DISSERTATION

ESCHERICHIA COLI O157:H7 ATTACHMENT, SURVIVAL, GROWTH, AND CONTROL ON STAINLESS STEEL AND IN MEAT BRINING SOLUTIONS AND ITS THERMAL INACTIVATION IN NON-INTACT BEEF

Submitted by

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ABSTRACT OF DISSERTATION

ESCHERICHIA COLI O157:H7 ATTACHMENT, SURVIVAL, GROWTH, AND CONTROL ON STAINLESS STEEL AND IN MEAT BRINING SOLUTIONS AND ITS THERMAL INACTIVATION IN NON-INTACT BEEF

The three studies described in this dissertation examined (1) the effects of the initial level of environmental hydration, nutrient density, natural flora, and fluid flow on the strength and attachment of *Escherichia coli* O157:H7 on stainless steel and the subsequent inactivation of pathogen within a biofilm through sanitizer exposure; (2) *E. coli* O157:H7 survival in model meat brines containing antimicrobials, natural flora, and meat residues; and (3) the extent of thermal inactivation of the pathogen at different depths of non-intact steaks under-cooked by pan-broiling and roasting to a 60°C geometric center temperature from either the frozen or thawed state.

In the first study, *E. coli* O157:H7, transferred to stainless steel and allowed to dry, exhibited stronger strength of attachment to the surface than pathogen cells that were kept hydrated, indicating that the pathogen's optimal physical removal would occur before the surface dried. Once on stainless steel, *E. coli* O157:H7 cells remained viable and were able to proliferate in a reduced nutrient substrate; however, when competing flora from beef were present, growth was limited and the pathogen demonstrated an increased strength of attachment. Given this, the inactivation of the pathogen within a biofilm through the use of sanitizers was studied. Peroxyacetic acid/octanoic acid, a

quaternary ammonium compound, and sodium hypochlorite based sanitizers were effective in inactivating greater than 99.99% (4 log CFU/cm^2) *E. coli* O157:H7 cells in biofilms with and without competing flora by 10 min of exposure; however, peroxyacetic acid/octanoic acid mixture was the most effective as it gave similar reductions after 1 min of exposure.

In the second study, the brining ingredients salt and phosphate in combination were sufficient to inhibit *E. coli* O157:H7 and natural flora growth for up to 48 h in meat brining solutions at 4 and 15°C with and without meat residues; however, both the pathogen and natural flora remained viable with the potential to spread contamination through recirculated brine solutions. Consequently, antimicrobials were studied for the inactivation of the pathogen in the brine solutions. Cetylpyridinium chloride and sodium metasilicate caused immediate and sustained cell reductions to below the detection limit (1.3 log CFU/ml) and were thus identified as to best reduce the probability of product cross-contamination through pathogen transfer in contaminated re-circulated brine injection.

In the third and final study, the 5-day storage of non-intact steaks either at 4 or -20°C did not alter their initial contamination level. Pathogen populations were similar in non-intact steaks in the frozen state or allowed to thaw at 4 or 25°C to simulate thawing in the refrigerator or on the kitchen countertop, respectively, suggesting that the microbial safety of non-intact beef was not decreased by the method of thawing as long as steaks were cooked immediately after the desired thawing temperature was reached. Steak size and cooking method affected the thermal inactivation of *E. coli* O157:H7 as thicker steaks and steaks cooked by pan-broiling had greater pathogen inactivation than thinner

and those cooked by roasting, respectively, even though non-intact steaks were cooked to the same internal geometric center temperature of 60°C. These data suggest that steak size and cooking method should be included in lethality guidelines that are designed to ensure the safe preparation of beef products, and when cooking non-intact steaks, pan-broiling is preferred to roasting to ensure their safe consumption.

Overall, the results of the studies reported in this dissertation may be useful in the development of cleaning and sanitization programs and improving brining recipes to control *E. coli* O157:H7 in brining solutions. Further, these data may be usefull in developing lethality guidelines for the safe preparation and consumption of non-intact beef products.

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CHAPTER 1

INTRODUCTION

Escherichia coli O157:H7 was identified as the causative agent of foodborne illness associated with consumption of undercooked hamburgers in 1982 (Riley et al., 1983). Between 1982 and 1993, 13 outbreaks of *E. coli* O157:H7 infection occurred in the United States with the transmission source, again, identified as hamburger or ground beef (Griffin, 1995). In 1993, a foodborne outbreak of *E. coli* O157:H7 associated with consumption of undercooked hamburgers in the Northwestern United States was responsible for 501 illnesses including 3 deaths (Bell et al., 1994). The 1993 outbreak of *E. coli* O157:H7 is generally recognized as the bellwether case that brought the pathogen to the forefront as a food safety concern. Currently, *E. coli* O157:H7 is estimated to be responsible for approximately 62,000 illnesses that result in 1,800 hospitalizations and 52 deaths per year in the United States (Mead et al., 1999). *E. coli* O157:H7 illnesses cost an estimated $460 million in the United States in 2008 (Frenzen et al., 2005; USDA-ERS, 2007).

In 1994, *E. coli* O157:H7 was declared an adulterant of ground beef by the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS); therefore, any ground beef product identified to contain *E. coli* O157:H7 may not enter commerce or is subject to a voluntary recall (USDA-FSIS, 1994). Following this

declaration, a microbiological testing plan was implemented in which the USDA-FSIS would test raw ground beef (25 g samples) for *E. coli* O157:H7 (USDA-FSIS, 1994). To increase testing sensitivity, in 1998 the USDA-FSIS increased the tested sample size from 25 to 325 g based on data showing that *E. coli* O157:H7 was infectious at low doses (USDA-FSIS, 1998). In 1999, the range of products considered adulterated by *E. coli* O157:H7 was broadened to include all non-intact beef products (USDA-FSIS, 1999). Non-intact beef products are defined by the USDA-FSIS as "ground beef; beef that has been mechanically tenderized by needling, cubing, frenching, or pounding devices; and beef that has been reconstituted into formed entrees" (USDA-FSIS, 1999). However, in 1999, USDA-FSIS noted in the Federal Register, "At this time, FSIS is not expanding its sampling program to include all types of non-intact beef products or intact cuts of muscle that are to be further processed into non-intact products prior to distribution" (USDA-FSIS, 1999). Even though the USDA-FSIS had declared non-intact beef cuts containing *E. coli* O157:H7 adulterated, at that time, the agency would not sample and test non-intact products for the presence of the pathogen.

