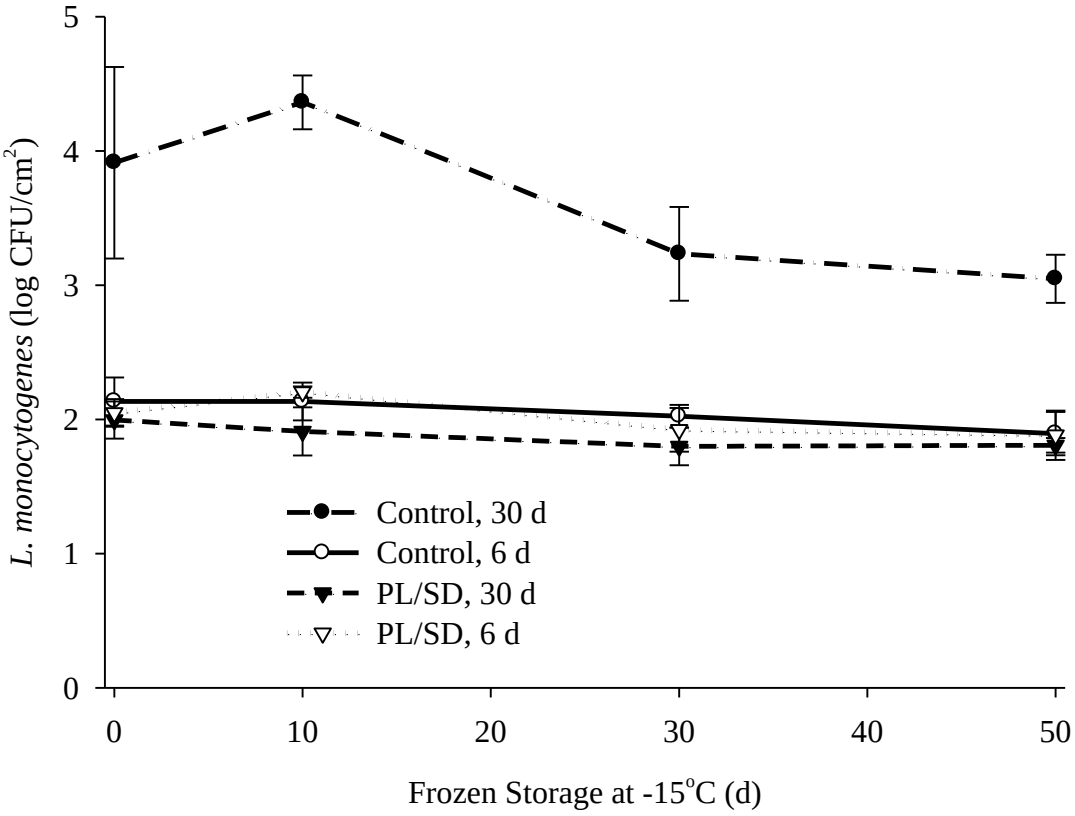

Figure 6.2 (Data in Appendix Tables 16-19). *Listeria monocytogenes* (log CFU/cm^2) recovered from vacuum-packaged frankfurters formulated without (control) or with 1.5% potassium lactate plus 0.1% sodium diacetate (PL/SD) which were stored at 4ºC for 6 or 30 d and then stored at -15°C for up to 50 d.

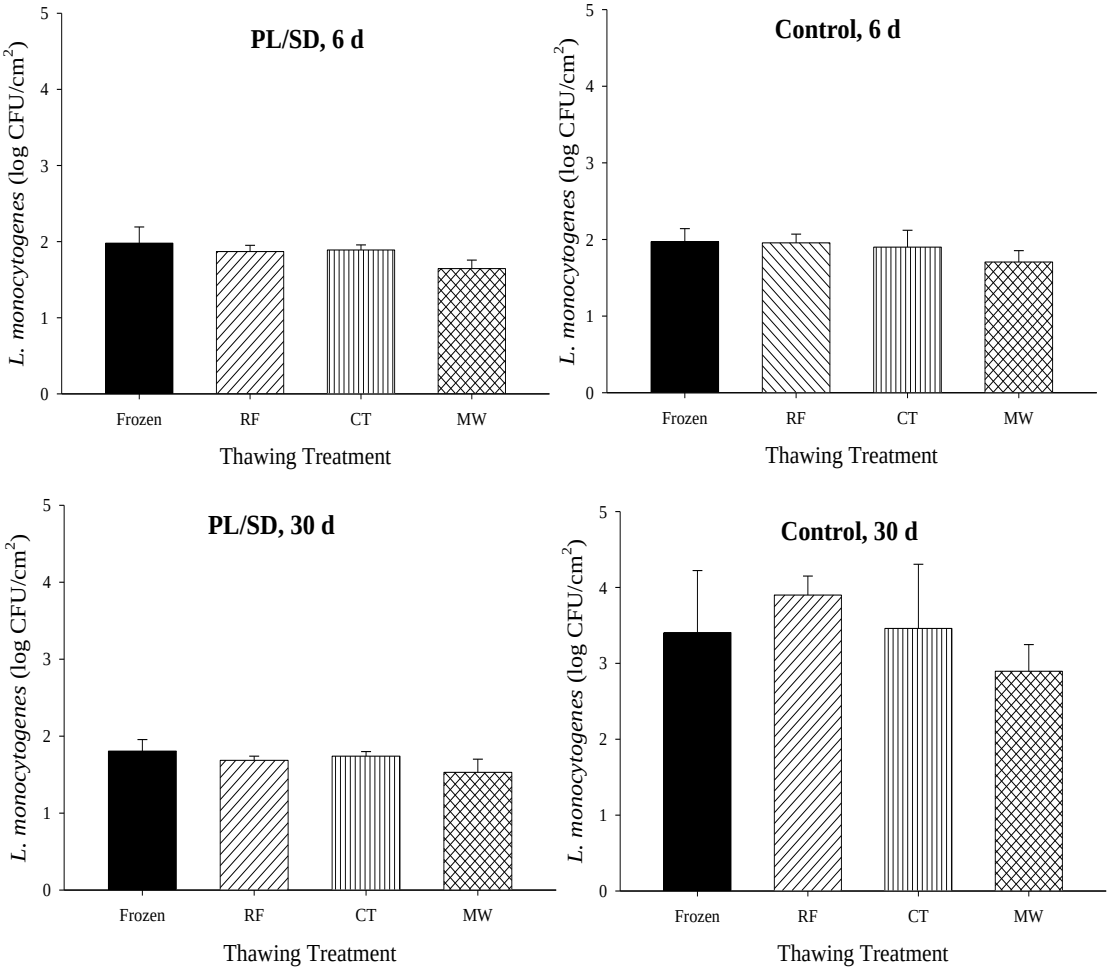

Figure 6.3 (Data in Appendix Tables 16-19). *Listeria monocytogenes* (log CFU/cm^2) recovered from frankfurters formulated without (control) or with 1.5% potassium lactate plus 0.1% sodium diacetate (PL/SD) which were stored at 4ºC for 6 or 30 d between inoculation and freezing, and then thawed using one of three methods: (i) holding packages under refrigeration (7ºC, 24 h; RF); (ii) placing packages directly on a countertop (23 ± 2ºC, 8 h; CT); or, (iii) microwave defrosting (2450 MHz, 1100 watts for 220 s, followed by 120 s holding; MW).