After outbreaks of *E. coli* O157:H7 foodborne illness associated with mechanically tenderized beef or moisture enhanced beef products in 2000 (2 cases; USDA-FSIS, 2005), 2003 (12 cases; Laine et al., 2005) and 2004 (4 cases; USDA-FSIS, 2005), the USDA-FSIS published a Notice in the Federal Register stating that all establishments producing mechanically tenderized (including moisture-enhanced beef product) should address biological hazards, specifically *E. coli* O157:H7, at the next annual reassessment of their HACCP plans (USDA-FSIS, 2005). In addition, in 2004, the USDA-FSIS amended its testing policy to include "Intact raw beef products

contaminated with *E. coli* O157:H7 that are intended to be processed into non-intact products are also adulterated" (USDA-FSIS, 2004). In 2009, all (even those produced at retail) beef manufactured trimmings and raw beef patty components that were to be ground or reformed were to be tested for the presence of *E. coli* O157:H7 and therefore considered adulterated upon detection of the pathogen (USDA-FSIS, 2009b). Currently, the USDA-FSIS tests non-intact beef products and intact raw beef products intended for the production of non-intact beef products for *E. coli* O157:H7 (USDA-FSIS, 2004). If a sample tests positive the batch or lot sampled is considered adulterated and must be removed from retail distribution or further processed to destroy the pathogen. Positive results are reported by the USDA-FSIS and give a prevalence rate of contaminated product. For example, in 2008, 0.81% (23 of 2,836) of intact raw beef products destined for the production of non-intact beef and 0.47% (54 of 11,607) of ground beef (non-intact beef) samples tested positive for *E. coli* O157:H7 (USDA-FSIS, 2009). Even with these low positive rates, the ultimate goal is to have a zero prevalence of pathogen and no product identified to contain *E. coli* O157:H7 in commerce. Given this, all possible sources of contamination through pathogen transfer should be analyzed and identified including processing surfaces, brining solutions and mechanical tenderizazation equipment used in the production of moisture-enhanced products (Sofos et al., 2008).

In 2002, the USDA-FSIS conducted a risk assessment regarding *E. coli* O157:H7 in non-intact beef to help support policy decision making (USDA FSIS, 2002b). Specifically, the agency wanted an evaluation of "the risk of illness per serving from intact versus non-intact (e.g., tenderized) beef steaks and roasts prepared using traditional cooking practices (grilling, broiling, and frying)" (USDA FSIS, 2002b). As a result, a

research need identified by the risk assessment was the "Survival of *E. coli* O157:H7 in core beef samples following cooking to specified temperatures, including data on the survival of *E. coli* O157:H7 in beef roasts compared to beef steaks" (USDA FSIS, 2002a).

The studies of this dissertation were designed to help fill the void of knowledge concerning *E. coli* O157:H7 attachment and growth on processing surfaces and its inactivation through sanitizer challenge, survival and inactivation by antimicrobials in brining solutions, and survival in non-intact meat products under-cooked to sub-lethal temperatures using different cooking methods. Specifically, the objectives of the studies were as follows:

1. Evaluate the effect of the level of environmental hydration, nutrient density, natural flora, and fluid flow on the strength of attachment and growth of *E. coli* O157:H7 on stainless steel, and the subsequent inactivation of the pathogen in biofilms through sanitizer exposure.
2. Evaluate survival of *E. coli* O157:H7 in model fresh (no natural flora or meat residues) or re-circulated (natural flora and meat residues) meat brines containing antimicrobials at temperatures associated with processing and storage of non-intact meat products.
3. Evaluate the thermal inactivation of *E. coli* O157:H7 at different depths of non-intact steaks cooked from frozen or after thawing at 4 or 25°C to a subleathal temperature using two different cooking methods.

CHAPTER 2

LITERATURE REVIEW

Escherichia coli O157:H7

Pathogenic *E. coli* O157:H7 contain a pathogenicity island within their genome called the locus of enterocyte effacement (LEE), which harbors the genes responsible for bacteria and intestinal host cell interactions (Beauchamp and Sofos, 2010; Elliott et al., 1998; Jerse et al., 1990) The LEE encodes a specialized type III secretion system *E. coli* O157:H7 uses for initial attachment and subsequent transfer of bacterial effector proteins into intestinal enterocytes (Knutton et al., 1998). Specifically, the initial binding of *E. coli* O157:H7 to intestinal epithelial cells is mediated through the EspA protein of the type III secretion system (Ebel et al., 1988). After initial attachment, *E. coli* O157:H7, inserts the bacterial intimin receptor Tir into the host cell wall (Hayward et al., 2006). The bacterial adhesion protein intimin (EaeA) binds Tir and initiates the degradation of the host cell microvilli and the reorganization of host cell cytoskeleton to form attaching and effacing (A/E) lesions (Torres et al., 2005). The formation of A/E lesions, which are characteristics of *E. coli* O157:H7 infection, promote further *E. coli* O157:H7 colonization of the intestine (Dean-Nystrom et al., 1998; Donnenberg et al., 1993; Donnenberg et al., 1998; Hayward et al., 2006). Once colonized in the intestine, *E. coli* O157:H7 infection causes abdominal cramps and non-bloody diarrhea. The more sever