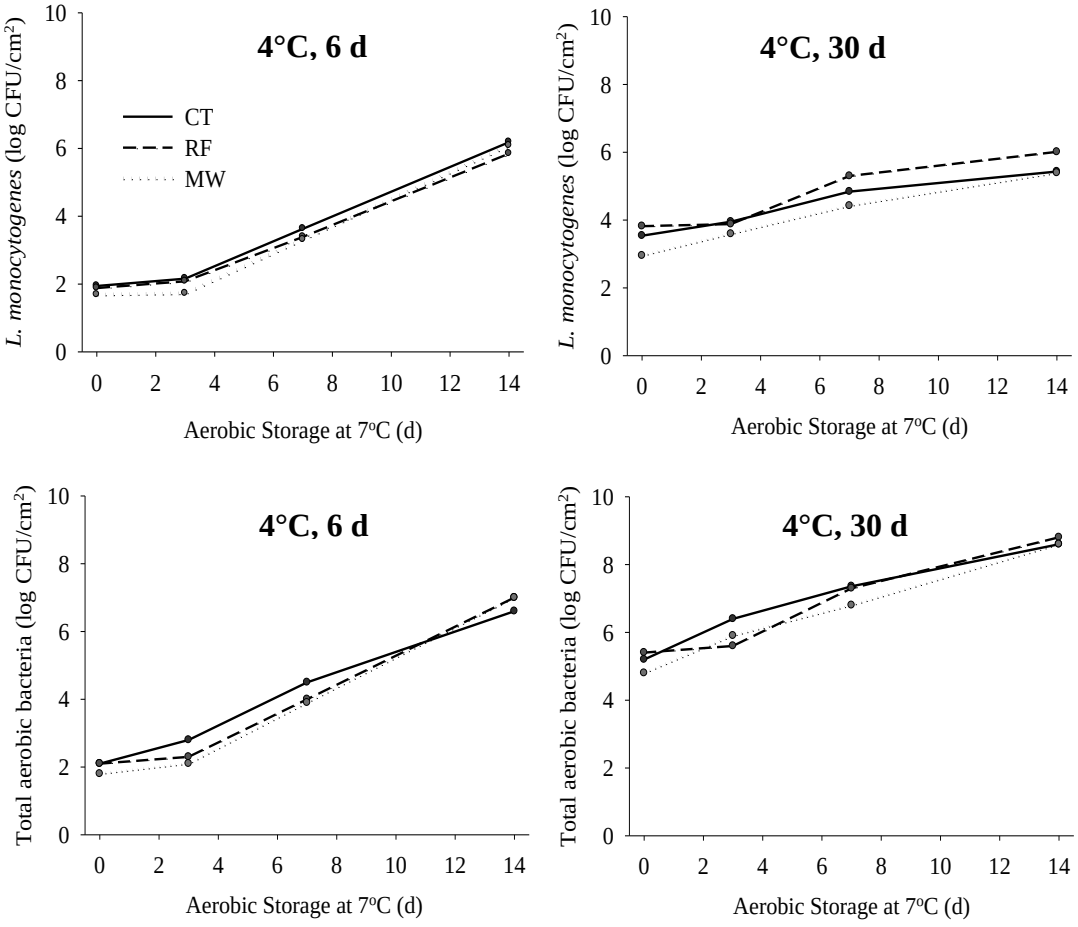

Figure 6.4 (Data in Appendix Tables 16-19). *Listeria monocytogenes* and total aerobic bacteria (log CFU/cm^2) recovered from control frankfurters (no antimicrobials) which were thawed under refrigeration (RF), on a countertop (CT) or by microwave defrosting (MW), and then stored aerobically for up to 14 d at 7ºC. Prior to frozen storage and thawing, inoculated frankfurters were stored for 6 or 30 d at 4ºC.

CHAPTER SEVEN

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CHAPTER EIGHT

Appendix

Appendix Table 1 (Data for Fig. 3.1A). pH values (mean ± standard deviation) of beef carcass runoff fluid trapped between fat and lean tissue of beef carcasses during chilling (4°C). Runoff fluid was adjusted to pH 3, 5 or 7 using lactic acid and then heated to 25, 45 or 65°C before being inoculated with *Escherichia coli* O157:H7 and poured into pockets of beef tissue and stored at 4°C for 48 h to simulate beef carcass chilling.

Treatment (pH/Temp)	pH during storage (std dev)				
	0 h	12 h	24 h	36 h	48 h
pH 3/25°C	3.08±0.06 aA	4.15± 0.18 bA	4.39±0.15 bA	4.64±0.12 bA	4.49±0.20 bA
pH 3/45°C	3.08±0.06 aA	4.01±0.17 bA	4.31±0.23 bA	4.43±0.33 bA	4.42±0.10 bA
pH 3/65°C	3.08±0.06 aA	4.17±0.22 bA	4.27±0.20 bcA	4.36±0.22 cA	4.40±0.20 cA
pH 5/25°C	4.90±0.01 aB	5.64±0.05 bB	5.41±0.07 bB	6.65±0.10 bB	5.53±0.15 bB
pH 5/45°C	4.90±0.01 aB	5.65±0.04 bB	5.61±0.08 bBC	5.79±0.13 bB	5.72±0.26 bB
pH 5/65°C	4.90±0.01 aB	5.66±0.28 bB	5.57±0.03 bB	5.78±0.28 bB	5.77±0.02 bB
pH 7/25°C	6.86±0.02 bC	5.78±0.01 aB	5.63±0.10 aBC	5.75±0.10 aB	5.58±0.08 aB
pH 7/45°C	6.86±0.02 cC	5.77±0.14 aB	5.86±0.22 aC	6.07±0.20 bB	5.78±0.20 aB
pH 7/65°C	6.86±0.02 bC	5.81±0.06 aB	5.72±0.03 aC	5.85±0.05 aB	5.72±0.10 aB

Least squares mean values with different lower case letters in the same row are different ($P < 0.05$).
Least squares mean values with different upper case letters in the same column are different ($P < 0.05$).

Appendix Table 2 (for Fig. 3.1B). Total aerobic bacteria populations (mean ± standard deviation) recovered on TSA from beef carcass runoff fluid trapped between fat and lean tissue of beef carcasses during chilling (4°C). Runoff fluid was adjusted to pH 3, 5 or 7 using lactic acid and then heated to 25, 45 or 65°C before being inoculated with *Escherichia coli* O157:H7 and poured into pockets of beef tissue and stored at 4°C for 48 h to simulate beef carcass chilling.

Treatment (pH/Temp)	log CFU/ml during storage				
	0 h	12 h	24 h	36 h	48 h
pH 3/25°C	3.9±0.2 aC	3.7±0.2 aA	3.6±0.2 aB	3.7±0.1 aB	4.7±0.4 bB
pH 3/45°C	3.2±0.4 aB	3.3±0.4 aA	2.9±0.3 aA	3.0±0.2 aA	3.5±0.2 aA
pH 3/65°C	<0.0 aA	3.4±0.0 aA	3.5±0.1 aB	3.6±0.1 aB	3.8±0.8 aA
pH 5/25°C	5.6±1.1 aD	5.4±0.6 aC	5.7±0.2 aD	6.1±0.1 abC	6.7±0.0 bD
pH 5/45°C	4.4±0.5 aC	5.0±0.7 aC	5.8±0.7 abD	5.5±1.4 abC	6.6±0.0 bD
pH 5/65°C	2.3±0.1 aB	3.9±0.3 aAB	4.8±0.3 cC	5.3±0.2 dC	6.0±0.3 cC
pH 7/25°C	5.5±0.3 aD	5.5±0.5 aC	5.4±0.5 aCD	5.5±0.4 aC	5.7±0.3 aC
pH 7/45°C	4.1±0.2 aC	4.4±0.0 aB	5.1±0.5 abC	6.3±0.7 bC	6.0±0.3 bC
pH 7/65°C	3.4±0.9 aB	4.3±0.3 abB	5.0±0.1 bC	5.1±0.6 bC	5.9±0.3 cC

Least squares mean values with different lower case letters in the same row are different ($P < 0.05$).
Least squares mean values with different upper case letters in the same column are different ($P < 0.05$).

Appendix Table 3 (for Fig. 3.1C). *Escherichia coli* O157:H7 populations (mean ± standard deviation) recovered on TSArif from beef carcass runoff fluid trapped between fat and lean tissue of beef carcasses during chilling (4°C). Runoff fluid was adjusted to pH 3, 5 or 7 using lactic acid and then heated to 25, 45 or 65°C before being inoculated with *E. coli* O157:H7 and poured into pockets of beef tissue and stored at 4°C for 48 h to simulate beef carcass chilling.

Treatment (pH/Temp)	log CFU/ml during storage				
	0 h	12 h	24 h	36 h	48 h
pH 3/25°C	3.8±0.0 aBC	3.4±0.04 aC	3.4±0.2 aC	3.3±0.5 aCD	3.5±0.2 aBC
pH 3/45°C	3.2±0.7 bB	2.2±0.6 aB	2.0±0.7 aB	1.9±0.0 aB	2.4±0.9 abB
pH 3/65°C	<0.0 aA	<0.0 aA	<0.0 aA	<0.0 aA	<0.0 aA
pH 5/25°C	4.3±0.4 aC	3.8±0.2 aD	4.0±0.1 aD	3.7±0.3 aD	3.8±0.2 aC
pH 5/45°C	4.0±0.1 aC	3.7±0.3 aD	3.6±0.1 aCD	3.6±0.2 aD	3.5±0.3 aBC
pH 5/65°C	2.7±0.3 bB	2.2±0.1 aB	2.0±0.2 aB	2.0±0.4 aB	2.2±0.1 aB
pH 7/25°C	3.9±0.1 aBC	4.0±0.1 aD	3.8±0.3 aD	3.7±0.2 aD	3.8±0.1 aC
pH 7/45°C	4.0±0.0 aC	3.9±0.1 aD	4.0±0.2 aD	3.9±0.3 aD	3.7±0.0 aC
pH 7/65°C	3.2±0.6 aB	3.0±0.0 aC	2.8±0.2 aC	3.0±0.1 aC	2.7±0.7 dB

Least squares mean values with different lower case letters in the same row are different ($P < 0.05$).
Least squares mean values with different upper case letters in the same column are different ($P < 0.05$).

Appendix Table 4 (for Fig. 3.2). Surviving *Escherichia coli* O157:H7 populations (mean ± standard deviation) recovered on TSArif from inoculated beef fat that was treated with sterile water (control), 5.0% lactic acid, 0.02% peracetic acid (PAA), or 0.5% cetylpyridinium chloride (CPC). Beef fat was inoculated with decontamination fluids that had been heated and/or acidified, inoculated with the pathogen and then stored at 4ºC for 48 h.

Cell type	log CFU/cm^2				
	No treatment	Water control	Lactic acid (5%)	PAA (0.02%)	CPC (0.5%)
pH 3/25°C	1.7±0.2 bB	1.7±0.2 bB	1.3±0.5 abB	1.6±0.3 bB	1.1±0.3 aA
pH 3/45°C	1.0±0.3 bB	0.6±0.7 abA	0.0±0.0 aA	0.1±0.2 aA	0.0±0.0 aA
pH 5/25°C	1.6±0.1 bB	1.5±0.1 bB	1.4±0.1 bB	1.5±0.1 bB	0.7±0.1 aAB
pH 5/65°C	0.4±0.3 aA	0.4±0.7 aA	0.0±0.0 aA	0.3±0.3 aA	0.2±0.4 aA
pH 7/25°C	1.5±0.1 bB	1.0±0.5 abAB	0.6±0.3 aA	1.1±0.0 aA	0.2±0.4 aA
pH 7/65°C	0.0±0.0 aA	0.0±0.3 aA	0.0±0.0 aA	0.0±0.0 aA	0.0±0.0 aA

Least squares mean values with different lower case letters in the same row are different ($P < 0.05$).
Least squares mean values with different upper case letters in the same column are different ($P < 0.05$).

Appendix Table 5 (for Fig. 4.1). Total aerobic bacterial populations (mean ± standard deviation; log CFU/cm^2), recovered with TSA, attached to stainless steel (SS), acetal, polypropylene (PP) and high density polyethylene (HDPE) coupons (2×5 cm) which were exposed (30 min, 15°C) to tryptic soy broth (TSB), beef fat-lean tissue homogenate (FLH), conveyor belt runoff fluid (CBRF), fresh ground beef or beef fat inoculated with a 6-strain *E. coli* O157:H7 composite.

Inoculation substrate	Food contact surfaces				
	Substrate	SS	acetal	PP	HDPE
TSB	6.1±0.1 aA	1.1±0.6 bD	1.5±0.6 bD	1.7±0.2 bC	1.5±0.2 bD
FLH	6.1±0.0 aA	2.4±0.2 bC	2.1±0.2 bC	2.0±0.4 bC	2.1±0.0 bC
CBRF	<0.3 aB	<0.3 aE	<0.3 aE	<0.3 aD	<0.3 aE
Ground beef	6.0±0.1 aA	2.8±0.3 cB	3.1±0.5 bcB	3.2±0.5 bB	2.8±0.5 cB
Beef fat	6.3±0.3 aA	4.5±0.2 bA	4.4±0.1bA	3.8±0.6 cA	4.3±0.2 bA

<0.3 = below the detection limit (0.3 log CFU/cm^2)
TSA: tryptic soy agar
Mean values with different lower case letters in the same row are different ($P < 0.05$)
Mean values with different upper case letters in the same column are different ($P < 0.05$)

Appendix Table 6 (for Fig. 4.1). *Escherichia coli* O157:H7 populations (mean ± standard deviation; log CFU/cm^2), recovered with TSA + rifampicin (100 µg/ml), attached to stainless steel (SS), acetal, polypropylene (PP) and high density polyethylene (HDPE) coupons (2×5 cm) which were exposed (30 min, 15°C) to tryptic soy broth (TSB), beef fat-lean tissue homogenate (FLH), conveyor belt runoff fluid (CBRF), fresh ground beef or beef fat inoculated with a 6-strain *E. coli* O157:H7 composite.

Inoculation Substrate	Food contact surfaces				
	Substrate	SS	acetal	PP	HDPE
TSB	6.4±0.7 aA	1.3±0.4 cD	1.2±0.4 cC	1.7±0.1 bC	1.6±0.2 bC
FLH	6.4±0.1 aA	2.2±0.3 bC	1.8±01.0 cB	1.8±0.2 cC	1.9±0.3 bcC
CBRF	<0.3 aB	<0.3 aE	<0.3 aD	<0.3 aD	<0.3 aD
Ground beef	5.9±0.1aA	2.7±0.3 bB	2.1±0.7 cB	2.2±0.6 cB	2.9±0.5 bB
Beef fat	6.1±0.3 aA	4.3±0.1 bA	4.1±0.1 bcA	3.9±0.2 cA	4.1±0.1 bcA

<0.3 = below the detection limit (0.3 log CFU/cm^2)
TSA: tryptic soy agar
Mean values with different lower case letters in the same row are different ($P < 0.05$)
Mean values with different upper case letters in the same column are different ($P < 0.05$)

Appendix Table 7 (for Fig. 4.2). Total aerobic bacterial populations (least squares mean; log CFU/cm^2) attached to coupons (2×5 cm) recovered with TSA. Dry/sterile coupons were inoculated by placing them between two pieces of inoculated (5 log CFU/cm^2) beef fat for 30 min. Inoculated coupons were then placed in sterile plastic tubes containing fresh beef fat homogenate (FH; 1:10 in sterile distilled water) or fresh conveyor belt runoff fluid (CBRF; dilute peroxyacetic/octanoic acid, pH 4.60) and incubated under static conditions at 15°C for 16 days.