effects of *E. coli* O157:H7 infection are linked to the cell's ability to produce shiga toxins which induce bloody diarrhea, hemorrhagic colitis, or hemolytic uremic syndrome (HUS) and, in the worst case scenario, death (Kapar and O'Brien, 1998; Griffin, 1995; Mead and Griffin, 1998; Tarr et al., 2005). The shiga toxins I and II are proteins encoded by the shiga toxin genes *stxI* and *stxII*; respectively (Beutin, 2006; Ritchie et al., 2003). These toxins target cells containing the Gb3 receptor, which are found extensively in the colon, vascular epithelium, and kidneys (Goldwater, 2007). Once the shiga toxins bind to their receptor they are internalized and cause cellular death by inhibiting cellular protein production (Goldwater, 2007). These toxins induce hemorrhaging in the intestine, which is the cause of bloody diarrhea, and can also migrate to the kidneys where they cause a localized thrombotic microangiopathy (TMA) that manifests itself as hemolytic uremic syndrome (Goldwater, 2007; Nangaku et al., 2007).

E. coli O157:H7 has been isolated from the feces or gastrointestinal tract of cattle among other species (Drasar and Barrow, 1985; Hancock et al., 1998; Heuvelink et al., 1999). The pathogen has been transferred, via fecal matter, and isolated throughout the beef production chain from hides, carcasses, sub-primals, and further processed meat products (Bonardi et al., 2001; Brichta-Harhay et al., 2007; Erickson and Doyle, 2007; Hussein, 2007; Hussein and Bollinger, 2005). Prevalence rates of *E. coli* O157 are in the range of 0.3 to 27.2% in feces (Alonso et al., 2007; Islam et al., 2008; Madden et al., 2007; Reinstein et al., 2009; Woerner et al., 2006), 3.8 to 73.8% on hides (Alonso et al., 2007; Arthur et al., 2007; Barkocy-Gallegher et al., 2004; Bosilevac et al., 2009; Duffy et al., 2006; Renter et al., 2008; Woerner et al., 2006), 0.0 to 40.8% on carcasses (Akkaya et al., 2008; Alonso et al., 2007; Barkocy-Gallagher et al., 2004; Bosilevac et al., 2009;

Duffy et al., 2006; Rahimi et al., 2008; Rigobelo et al., 2008; Varela-Hernandez et al., 2007; Woerner et al., 2006), 0.0 to 1.4% on post-evisceration carcasses (Rigobelo et al., 2008; Woerner et al., 2006), 0.083 to 0.2% on sub-primals (Heller et al., 2007; Kennedy et al., 2006), 1.1 to 36.0% on various retail cuts (Hussein, 2007), and 0.1 to 54.2% in ground beef (Hussein, 2007).

Biofilms

In their natural state bacteria prefer to grow in complex communities called biofilms, as opposed to in suspension (Costerton, 2007; Costerton et al., 1994, 1995). The biofilm state increases the survival of bacteria in harsh environmental conditions and its formation is promoted by limited nutrients in the environment (Costerton et al., 1994, 1995; Chmielewski and Frank, 2003). Initially, more energy is used in forming a biofilm than used growing in a suspension; however, overtime bacteria in a biofilm use fewer nutrients than their planktonic counterparts (Costerton, 2007). For biofilm formation, there must first be attachment of the bacteria to a surface, which can be broken into two stages: reversible and irreversible adhesion (Mittelman, 1998). Reversible adhesion is based on van der Waals forces, electric charge, hydrophobicity, and free energy of the surface; whereas, irreversible adhesion is the dipole-dipole interaction, hydrogen, ionic, and covalent bonding between the bacteria and the surface (Chmielewski and Frank, 2003). Once attached to a surface, bacteria start to grow and divide to form secondary structures and protective barriers (Costerton, 2007). As the bacteria in the biofilm grow, there becomes a dense inner core of slow growing cells that form open channels for fluid passage (Costerton, 2007). The fluid channels allow for the import and transfer of

nutrients and the export of waste materials (Costerton, 2007). The dense inner core is surrounded by faster growing cells that form vertical columns of bacteria called pillars, stalks, or mushrooms (Costerton, 2007). As the bacteria divide within the vertical columns they may slough off the biofilm to reattach elsewhere and start new biofilms (Costerton, 2007). Microscopically, as is the case with *E. coli* O157:H7, biofilms may be identified by an external layer of exopolysaccharide (EPS) (Costerton, 2007). The EPS layer protects members of the biofilm from chemical and physical inactivation and removal (Costerton, 2007).

Environmental conditions, such as temperature and hydration, have been shown to affect the optimal binding of pathogens to processing surfaces (Kusumaningrum et al., 2003; Norwood and Gilmore, 2001; Skandamis et al., 2009). Once established in the processing environment, pathogen containing biofilms present an opportunity for cross-contamination of product (Chmielewski and Frank, 2003). In fact, Rodriguez and McLandsborough (2007) observed that biofilms transferred more bacterial contamination to product than attached cells (single cells not in a biofilm). Also in the Rodriquez and McLandsborough (2007) study, when comparing surfaces, the potential for cross-contamination from stainless steel was greater than that of polyethylene surfaces as more cells were transferred from stainless steel than polyethylene.

Moisture enhanced non-intact beef

A 2003 survey of 200 meat processors conducted by the North American Meat Processors and Food Safety Systems on behalf of the National Cattlemen's Beef Association and the Cattlemen's Beef Board found that 24% of the surveyed processors used meat enhancement solutions, which equaled to over 90,000,000 kg of beef being

moisture-enhanced (NCBA, 2006). In 2007, the USDA-FSIS indicated that 372 establishments enhanced beef product either by tumbling, massaging or injection with solutions, producing a monthly volume of 25,000,000 kg (290,000,000 kg yearly; USDA-FSIS, 2008). The usual range of moisture enhancement of meat products is 7 to 18% (Baublits et al., 2005, 2006; Miller, 1998).