Substrate	Semi/Fully Submerged	Storage days[a]					
		1	2	5	8	12	16
FH	Semi-submerged	4.5 cA	3.2 dA	7.1 bA	7.6 aA	7.4 abA	7.7 aA
	Fully-submerged	4.5 cA	3.1 dA	6.4 bB	7.0 aB	6.8 aB	6.7 abB
CBRF	Semi-submerged	1.4 cB	1.6 cB	3.8 aC	2.5 bC	4.0 aC	2.3 bC
	Fully-submerged	0.8 dC	1.6 bcB	1.7 bcD	1.8 bD	2.9 aD	1.4 cD

[a] Initial (day-0) counts (log CFU/ml or cm^2) recovered on TSA were 4.2±0.4 for FH and <0.3 for CBRF.
TSA: tryptic soy agar
Least squares mean values with different lower case letters in the same row are different ($P < 0.05$).
Least squares mean values with different upper case letters in the same column are different ($P < 0.05$).

Appendix Table 8 (for Fig. 4.2). *Escherichia coli* O157:H7 populations (least squares mean; log CFU/cm^2) attached to coupons (2×5 cm) and recovered with TSA + rifampicin. Dry/sterile coupons were inoculated by placing them between two pieces of inoculated (5 log CFU/cm^2) beef fat for 30 min. Inoculated coupons were then placed in sterile plastic tubes containing fresh beef fat homogenate (FH; 1:10 in sterile distilled water) or fresh conveyor belt runoff fluid (CBRF; dilute peroxyacetic/octanoic acid, pH 4.60) and incubated under static conditions at 15°C for 16 days.

Substrate	Semi/Fully Submerged	Storage days[a]					
		1	2	5	8	12	16
FH	Semi-submerged	2.3 dA	2.7 cA	3.5 bA	3.9 aA	3.7 abA	3.7 bA
	Fully-submerged	2.0 dB	2.5 bcA	2.7 abB	2.3 cB	2.5 bcB	2.8 aB
CBRF	Semi-submerged	<0.3 aC	0.3 aB	0.5 aC	<0.3 aC	<0.3 aC	0.4 aC
	Fully-submerged	<0.3 aC	0.3 aB	<0.3 aC	<0.3 aC	<0.3 aC	<0.3 aC

[a] Initial (day-0) counts (log CFU/ml or cm^2) recovered on TSA were 4.2±0.4 for FH and <0.3 for CBRF.
[b] Detection limit = 0.3 log CFU/cm^2
TSA: tryptic soy agar
Least squares mean values with different lower case letters in the same row are different ($P < 0.05$).
Least squares mean values with different upper case letters in the same column are different ($P < 0.05$).

Appendix Table 9 (for Fig. 4.2). *Escherichia coli* O157:H7 populations (mean ± standard deviation; log CFU/cm^2) attached to coupons (2x5 cm) and recovered with SMACct. Dry/sterile coupons were inoculated by placing them between two pieces of inoculated (5 log CFU/cm^2) beef fat for 30 min. Inoculated coupons were then placed in sterile plastic tubes containing fresh beef fat homogenate (FH; 1:10 in sterile distilled water) or fresh conveyor belt runoff fluid (CBRF; dilute peroxyacetic/octanoic acid, pH 4.60) and incubated under static conditions at 15°C for 16 days.

Substrate	Semi/Fully Submerged	Storage days[a]					
		1	2	5	8	12	16
FH	Semi-submerged	2.4 dA	2.0 eA	5.4 cA	5.9 bA	6.2 bA	6.5 aA
	Fully-submerged	2.5 cA	2.2 cA	4.6 bB	5.8 aA	5.5 aB	5.6 aB
CBRF	Semi-submerged	<0.3 cB	0.5 cB	2.6 aC	0.6 cB	2.0 bC	0.6 cC
	Fully-submerged	<0.3 cB	0.5 cB	0.6 bcD	0.8 abB	0.9 aD	0.4 cC

[a] Initial (day 0) counts (log CFU/ml or cm^2) recovered on SMACct were 0.7±0.1 for FH and <0.3 for CBRF.
[b] Detection limit = 0.3 log CFU/cm^2
TSA: tryptic soy agar
Least squares mean values with different lower case letters in the same row are different ($P < 0.05$).
Least squares mean values with different upper case letters in the same column are different ($P < 0.05$).

Appendix Table 10 (for Fig. 4.3). Total aerobic bacterial populations (mean ± standard deviation; log CFU/cm^2), recovered with TSA, and attached to stainless steel (SS) or acetal coupons (2×5 cm). Dry/sterile coupons were place between two pieces of beef fat inoculated with a 6-strain *E. coli* O157:H7 composite (cells suspended in beef tissue homogenate, applied to tissue which was then held for 24 h at 4ºC), and pressure was applied for 30 min. Inoculated coupons were then placed in sterile plastic tubes containing tryptic soy broth (TSB), fresh beef fat-lean tissue homogenate (FLH; 1:10 in sterile distilled water) or fresh conveyor belt runoff fluid (CBRF; dilute peroxyacetic/octanoic acid, pH 4.60) and incubated under static conditions for 10 days at 15°C.

Substrate	Surface material	Storage days				
		0	1	3	7	10
TSB	SS	3.6±0.4 aA	4.2±0.3 aC	6.8±0.1 bB	6.7±0.4 bC	7.3±1.0 bB
	Acetal	3.4±0.3 aA	3.6±0.4 aC	7.0±0.2 bB	7.0±0.4 bC	6.7±0.4 bB
FLH	SS	3.7±0.1 aA	4.0±0.3 aC	6.6±0.1 bB	7.3±0.2 bC	7.0±0.3 bB
	Acetal	3.3±0.1 aA	3.5±0.1 aC	7.0±0.1 bB	6.9±0.1 bC	6.9±0.1 bB
CBRF	SS	3.6±0.2 aA	1.7±0.4 bB	3.0±1.5 abA	1.7±0.7 bB	2.5±1.2 abA
	acetal	3.4±0.0 aA	0.6±0.6 bcA	4.3±1.2 dA	< 0.3 cA	1.3±1.2 bA

<0.3 = below the detection limit (0.3 log CFU/cm^2)
TSA: tryptic soy agar
Mean values with different lower case letters in the same row are different ($P < 0.05$)
Mean values with different upper case letters in the same column are different ($P < 0.05$)

Appendix Table 11 (for Fig. 4.3). *Escherichia coli* O157:H7 populations (mean ± standard deviation; log CFU/cm^2), recovered with TSA + rifampicin (100 µg/ml), attached to stainless steel (SS) or acetal coupons (2×5 cm). Dry/sterile coupons were place between two pieces of beef fat inoculated with a 6-strain *E. coli* O157:H7 composite (cells suspended in beef tissue homogenate, applied to tissue which was then held for 24 h at 4ºC), and pressure was applied for 30 min. Inoculated coupons were then placed in sterile plastic tubes containing tryptic soy broth (TSB), fresh beef fat-lean tissue homogenate (FLH; 1:10 in sterile distilled water) or fresh conveyor belt runoff fluid (CBRF; dilute peroxyacetic/octanoic acid, pH 4.60) and incubated under static conditions for 10 days at 15°C.

Substrate	Surface material	Storage days				
		0	1	3	7	10
TSB	SS	3.6±0.4 aAB	3.8±0.2 aAB	6.6±0.2 bA	6.7±0.2 bA	6.6±0.2 bA
	Acetal	3.4±0.1 aAB	3.6±0.3 aAB	6.5±0.5 bA	6.7±0.7 bA	6.0±0.4 bA
FLH	SS	3.9±0.2 aA	4.0±0.4 aA	4.9±0.1 bcB	4.6±0.2 bB	5.3±0.4 cB
	Acetal	3.4±0.1 aB	3.0±0.4 aB	4.3±0.7 bB	4.6±0.3 bB	4.7±0.2 bB
CBRF	SS	3.4±0.5 aB	< 0.3 cC	1.2±0.8 bC	0.6±1.3 bC	2.1±0.7 bC
	acetal	3.3±0.0 aB	0.3±0.0 bC	2.3±1.3 bC	< 0.3 bC	1.0±0.9 bC

<0.3 = below the detection limit (0.3 log CFU/cm^2)
TSA: tryptic soy agar
Mean values with different lower case letters in the same row are different ($P < 0.05$)
Mean values with different upper case letters in the same column are different ($P < 0.05$)

Appendix Table 12 (for Fig 5.1). Reductions in aerobic bacterial populations (mean ± standard deviation; log CFU/cm^2), recovered with TSA, attached to stainless steel, acetal or high-density polyethylene (HDPE) coupons (2x5 cm) after sanitizer treatment. Fat-inoculated coupons were placed in tubes containing fresh beef fat-lean tissue homogenate (1:10 in sterile distilled water) and incubated for 3 d at 15°C. Coupons were then placed in minimum or high recommended concentrations of sanitizing solution or in sterile distilled water (control) for 1 or 10 min. Sanitizers included acidified sodium chlorite (ASC), sodium hypochlorite (SH), two quaternary ammonium compounds (QUAT-A, QUAT-B), peroxyacetic acid (PAA), a commercial peroxyacetic acid/octanoic acid mixture (PA/OA) and potassium peroxymonosulfate (PP/SC).

Sanitizer	Level	SS		Acetal		HDPE	
		1 min	10 min	1 min	10 min	1 min	10 min
Control		0.1±0.3 a	0.2±0.2 a	0.2±0.2 a	0.2±0.2 a	0.2±0.2 a	0.2±0.2 a
ASC	low	2.7±0.8 aA	2.7±0.4 aA	1.6±0.4 aA	2.4±0.7 aA	2.6±1.2 aA	2.7±0.2 aA
	high	2.9±0.8 aA	3.3±1.2 aB	1.6±0.5 aA	3.7±1.2 bB	2.8±0.3 aA	2.8±0.4 aA
SH	low	0.9±0.3 aA	1.5±0.8 aA	1.2±0.3 aA	1.4±0.7 aA	1.2±0.3 aA	1.4±0.9 aA
	high	1.0±0.3 aA	1.5±0.3 aA	1.5±0.3 aA	1.6±0.4 aA	1.2±0.6 aA	1.3±0.4 aA
QUAT-A	low	1.1±0.8 bA	0.7±0.2 aA	1.0±1.2 aA	0.6±0.2 aA	1.2±0.7 aA	0.7±0.4 aA
	high	1.2±0.5 aA	1.0±0.4 aA	0.8±0.5 aA	1.3±0.6 aB	1.4±0.7 aA	1.2±0.5 aA
QUAT-B	low	1.3±0.4 aA	1.3±0.4 aA	1.4±0.1 aA	1.6±0.4 aA	1.1±0.6 aA	1.3±0.2 aA
	high	1.9±0.1 aB	1.9±0.6 aA	1.9±0.4 aB	1.6±0.4 aA	1.5±0.5 aA	1.2±0.2 aA
PAA	low	1.0±0.2 aA	2.6±0.9 bA	1.0±0.3 aA	2.5±0.9 bA	1.6±0.4 aA	1.9±1.3 aA
	high	1.5±0.6 aB	2.3±0.2 aA	1.6±0.2 aA	2.3±0.5 aA	1.7±0.5 aA	2.2±0.6 aA
PA/OA	low	0.9±0.3 aA	1.7±1.2 aA	1.2±0.5 aA	2.3±0.8 bA	1.4±0.7 aA	1.6±1.1 aA
	high	1.9±0.4 bB	1.5±0.3 aA	1.5±0.8 aA	2.1±0.6 aA	1.6±0.3 aA	1.9±0.8 aA
PP/SC	low	1.7±0.5 aA	1.7±0.4 aA	1.9±0.3 aA	2.5±0.4 aA	1.5±0.4 aA	1.9±0.5 aA
	high	2.3±0.4 aA	2.3±0.3 aA	2.4±0.2 aA	3.2±0.3 aB	2.0±0.3 aB	2.1±0.3 aA

Initial counts on untreated SS, acetal and HDPE coupons were 6.5±0.7, 6.4±0.6 and 6.4±0.7 log CFU/cm^2, respectively.
Least squares mean values with different lower case letters in the same row and within each surface material are different ($P < 0.05$).
Least squares mean values with different upper case letters in the same column and within each sanitizer are different ($P < 0.05$).

Appendix Table 13 (for Fig 5.2). Reductions in *Escherichia coli* O157:H7 counts (mean ± standard deviation; log CFU/cm^2), recovered with TSA plus rifampicin (100 µg/ml), attached to stainless steel, acetal or high-density polyethylene (HDPE) coupons (2x5 cm) after sanitizer treatment. Fat-inoculated coupons were placed in tubes containing fresh beef fat-lean tissue homogenate (1:10 in sterile distilled water) and incubated for 3 d at 15°C. Coupons were then placed in low or high concentrations of sanitizing solution or in sterile distilled water (control) for 1 or 10 min. Sanitizers included acidified sodium chlorite (ASC), sodium hypochlorite (SH), two quaternary ammonium compounds (QUAT-A, QUAT-B), peroxyacetic acid (PAA), a commercial peroxyacetic acid/octanoic acid mixture (PA/OA) and potassium peroxymonosulfate (PP/SC).