Moisture enhancement through brine injection increases the value of whole muscle beef cuts by increasing their tenderness, juiciness, and flavor and decreasing their fat content(Grobbel, 2008; Sofos et al., 2008; Vote et al., 2000). Two common ingredients in brining solutions, salt (NaCl) and phosphate, contribute to the increase in perceived tenderness and juiciness of moisture enhanced products (Hedrick et al., 1994). The sodium ion (Na^+) of sodium chloride binds to the peptide side chains of the amino acids that comprise the proteins of muscle and displaces a divalent cation thus changing the isoelectric point of the meat and freeing up one positively charged reactive group to bind the polar (charged) molecule of water (Hedrick et al., 1994). The predominant phosphate used in brining solutions is the sodium or potassium salt of tripolyphosphate (Miller, 1998), but other forms of phosphates can be added alone or in combination including sodium pyrophosphate, tetrasodium pyrophosphate, and sodium hexametoposphate (Baublits et al., 2005, 2006; Miller, 1998). Phosphate raises the pH of the muscle which increases the net charge of the muscle and its ability to bind water (Hedrick et al., 1994). Phosphate itself also binds water inside the meat (Hedrick et al., 1994). By increasing the amount of bound water, NaCl and phosphate increase the juiciness and perceived tenderness and decrease the cooking loss of moisture-enhanced products (Hedrick et al., 1994). Other ingredients may be added to the brining solutions

that increase the color, juiciness, tenderness, and flavor, including beef broth (Lawrence et al., 2004), bromelain (Kolle et al., 2004), calcium, potassium, or sodium salts of the organic acids acetate, citrate, and lactate (Barmphalia et al., 2004; Davis et al., 2004; Kim et al., 2006; Knock et al., 2006; Lawrence et al., 2004; Mukherjee et al., 2008, 2009; Stephens et al., 2006); calcium chloride (Boles and Swan, 1997), glucose (Boles and Swan, 1997; Kim et al., 2006), kappa carrageenan (Lawrence et al., 2004), rosemary extract (Lawrence et al., 2004; Sanchez-Escalante et al., 2003; Seyfret et al., 2005), and soy protein isolates (Xiong, 2005).

During moisture enhancement, brine that does not stay within the injected meat is collected, re-circulated, and re-injected into other whole muscle cuts (Greer et al., 2004 Pfeiffer et al., 2008; Wicklund et al., 2005). Even though the quality of the meat has been increased, brine injection may introduce pathogens to the interior of the moisture enhanced product (Bohaychuk and Greer, 2003). There are two potential mechanisms by which bacteria can be introduced into meat during moisture enhancement. First, the injection needles may come in contact with the bacteria on the surface of the meat and push the cells into the meat. Wicklund et al. (2007) injected surface contaminated meat with fresh brines (brines that did not contain natural microbiological contamination) and was able to isolate the inoculum within the meat tissues. Secondly, the brine may become contaminated with bacteria and the bacteria may be injected into the meat through the brine (Greer et al., 2004; Heller et al., 2007). Greer et al. (2004) showed the accumulation of bacteria in brining solutions and detected Enterobacteriaceae (2.5 log CFU/ml) in pork injection brines after 2.5 h of enhancement. In another study, Heller et al. (2007) demonstrated a larger prevalence of *E. coli* O157:H7 after tenderization in cuts of meat

that were moisture enhanced with re-circulated brine (73 of 76) as opposed to blade tenderized (3 of 76).

Bacteria that are injected into the meat within the brine are found in and between meat fibers (Gill et al., 2008). Interestingly, the brine and not the bacteria were able to disperse into the muscle indicating that the meat filtered the brine of the bacteria (Gill et al., 2008). There are conflicting reports concerning the correlation with bacterial concentration of brining solutions and the amount of bacteria transferred into meat through brine injection. In one study, the amounts of bacteria transferred into deep tissues during moisture enhancement were reflective of the bacterial concentration of the brines (Gill and McGinnis, 2004). However, others have only detected *E. coli* in steaks that were moisture enhanced with a brining solution containing 6 log CFU/ml of *E. coli* as opposed to 3 log CFU/ml (Wicklund et al., 2006), suggesting that there is a minimum concentration of bacteria that is necessary in brines for transfer into meat during moisture enhancement.

Limited studies have been performed concerning the control of bacterial populations in brining solutions. However, antimicrobial compounds have been incorporated into processed meat and meat brining formulations and tested for their ability to control bacterial growth on or within the final meat product, suggesting their potential use in bring solutions, and include cetylpyridinium chloride (Breen et al., 1997; Dupard et al., 2006; Jimenez-Villarreal et al., 2003; Kim and Slavik, 1996; Li et al., 1996; Ozdemir et al., 2006; Singh et al., 2005), hops beta acids (Shen and Sofos, 2008; Shen et al., 2009), nisin (Cabo et al., 2009; Penna and Moraes, 2002), pediocin (Cabo et al., 2009; Chen et al., 2004), and sodium metasilicate (Pohlman et al., 2009; Quilo et al.,

2006). The organic acids acetate, citrate, and lactate also can be added due to their antimicrobial activity, in addition to increasing tenderness (Barmpalia et al., 2004; Mukherjee et al., 2008, 2009). Cetylpyridinium chloride is a cationic quaternary ammonium compound that is approved by the USDA-FSIS "to treat the surface of raw poultry carcasses prior to or after chilling" (USDA-FSIS, 2009a), and was shown to be effective in reducing *Salmonella* Typhimurium, coliforms including *E. coli*, and aerobic bacteria on beef before grinding (Pohlman et al., 2002). Sodium metasilicate is an alkaline antimicrobial that is approved by the USDA-FSIS as a "component of marinades used for raw meat and poultry products" (USDA-FSIS, 2009a) and has been shown to be able to reduce *E. coli* O157:H7 populations in an aqueous solution (Weber et al., 2004). Nisin and pediocin are bacteriocins (antimicrobial peptides) that have better efficacy against Gram-positive bacteria as opposed to Gram-negative; however, their efficacy against Gram-negative bacteria may be improved in the presence chelators, such as ethylenediaminetetraacetic acid (EDTA), that permiabilize the bacterial outer membrane (Cutter and Siragusa, 1995; Galvez et al., 2007; Thomas and Delves-Broughton, 2005). Of the two bacteriocins, only nisin is classified at this time as a safe and suitable ingredient that may be used in the production of meat, poultry and egg products (USDA-FSIS, 2009a); however, pediocin has been shown to be able to reduce *Listeria monocytogenes* on meat products (Chen et al., 2004). Hops beta acids have been shown to reduce *L. monocytogenes* populations in a broth medium (Shen et al., 2008) and on frankfurters (Shen et al., 2009) and is approved for use on ready-to-eat meats and in salad dressings (USDA-FSIS, 2009a).