Sanitizer	Level	SS		acetal		HDPE	
		1 min	10 min	1 min	10 min	1 min	10 min
Control		0.0±0.2 a	0.1±0.6 a	0.1±0.2 a	0.1±0.2 a	0.1±0.3 a	0.2±0.3 a
ASC	low	1.3±0.8 bA	0.1±0.3 bA	0.8±0.6 aA	0.5±0.5 aA	1.7±0.6 aA	1.2±0.3 aA
	high	1.4±0.8 aA	2.2±1.1 bB	1.4±0.4 aA	2.3±1.1 bB	1.5±0.2 aA	1.5±0.6 aA
SH	low	0.1±0.3 aA	0.0±0.5 aA	0.2±0.8 aA	0.0±0.7 aA	0.1±0.6 aA	0.0±0.8 aA
	high	0.1±0.6 aA	0.0±0.3 aA	0.6±0.2 aA	0.4±0.5 aA	0.1±0.6 aA	0.0±0.4 aA
QUAT-A	low	0.0±0.6 aA	0.0±0.2 aA	0.0±0.6 aA	0.0±0.2 aA	0.2±0.5 aA	0.0±0.4 aA
	high	0.0±0.7 aA	0.8±0.4 aB	0.2±0.6 aA	0.5±0.4 aA	0.0±0.4 aA	0.0±0.2 aA
QUAT-B	low	0.1±0.4 aA	0.0±0.2 aA	0.2±0.2 aA	0.2±0.4 aA	0.1±0.8 aA	0.0±0.3 aA
	high	0.6±0.5 aA	0.3±0.4 aA	0.7±0.6 aA	0.7±0.5 aA	0.4±0.6 aA	0.2±0.2 aA
PAA	low	0.8±0.3 aA	0.9±0.6 aA	0.6±0.3 aA	0.7±0.5 aA	0.7±0.8 aA	0.9±0.2 aA
	high	0.5±0.5 aA	0.8±0.3 aA	0.8±0.4 aA	1.3±0.5 aA	1.0±0.5 aA	0.5±0.7 aB
PA/OA	low	0.4±0.4 aA	0.2±0.4 aA	0.3±0.7 aA	0.8±0.3 aA	0.4±0.7 aA	0.3±0.8 aA
	high	1.0±0.3 aA	0.7±0.5 aA	0.9±0.7 aA	1.0±0.3 aA	0.7±0.3 aA	0.8±0.5 aA
PP/SC	low	0.5±0.3 aA	0.6±0.3 aA	1.0±0.4 aA	1.3±0.4 aA	0.7±0.3 aA	0.2±0.4 aA
	high	0.8±0.5 aA	0.3±1.0 aA	0.9±0.3 aA	1.4±0.1 aA	0.9±0.5 aA	0.5±0.4 aA

Initial counts on untreated SS, acetal and HDPE coupons were 4.3±0.6, 4.4±0.7 and 4.3±0.6 log CFU/cm^2, respectively.
Least squares mean values with different lower case letters in the same row and within each surface material are different ($P < 0.05$).
Least squares mean values with different upper case letters in the same column and within each sanitizer are different ($P < 0.05$).

Appendix Table 14 (for Fig. 5.3). Reductions in total aerobic bacteria counts (mean ± standard deviation; log CFU/cm^2) attached to coupons (2x5 cm), recovered with TSA following sanitizer treatment. Fat-inoculated coupons were incubated for 0, 3 or 7 d ays (15°C) in fresh beef fat-lean tissue homogenate and then placed in low or high concentrations of sanitizing solution or in sterile distilled water (control) for 1 or 10 min. Sanitizers included sodium hypochlorite (SH), quaternary ammonium compound (QUAT-A) and a commercial peroxyacetic acid/octanoic acid mixture (PA/OA).

Sanitizer	Level	Exposure					
		0 d		3 d		7 d	
		1 min	10 min	1 min	10 min	1 min	10 min
Control		0.1±0.5 aA	0.0±0.3 aA	0.2±0.2 aA	0.2±0.2 aA	0.2±0.4 aA	0.2±0.3 aA
SH	Low	0.6±0.2 aB	0.9±0.5 aB	1.1±0.1 aB	1.4±0.1 aB	0.6±0.5 aAB	1.3±0.4 bBC
	High	0.6±0.5 aB	1.0±0.7 aB	1.2±0.3 aB	1.4±0.2 aB	1.1±0.3 aB	1.8±0.9 bCD
QUAT-A	Low	0.6±0.3 aB	1.0±0.8 aB	1.1±0.1 bB	0.7±0. aAB	0.4±0.3 aA	0.6±0.2 aAB
	High	0.8±0.7 aB	1.1±0.5 aB	1.1±0.3 aB	1.2±0.2 aB	0.5±0.3 aA	1.0±0.3 aB
PA/OA	Low	0.9±0.5 aB	1.5±0.7 bBC	1.2±0.3 aB	1.9±0.3 bC	0.6±0.3 aAB	1.5±0.4 bBC
	High	1.0±0.9 aB	1.8±0.7 bC	1.4±0.5 aB	1.8±0.3 aBC	1.0±1.0 aB	3.2±2.2 bD

Initial counts on untreated coupons at d-0, 3 and 7 were 5.0±0.2, 6.4±0.7 and 7.7±0.3 log CFU/cm^2, respectively.
Least squares mean values with different lower case letters in the same row and within each treatment day are different ($P < 0.05$).
Least squares mean values with different upper case letters in the same column are different ($P < 0.05$).

Appendix Table 15 (for Fig. 5.4). Reductions in *Escherichia coli* O157:H7 counts (mean ± standard deviation; log CFU/cm^2) attached to coupons (2x5 cm), recovered with TSA plus rifampicin (100 µg/ml) following sanitizer treatment. Fat-inoculated coupons were incubated for 0, 3 or 7 days (15°C) in fresh beef fat-lean tissue homogenate and then placed in low or high concentrations of sanitizing solution or in sterile distilled water (control) for 1 or 10 min. Sanitizers included sodium hypochlorite (SH), quaternary ammonium compound (QUAT-A) and a commercial peroxyacetic acid/octanoic acid mixture (PA/OA).

Sanitizer	Level	Exposure					
		0 d		3 d		7 d	
		1 min	10 min	1 min	10 min	1 min	10 min
Control		0.2±0.6 aA	0.0±0.6 aA	0.1±0.2 aA	0.1±0.2 aA	0.0±0.4 aA	0.2±0.4 aA
SH	Low	0.1±0.6 aA	0.4±0.6 aAB	0.1±0.4 aA	0.0±0.8 aA	0.1±0.6 aA	0.2±0.4 aA
	High	0.2±0.2 aA	0.6±0.7 bB	0.3±1.4 aA	0.2±04 aA	0.0±0.6 aA	0.2±0.7 aA
QUAT-A	Low	0.1±0.3 aA	0.3±0.6 aA	0.1±0.5 aA	0.0±0.4 aA	0.0±0.4 aA	0.0±0.5 aA
	High	0.3±0.5 aAB	0.8±0.5 bB	0.1±0.7 aA	0.4±0.2 aAB	0.0±0.3 aA	0.1±0.7 aA
PA/OA	Low	0.6±0.8 aB	1.2±0.8 bC	0.4±0.7 aAB	0.4±0.5 aAB	0.0±0.4 aA	0.4±0.8 aA
	High	0.5±0.1 aAB	1.4±0.9 bC	0.8±0.6 aB	0.8±0.5 aB	0.3±0.5 aA	0.9±0.5 bB

Initial counts on untreated coupons at d-0, 3 and 7 were 3.6±0.4, 4.3±0.6 and 3.7±0.5 log CFU/cm^2, respectively.
Least squares mean values with different lower case letters in the same row and within each treatment day are different ($P < 0.05$).
Least squares mean values with different upper case letters in the same column are different ($P < 0.05$).

Appendix Table 16 (for Fig. 6.1-6.3). *L. monocytogenes* (log CFU/cm^2) recovered from frankfurters formulated without antimicrobials. Frankfurters (n = 8 per package) were inoculated, stored for 6 or 30 d at 4ºC to generate two inoculum levels (approximately 2.2 log or 4.2 log CFU/cm^2, respectively), and then frozen (-15ºC) for 10, 30, or 50 d. Frozen packages were thawed by microwave defrosting (220 sec), in a refrigerator (7ºC, 24 h), or on a countertop (23ºC, 8 h); immediately following thawing treatments and throughout aerobic storage (7ºC) frankfurters (n = 2) were systematically removed from packages for microbiological analysis.

Length of storage at 4ºC (d)	Length of frozen storage (d)	Storage day	Thawing method			
			Control	Microwave	Refrigerator	Countertop
6	10	0	2.1 ± 0.1 a	1.7 ± 0.9 Aa	2.1 ± 2.3 Aa	2.1 ± 0.1 Aa
		3		1.9 ± 0.3 Aa	2.3 ± 0.2 Aa	2.4 ± 0.3 Aa
		7		3.0 ± 0.3 Ba	3.5 ±0.4 Ba	3.2 ± 0.8 Aa
		14		5.7 ± 0.5 Ca	5.9 ± 0.3 Ca	6.0 ± 0.4 Ba
	30	0	1.9 ± 0.3 a	1.8 ± 0.2 Aa	2.0 ± 0.1 Aa	2.0 ± 0.2 Aa
		3		1.7 ± 0.3 Aa	1.9 ± 0.2 Aa	2.1 ± 0.3 Aa
		7		3.6 ± 0.2 Ba	3.3 ± 0.4 Ba	3.9 ± 0.2 Ba
		14		6.2 ± 0.5 Ca	5.7 ± 0.9 Ca	6.2 ± 1.0 Ca
	50	0	1.9 ± 0.2 a	1.5 ± 0.2 Aa	1.7 ± 0.1 Aa	1.8 ± 0.1 Aa
		3		1.6 ± 0.2 Aa	2.0 ± 0.2 Aa	2.1 ± 0.2 Aa
		7		3.3 ± 0.6 Ba	3.4 ± 0.5 Ba	3.8 ± 0.3 Ba
		14		6.3 ± 0.5 Ca	6.0 ± 0.4 Ca	6.0 ± 0.7 Ca
30	10	0	4.4 ± 0.2 b	3.1 ± 0.9 Aa	3.8 ± 0.7 Aab	4.4 ± 0.5 Ab
		3		3.7 ± 1.0 Aa	3.9 ± 1.2 A ab	5.0 ± 1.3 Aba
		7		4.7 ± 1.9 ABa	5.3 ± 1.9 ABa	5.8 ± 2.5 Ba
		14		5.5 ± 3.0 Ba	6.0 ± 2.9 Ba	6.4 ± 2.3 Ba
	30	0	3.4 ± 0.4 b	2.6 ± 0.4 Aa	3.8 ± 0.7 Ab	3.5 ± 0.3 Ab
		3		3.4 ± 0.6 Aa	3.9 ± 0.8 Aa	3.7 ± 0.7 Aa
		7		3.8 ± 1.1 ABa	4.9 ± 1.5 ABa	4.7 ± 1.6 Aba
		14		4.6 ± 3.0 Ba	5.4 ± 2.4 Ba	5.6 ± 2.7 Ba
	50	0	3.4 ± 0.8 ab	3.2 ± 0.7 Aab	3.8 ± 0.7 Ab	2.8 ± 0.6 Aa
		3		3.8 ± 0.8 ABa	3.9 ± 1.0 Aa	3.2 ± 0.9 Aba
		7		4.8 ± 0.6 Ba	5.7 ± 1.4 Ba	4.0 ± 1.8 Ba
		14		6.1 ± 1.8 Ca	3.3 ± 2.2 Aa	4.4 ± 1.3 Ba

Least squares mean values with different lower case letters in the same row are different ($P < 0.05$). Least squares mean values with different upper case letters in the same column and within the same treatment are different ($P < 0.05$).

Appendix Table 17 (for Fig. 6.1-6.3). *L. monocytogenes* (log CFU/cm^2) recovered from frankfurters formulated with antimicrobials. Frankfurters (n = 8 per package) were inoculated, stored for 6 or 30 d at 4ºC to generate two inoculum levels (approximately 2.2 log or 4.2 log CFU/cm^2, respectively), and then frozen (-15ºC) for 10, 30, or 50 d. Frozen packages were thawed by microwave defrosting (220 sec), in a refrigerator (7ºC, 24 h), or on a countertop (23ºC, 8 h); immediately following thawing treatments and throughout aerobic storage (7ºC) frankfurters (n = 2) were systematically removed from packages for microbiological analysis.

Length of storage at 4ºC (d)	Length of frozen storage (d)	Storage day	Thawing method			
			Control	Microwave	Refrigerator	Countertop
6	10	0	2.2 ± 0.1 a	1.7 ± 0.2 Aa	1.9 ± 0.1 Aa	2.0 ± 0.1 Aa
		3		1.7 ± 0.1 Aa	1.8 ± 0.1 Aa	1.8 ± 0.2 Aa
		7		1.6 ± 0.2 Aa	1.9 ± 0.1 Aa	2.1 ± 0.7 Aa
		14		1.6 ± 0.3 Aa	1.9 ± 0.1 Aa	1.8 ± 0.1 Aa
	30	0	1.9 ± 0.3 a	1.5 ± 0.3 Aa	1.9 ± 0.1 Aa	1.9 ± 0.1 Aa
		3		1.6 ± 0.2 Aa	1.8 ± 0.2 Aa	1.5 ± 0.5 Aa
		7		1.6 ± 0.2 Aa	1.8 ± 0.1 Aa	1.7 ± 0.3 Aa
		14		1.5 ± 0.3 Aa	1.8 ± 0.1 Aa	1.8 ± 0.2 Aa
	50	0	1.9 ± 0.2 a	1.8 ± 0.1 Aa	1.8 ± 0.1 Aa	1.8 ± 0.1 Aa
		3		1.7 ± 0.7 Aa	1.9 ± 0.1 Aa	1.8 ± 0.1 Aa
		7		1.9 ± 0.4 Aa	2.0 ± 0.7 Aa	1.7 ± 0.2 Aa
		14		1.6 ± 0.2 Aa	1.5 ± 0.2 Aa	1.4 ± 0.2 Aa
30	10	0	1.9 ± 0.2 b	1.4 ± 0.1 Aa	1.6 ± 0.2 Aab	1.8 ± 0.1 Aab
		3		1.3 ± 0.2 Aa	2.0 ± 0.2 Ab	1.6 ± 0.2 Aab
		7		1.4 ± 0.1 Aa	1.9 ± 0.1 Aa	1.8 ± 0.1 Aa
		14		1.5 ± 0.8 Aa	2.0 ± 0.7 Aa	1.8 ± 0.5 Aa
	30	0	1.6 ± 0.6 a	1.5 ± 0.1 Aa	1.6 ± 0.1 Aa	1.7 ± 0.1 Aa
		3		1.6 ± 0.1 Aa	1.7 ± 0.1 Aa	1.6 ± 0.1 Aa
		7		1.2 ± 0.3 Aa	1.6 ± 0.1 Aa	1.6 ± 0.1 Aa
		14		1.3 ± 0.6 Aa	1.6 ± 0.3 Aa	1.8 ± 0.5 Aa
	50	0	1.6 ± 0.4 a	1.6 ± 0.2 Aa	1.7 ± 0.1 Aa	1.5 ± 0.5 Aa
		3		1.8 ± 0.4 Aa	1.7 ± 0.1 Aa	1.6 ± 0.2 Aa
		7		1.3 ± 0.3 Aa	1.7 ± 0.1 Aa	1.6 ± 0.1 Aa
		14		1.6 ± 0.5 Aa	1.4 ± 0.2 Aa	1.5 ± 0.5 Aa

Least squares mean values with different lower case letters in the same row are different ($P < 0.05$).
Least squares mean values with different upper case letters in the same column and within the same treatment are different ($P < 0.05$).

Appendix Table 18 (for Fig. 6.1-6.3). Total aerobic bacteria (log CFU/cm^2) recovered from frankfurters formulated without antimicrobials. Frankfurters (n = 8 per package) were inoculated, stored for 6 or 30 d at 4ºC to generate two inoculum levels (approximately 2.2 log or 4.2 log CFU/cm^2, respectively), and then frozen (-15ºC) for 10, 30, or 50 d. Frozen packages were thawed by microwave defrosting (220 sec), in a refrigerator (7ºC, 24 h), or on a countertop (23ºC, 8 h); immediately following thawing treatments and throughout aerobic storage (7ºC) frankfurters (n = 2) were systematically removed from packages for microbiological analysis.