Wicklund et al. (2005) showed that heating (50 to 70°C) brining solutions containing antimicrobials is an effective way of decreasing their bacterial populations. More importantly, a greater reduction in *E. coli* K 12 populations was observed when sodium lactate (33% in brine for a target of 3% in injected product) and sodium diacetate (2.75% in brine for a target of 2.5% in injected product) were added to brining solutions heated to 50°C. These data illustrate that heat and antimicrobial agents are effective in controlling bacterial populations in brining solutions and may have synergistic effects in the reduction of bacterial populations in brining solutions. Given the scarcity of data, there is a need to evaluate the effect of antimicrobial compounds in brining solutions as a means of reducing bacterial populations.

Translocation of *E. coli* O157:H7 by blade tenderization

If the external surface of the sub-primals contains pathogens, such as E. *coli* O157:H7, they may be transloctated to the interior of blade tenderized beef cuts (BIFSCO, 2006; Gill et al., 2005b; Hajmeer et al., 2000; Krizner, 1999; Lambert et al., 2001; Luchansky et al., 2008a,b; Phebus et al., 2000; Thippareddi, 2000). Sporing (1999) observed that 3-4% of surface contamination was transferred to the interior of the meat during mechanical tenderization. Recently, Luchansky et al. (2009) demonstrated that blade tenderization transferred 40% of surface inoculated *E. coli* O157:H7 into beef sub-primals. While it has been shown that blade tenderization transfers *E. coli* O157:H7 primarily to the topmost 1 cm, it also transfers pathogen cells to deep tissues (Luchansky and Call, 2008; Luchansky et al., 2008, 2009). Sporing (1999) reported that the amount of *E. coli* O157:H7 contamination transferred to the interior of meat decreased with increasing depth of

penetration; specifically, there was a 0.5 log decrease in *E. coli* O157:H7 populations for each 1 cm penetration by tenderizing blades or needles. This was later supported by Luchansky et al. (2009) who observed the percent translocation of bacteria to be 33.00, 3.04, 3.09, 0.44, 0.10, and 0.82% at 0-1, 1-2, 2-3, 3-4, 4-6, and 6-8 cm of depths, respectively.

There are conflicting reports, even by the same authors, that describe the amount of bacteria transferred into the interior of the muscle in relation to the concentrations of bacteria on the meat surface during mechanical tenderization. It has been reported that the amount of internalized bacteria due to blade tenderization was not proportional to the amount of surface bacteria (Gill and McGinnis, 2004; Gill et al., 2005a,b). However, others have observed the opposite and concluded that the translocation of bacteria from the surface to the interior of beef cuts during blade tenderization was dependent on the amount of bacteria on the surface of the muscle (Gill and McGinnis, 2005; Hajmeer et al., 2000; Luchansky et al. 2008b; Sporing, 1999). This discrepancy in the data suggests that there are other factors contributing to the amount of bacteria transferred into the interior of the meat due to mechanical tenderization other that the surface population of the bacteria. Factors identified in the aforementioned papers that may influence the amount of transferred bacteria during mechanical tenderization, but not necessarily studied, include product storage time and temperature post inoculation before blade tenderization, shape and penetration depth of the tenderization blades or needles, size of sub-primal being blade tenderized, and the species and growth of the bacteria on meat that is tenderized (Gill and McGinnis, 2005; Gill et al., 2005a,b; Hajmeer et al., 2000; Luchansky et al. 2008b; Sporing, 1999).

Another factor affecting the amount of bacteria transferred into meat during mechanical blade tenderization is the number of passes through the tenderizing machine or the number of blade or needle penetrations into the meat cut (Sofos and Geornaras, 2010). Gill and McGinnis (2005) and Sporing (1999) observed that multiple passes of a blade tenderizer over product did not significantly transfer more surface contamination than a single pass. Gill and McGinnis (2005) reasoned that there is no observed increase in the amount of bacteria transferred with multiple tenderizing events because the data (CFU/g) were log transformed and therefore any additive increase in bacterial numbers would be hard to detect after log transformation.

Bacteria can also penetrate into meat between muscle fibers to depths of 0.2-0.4 cm during storage through the production of proteolytic enzymes, which are produced in the logarithmic phase of bacterial growth (Gill and Penny, 1977, Gill et al., 2008). The depth of penetration is dependent on the organism, its ability to produce proteolytic enzymes, and the type and structure of the meat (Elmossalami and Wassef, 1971; Gill and Penney, 1977, 1982; Maxcy, 1981; Sikes and Maxcy, 1980; Thomas, 1966). Mechanical tenderization may exacerbate the cell's natural tendency to penetrate into meat and allow access into muscle fibers damaged by tenderization (Gill et al., 2008). The pathogen *Salmonella* Newport at low levels (2 log CFU/cm^2) was able to transfer from the surface of muscle into the interior without assistance from a physical process (e.g., blade tenderization); however, roasts that were blade tenderized contained 1 log greater level of *Salmonella* Newport than untenderized roasts (Johnston et al., 1978). Thus, mechanical tenderization may transfer more bacteria into the tenderized product than the bacteria acting alone.

Thermal inactivation of *E. coli* O157:H7 in non-intact beef

Blade tenderizing and moisture enhancing through brine injection of beef sub-primals before portioning increases the perceived tenderness of beef, thus, increasing the value of tougher muscles within the carcass (Sofos et al., 2008). After mechanical tenderization, whole muscle cuts are portioned into non-intact steaks. These non-intact steaks may appear intact and consequently be cooked to rare and medium-rare temperatures which are insufficient to inactivate internalized pathogens (Sofos et al., 2008). Between 25 and 53% of consumers prefer to consume their meat cooked to an endpoint internal temperature of medium-rare (63°C) or rare (60°C) (Cox et al., 1997; Schmidt et al., 2002; USDA-FSIS, 2002a).

Lethality of pathogens during and after cooking is a function of cooking temperature and method, meat thickness, and the amount and location of the bacteria within the meat (Shen et al., 2010; USDA-FSIS, 2002b). For non-intact steaks that were broiled in a conventional oven, an internal temperature of 60°C was sufficient to inactivate surface inoculated *E. coli* O157:H7 on intact and non-intact steaks and internalized *E. coli* O157:H7 transferred during mechanical tenderization (Phebus et al., 2000; Sporing, 1999). Based on these data the National Advisory Committee on Microbiological Criteria for Foods (NACMCF, 2002) recommended cooking non-intact blade tenderized steaks to 60°C by broiling, suggesting that mechanically tenderized steaks broiled to 60°C present no greater risk for foodborne illness than intact steaks cooked to the same endpoint temperature (Sporing, 1999; USDA-FSIS, 2002a). However, Gill et al. (2009) concluded that the recommended internal cooking parameters (63°C throughout the steak) as set forth by the United States Food and Drug Administration are

insufficient to inactivate *E. coli* on the surface or in non-intact steaks when cooked by pan-broiling (USFDA, 2005). As an alternative the authors recommended that cooking by pan-broiling to 65°C would be sufficient to assure the safe consumption of non-intact beef steaks. This recommendation was based on an observed reduction of ≥7 log CFU/ steak of internalized *E. coli* O157:H7 in 3 cm steaks cooked to an internal temperature of 65°C. Others have also proposed that cooking by grilling, roasting, or pan-broiling to 65°C is recommended as an endpoint cooking temperature to assure the safe consumption of non-intact products (Luchansky and Call, 2008a; NACMCF, 2002; Phebus et al., 2000; USDA, 2002a). Gill and McGinnis (2004) further proposed "cooking to a medium rare condition may be adequate for assuring the microbiological safety of mechanically tenderized beef that is prepared without excessive contamination of deep tissues." In the same study, Gill and McGinnis (2004) observed that the temperatures of steaks continued to rise from 63 to 68°C even after they were removed from the cooking apparatus when the geometric temperature reached 61°C. In later work, Gill et al. (2005a) explained that moisture-enhanced pork removed from the heat source at an internal temperature of 61°C can be microbiologically safe for consumption most likely due to the increase in internal temperature (64.8°C) observed after the meat was removed from the heating source, which indicates that the increase in temperature during hold time post cooking may affect bacterial populations. In general, it has been observed that the thicker the non-intact steak the greater the pathogen reduction during cooking (Shen et al., 2010; Sporing, 1999; Thippareddi et al., 2000). Interestingly, Shen et al. (2010) observed that greater reductions were observed during cooking in surface inoculated then blade tenderized steaks (Sporing, 1999) than restructured non-intact steaks with inocula evenly distributed

throughout and concluded that the amount of thermal destruction of *E. coli* O157:H7 was dependent on location of the inoculum.

Cold storage of meat containing *E. coli* O157:H7 may place a cold stress on the pathogen (Jones et al., 1987) and thus alter the thermal inactivation of pathogen during cooking (Jackson et al., 1996). The *E. coli* cold stress response includes the induction of cold shock proteins (VanBogelen and Neidhardt, 1990) that are involved in transcription and translation (Jones et al., 1987) and thought to overcome the partial block in protein synthesis that is observed at low temperatures (Jones and Inouye, 1994). Also, in response to a cold stimulus, bacteria will increase the proportion of unsaturated fatty acids in their cell membrane (Beals, 2004; Berry and Foegeding, 1997); which may possibly affect the thermal tolerance of bacteria during cooking. Specifically for *E. coli* O157:H7, in broth a cold stress increased the pathogen's susceptibility to heat inactivation (Leenanon and Drake, 2001; Semanchek and Golden, 1998; Stringer et al., 2000). In ground beef there are conflicting reports regarding the effect of cold stress on the thermal inactivation of *E. coli* O157:H7 (Jackson et al., 1995; Juneja et al., 1997; Sage and Ingham, 1998). Jackson et al. (1996) observed that *E. coli* O157:H7 was more heat resistant in ground beef patties cooked from the frozen state (-18°C) than those cooked from refrigerated storage (3°C). Also, Jackson et al. (1996) observed that thawing (holding patties at 21 or 30°C prior to cooking) increased the pathogen's heat sensitivity. Conversely, Juneja et al. (1997) observed similar thermal inactivation of *E. coli* O157:H7 in refrigerated and frozen ground beef samples after cooking, and Sage and Ingham (1998) observed that "no thawing method had consistently and significantly greater lethality". Juneja et al. (1997) further explained that the observed difference between

their and data presented by Jackson et al. (1996) may be attributed to differences in sample size, heating rates, and storage time between the two papers.

Ingredients in the meat may also affect the thermal inactivation of pathogens during cooking. Cooking a 2.54 thick pork steak to an internal temperature of 71.1°C resulted in a 4 log CFU/g reduction in internalized *Salmonella* Typhimurium Thippareddi, 2000). However, steaks with a spice rub needed to be cooked to 76.6°C to achieve the same reduction indicating that the spice rub may exert a protective effect on the pathogen (Thippareddi, 2000). The addition of salt to ground beef also afforded *L. monocytogenes* protection from heat (Mackey, 1990). Heat protection of pathogens by spices or salt may be due to the lowering of the water activity of the beef by the salt.