Length of storage at 4ºC (d)	Length of frozen storage (d)	Storage day	Thawing method			
			Control	Microwave	Refrigerator	Countertop
6	10	0	2.5 ± 0.2 b	1.8 ± 0.2 Aa	2.0 ± 0.2 Aab	2.3 ± 0.4 Aab
		3		2.2 ± 0.5 Aa	2.4 ± 0.4 Aa	2.8 ± 0.6 Aa
		7		3.4 ± 1.0 Ba	3.3 ± 0.3 Ba	3.2 ± 0.8 Aba
		14		5.9 ± 1.1 Ca	6.2 ± 0.6 Ca	4.5 ± 1.5 Ba
	30	0	2.3 ± 0.1 a	1.9 ± 0.3 Aa	2.1 ± 0.5 Aa	1.9 ± 0.1 Aa
		3		2.0 ± 0.2 Aa	2.4 ± 0.5 Aa	2.6 ± 0.7 Aa
		7		4.0 ± 0.9 Ba	4.4 ± 0.9 Ba	4.9 ± 0.9 Ba
		14		7.4 ± 0.8 Ca	7.5 ± 0.6 Ca	7.8 ± 0.6 Ca
	50	0	2.3 ± 0.3 b	1.7 ± 0.2 Aa	2.0 ± 0.1 Aab	2.0 ± 0.1 Aab
		3		2.0 ± 0.4 Aa	2.2 ± 0.3 Aa	3.0 ± 0.8 Aa
		7		4.2 ± 1.3 Ba	4.3 ± 1.9 Ba	5.3 ± 0.5 Ba
		14		7.7 ± 1.1 Ca	7.4 ± 0.9 Ca	7.4 ± 1.1 Ca
30	10	0	5.8 ± 1.5 a	5.2 ± 1.9 Aa	5.7 ± 1.8 Aa	4.9 ± 0.9 Aa
		3		6.2 ± 1.8 Aa	5.6 ± 2.1 Aa	7.0 ± 1.0 Ba
		7		7.7 ± 0.7 ABa	7.7 ± 1.0 ABa	7.8 ± 0.9 BCa
		14		8.6 ± 0.2 Ba	8.8 ± 0.2 Ba	8.6 ± 0.1 Ca
	30	0	5.4 ± 1.0 a	5.0 ± 2.1 Aa	5.7 ± 1.9 Aa	5.6 ± 1.7 Aa
		3		6.1 ± 2.3 Aa	6.4 ± 2.3 ABa	6.6 ± 1.9 ABa
		7		7.3 ± 1.7 ABa	7.7 ± 1.1 ABa	7.7 ± 1.1 ABa
		14		8.6 ± 0.6 Ba	8.6 ± 0.3 Ba	8.6 ± 0.1 Ba
	50	0	5.5 ± 1.0 b	4.3 ± 1.6 Aa	4.8 ± 1.2 Aab	5.0 ± 2.5 Aab
		3		5.4 ± 1.5 Aa	5.0 ± 1.7 Aa	5.5 ± 2.7 Aa
		7		5.5 ± 1.2 Aa	6.6 ± 1.3 Aa	6.6 ± 2.5 ABa
		14		8.6 ± 0.5 Ba	8.9 ± 0.1 Ba	8.5 ± 0.5 Ba

Least squares mean values with different lower case letters in the same row are different ($P < 0.05$).
Least squares mean values with different upper case letters in the same column and within the same treatment are different ($P < 0.05$).

Appendix Table 19 (for Fig. 6.1-6.3). Total aerobic bacteria (log CFU/cm^2) recovered from frankfurters formulated with antimicrobials. Frankfurters (n = 8 per package) were inoculated, stored for 6 or 30 d at 4ºC to generate two inoculum levels (approximately 2.2 log or 4.2 log CFU/cm^2, respectively), and then frozen (-15ºC) for 10, 30, or 50 d. Frozen packages were thawed by microwave defrosting (220 sec), in a refrigerator (7ºC, 24 h), or on a countertop (23ºC, 8 h); immediately following thawing treatments and throughout aerobic storage (7ºC) frankfurters (n = 2) were systematically removed from packages for microbiological analysis.

Length of storage at 4ºC (d)	Length of frozen storage (d)	Storage day	Thawing method			
			Control	Microwave	Refrigerator	Countertop
6	10	0	2.2 ± 0.3 a	1.8 ± 0.1 Aa	2.0 ± 0.4Aa	2.2 ± 0.8Aa
		3		1.8 ± 0.1 Aa	1.9 ± 0.1Aa	2.2 ± 0.6Aa
		7		1.8 ± 0.4Aa	2.0 ± 0.1Aa	2.4 ± 0.7Aa
		14		1.8 ± 0.1Aa	2.0 ± 0.1 Aa	2.7 ± 0.6Ab
	30	0	2.1 ± 0.2 a	1.7 ± 0.2Aa	2.0 ± 0.1Aa	1.9 ± 0.1Aa
		3		1.6 ± 0.1Aa	1.9 ± 0.2Aa	1.9 ± 0.1Aa
		7		1.8 ± 0.1Aa	1.9 ± 0.2Aa	2.0 ± 0.3Aa
		14		1.8 ± 0.2Aa	2.0 ± 0.2Aa	2.0 ± 0.2Aa
	50	0	2.2 ± 0.4 a	1.9 ± 0.1Aa	1.9 ± 0.1Aa	1.9 ± 0.1Aa
		3		1.8 ± 0.3Aa	2.0 ± 0.1Aa	1.9 ± 0.1Aa
		7		1.9 ± 0.5Aa	2.1 ± 0.3Aa	1.8 ± 0.1Aa
		14		2.5 ± 2.0Aa	2.0 ± 0.1Aa	2.7 ± 0.6Ba
30	10	0	2.4 ± 0.4 b	1.5 ± 0.3Aa	2.0 ± 0.4Ab	1.9 ± 0.1Ab
		3		1.6 ± 0.5Aa	2.7 ± 1.5Ab	2.0 ± 0.3Aab
		7		2.7 ± 1.0Aa	3.6 ± 1.8Ba	2.7 ± 0.9ABa
		14		4.6 ± 2.8Ba	4.9 ± 3.3Ba	4.6 ± 3.4Ba
	30	0	2.6 ± 1.3 b	1.7 ± 0.3Aa	1.9 ± 0.1Aa	2.5 ± 1.3Ab
		3		1.8 ± 0.3Aa	1.8 ± 0.2Aa	2.0 ± 0.3Aa
		7		2.2 ± 1.8Aa	2.1 ± 0.5Aa	2.0 ± 0.4Aa
		14		4.2 ± 3.1Ba	4.9 ± 3.4Ba	4.7 ± 2.9Ba
	50	0	2.0 ± 0.1 a	1.7 ± 0.2Aa	1.9 ± 0.1Aa	2.0 ± 0.3Aa
		3		2.1 ± 0.6Aa	2.0 ± 0.1Aa	2.5 ± 0.9Aa
		7		1.8 ± 0.4Aa	2.3 ± 0.7Aa	2.6 ± 2.0Aa
		14		3.8 ± 2.9Ba	3.4 ± 2.8Ba	3.5 ± 2.7Ba

Least squares mean values with different lower case letters in the same row are different ($P < 0.05$).
Least squares mean values with different upper case letters in the same column and within the same treatment are different ($P < 0.05$).