Protective enclaves/structures could form on or in steaks as they cook providing a protective environment and shielding the pathogen from the heat of cooking (Thippareddi et al., 2000; USDA-FSIS, 2002a; USDA-FSIS, 2002b). Gill et al. (2008) found that non-intact beef retained as much as 1000-fold more bacteria if it were tenderized and then injected with contaminated brine as opposed to injection followed by blade tenderization. Inside meat, *E. coli* O157:H7 may be shielded from the heat of cooking (USDA-FSIS, 2002b) or the brining ingredients may protect pathogens from thermal inactivation (Sofos et al., 2008). Also during cooking an evaporative phenomenon may occur that would allow for the survival of surface bacteria (Thippareddi et al., 2000). The heat may dry the surface and lower the water activity of the surface of the steak. Also, as the surface dries the moisture would evaporate thus lowering the immediate temperature to inactivate bacteria. The combination of low water activity and evaporative heat loss may allow for the survival of pathogens during cooking. Juices flowing to the surface would also

contribute to the evaporative protection of bacteria (Blankenship, 1978; Blankenship and Craven, 1982; Blankenship et al., 1980; Harrison and Carpenter, 1989).

CHAPTER 3

Attachment and growth of *Escherichia coli* O157:H7 on stainless steel as affected by initial level of hydration, nutrient density, ground beef residues, natural flora, and fluid flow and the pathogen's inactivation with sanitizers

ABSTRACT

This study evaluated the effect of initial level of hydration, nutrient density, ground beef residues, natural flora, and fluid flow on the strength of attachment and growth of *E. coli* O157:H7 on stainless steel and the pathogen's subsequent inactivation through sanitizer exposure. Stainless steel coupons (2 × 5 cm) inoculated with *E. coli* O157:H7 (5 strains; 3 log CFU/cm^2) by applying the inoculum directly to the surface followed by drying or by submersion in inoculum were incubated (4 days) at 15°C in 10-fold diluted tryptic soy broth (TSB), water or beef-grinder washings (filter-sterilized or unsterilized) either statically or under agitation (60 rpm), to simulate a fluid flow. Four day old biofilms were either dried for 30 min or kept hydrated and challenged with peroxyacetic acid/octanoic acid mixture (PAOA), quaternary ammonium compound (QUAT), or sodium hypochlorite (SH). Loosely and firmly attached *E. coli* O157:H7 and total bacterial cells were removed with vortexing, enumerated, and used to calculate strength of attachment (Sr). *E. coli* O157:H7 allowed to air dry onto stainless steel coupons had a stronger (P<0.05) Sr than cells kept hydrated during inoculation. Attached *E. coli* O157:H7 counts

were <2 log CFU/cm^2 in unsterilized beef-grinder washings (P<0.05), while total microbial flora increased from initial levels of 3.0 log CFU/cm^2 to 6.1 ± 0.4 log CFU/cm^2. Planktonic *E. coli* O157:H7 counts in unsterilized beef-grinder washings were lower (P<0.05) than counts in TSB and sterilized washings, indicating that the competitive flora decreased attached populations of *E. coli* O157:H7. The higher Sr (0.32 ± 0.09) of *E. coli* O157:H7 in beef-grinder washings suggested that the natural flora or beef residues may aid in pathogen attachment to stainless steel. Exposure to PAOA caused reductions in *E. coli* O157:H7 counts to below the detection level (0.6 log CFU/ cm^2) within 1 min, while the other sanitizers caused such reductions at 10 min. These data suggest that, on stainless steel surfaces present in beef processing facilities, although natural flora and meat residues may provide an environment for stronger cell attachment, natural flora may outgrow *E. coli* O157:H7. PAOA may be preferred to QUAT and SH in controlling *E. coli* O157:H7 populations on contaminated stainless steel surfaces.

INTRODUCTION

Previous research has shown that *E. coli* O157:H7 can attach, grow, and form biofilms on stainless steel surfaces (Rivas et al., 2007a,b; Ryu et al., 2004a,b). For the initial attachment, *E. coli* O157:H7 uses several intrinsic factors (surface's electro negativity, hydrophobicity and energy) for attachment and biofilm formation (Torres et al., 2005). These factors are a part of a redundant attachment system that *E. coli* O157:H7 uses where no one factor in isolation can be used to explain pathogen attachment to surfaces

(Van Houdt and Michiels, 2005); this demonstrates the importance of biofilm formation to the survival of bacteria (Van Houdt and Michiels, 2005).

Gram-negative motile bacteria, such as *E. coli* O157:H7, exhibited a greater attachment rate to meat than gram-positive bacteria (Butler et al., 1979). Cabedo et al. (1997) showed that *E. coli* O157:H7 could attach to beef if the two come in contact. Therefore, once established in the processing environment, pathogen-containing biofilms constitute a source for cross-contamination of product (Bradford et al., 1997; Chmielewski and Frank, 2003; Donlan, 2002; Farrell et al., 1998; Gilbert et. al, 2003; Norwood and Gilmore, 2001). In fact, Rodriguez and McLandsborough (2007) observed that biofilms transferred more bacterial contamination to product than attached cells not in a biofilm. They also observed, when comparing surfaces, that the potential for cross-contamination from stainless steel was greater than that from polyethylene surfaces as more cells were transferred from stainless steel than polyethylene (Rodriguez and McLandsborough, 2007). Cold temperatures, such as those found in the processing environment, affect the attachment to and subsequent growth of pathogen cells on surfaces. *E. coli* K-12 exhibited a cold stress response at 23°C that activated transcription of several biofilm genes (White-Zigler et al., 2008) indicating a potential physiological response to attach and form biofilm at this temperature. Drying of surfaces containing pathogen cells within a biofilm reduced initial population numbers; however, viable pathogen cells were still recovered from the dried surfaces for up to 4 days and 12 h post drying for *L. monocytogenes* and *E. coli* O157:H7, respectively (Kusumaningrum et al., 2003; Skandamis et al., 2009). Drying also injures pathogens and subsequently makes pathogen recovery more difficult using selective media (Bremer et al., 2001). Thus,

pathogens may remain viable on dried surfaces and serve as sources for the contamination of food while being undetectable by standard detection procedures. It has been proposed that drying weakens cell-to-cell and cell-to-surface interactions (Rodriquez and McLandsborough, 2007). Given this, mature dry biofilms might be more likely to be transferred to and contaminate product. Also, flowing fluids and the ionic strength of the fluids have been shown to affect the amount of attachment of organic materials to surfaces (Sharma et al., 1992), indicating that a fluid flow may be able to wash surfaces and inhibit pathogen reattachment.

In the Draft Compliance Guideline for Sampling Beef Trimming for *E. coli* O157:H7, the United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS) has recommended testing surfaces for the presence of *E. coli* O157:H7 (USDA-FSIS, 2008). Therefore, data concerning the attachment and growth of *E. coli* O157:H7 on surfaces found in the plant environment are needed. The purpose of this study was to evaluate the effect of the level of environmental hydration, nutrient density, natural flora, and fluid flow on the strength of attachment and growth of *E. coli* O157:H7 on stainless steel, and the subsequent inactivation of the pathogen in a biofilm through sanitizer exposure.

MATERIALS AND METHODS

E. coli O157:H7 strain selection and inoculum preparation

A 5-strain *E. coli* O157:H7 composite was used in all experiments of this study; however, not all strains used were the same in every experiment. In order to examine *E. coli*

O157:H7 growth at different levels of initial environmental hydration, strains ATCC 43889, ATCC 43894, ATCC 51658, ATCC 43895/ISEHGFP (Noah et al., 2005), and EO-139 (venison jerky isolate kindly provided by M. P. Doyle, University of Georgia, Griffin) were used based on their previous use in our laboratory (Stopforth et al., 2003b, Yoon et al., 2005). To examine the effects of natural flora on the attachment and growth of *E. coli* O157:H7 on stainless steel coupons and inactivation of the pathogen by sanitizers, rifampicin-resistant *E. coli* O157:H7 strains were used to allow for selective isolation of the inoculum in the presence of natural contaminating flora. Thus, rifampicin-resistant isolates of strains ATCC 51657, ATCC 51658, ATCC 43895/ISEHGFP, F284, and F469 were selected based on the methods of Kaspar and Tamplin (1993). Strains F284 and F469 were isolated in a previous study by Woerner et al. (2006). Strains ATCC 51657, F284, and F469 replaced strains ATCC 43889, ATCC 43894, and EO-139 in experiments containing natural flora because these strains demonstrated an increased ability to form biofilms in a concurrent study performed in our laboratory (unpublished data).

All strains were activated (35°C, 24 ± 2 h) in 10 ml tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) from stock cultures stored at -70ºC and sub-cultured (35°C, 24 ± 2 h) in 10 ml TSB. Rifampicin (Sigma, St. Louis, MO) was added (100 µg/ml) to TSB (TSB + rif) for all rifampicin-resistant strains. An aliquot (0.1 ml) of each strain was then combined into 10 ml of sterile distilled water. The combined strains were further diluted 1:100 and 1:10,000 in sterile distilled water for use as inoculum. Sterile distilled water was used to negate the effects that salts or proteins from the substrate

could have on the electrostatic or hydrophobic interactions between bacteria and stainless steel (Dewanti and Wong, 1995; Sharma et al., 1992).

Preparation of substrates

To simulate fluids with similar nutrient and natural flora content found in beef processing plants, waste fluids from water-rinsing of a grinder immediately after grinding beef were collected (Meat Science Laboratory, Colorado State University). Specifically, following grinding of approximately 100 kg of beef (80:20 lean to fat content) and before cleaning, 4 L of sterile distilled water was deposited into the grinder. The grinder was powered on and the non-sterile beef-grinder washings (NSBGW) was collected in a sterile carboy as it came out of the grinding port. Large particles of organic material were removed by passing NSBGW through four layers of cheese-cloth. Afterwards, the NSBGW was distributed into sterile 500 ml bottles and stored at -20 °C and used within 30 days. Before use, NSBGW was thawed for 24 h at 4 °C.

To test and differentiate the effect of natural flora in NSBGW on the attachment and growth of *E. coli* O157:H7 on stainless steel surfaces, 500 ml of NSBGW was sterilized by sequentially passing through 25 μm (#4 Qualitative Whatman Filter, Whatman Inc., Florham Park, NJ) and 1.2 μm (GF/C Whatman Filter) pore size exclusion filters, and a final 0.22 μm (Stericup; Millipore, Bedford, MA) filter (Hood and Zottola, 1997). A 1-ml sample of the filtrate was incubated (24 h, 35 °C) in TSB (9 ml) to confirm sterility, sterile filtrates were designated filter-sterilized beef-grinder washing (FSBGW). The general growth medium TSB was used as a control substrate after a 10-fold dilution with sterile distilled water (dTSB). The dilution was done because studies have found

poor initial attachment of *E. coli* O157:H7 on stainless steel in TSB, while attachment was induced in restricted nutrient environments (Dewanti and Wong, 1995; Ryu et al., 2004a). In order to estimate the nutrient content of the beef-grinder washings, growth of *E. coli* O157:H7 in FSBGW and dTSB was compared (Hood and Zottola, 1997; Ryu et al., 2004a,b).

Preparation of stainless steel coupons

Previous studies have concluded that abraded or rough surfaces allow attachment of greater amounts of bacteria than smooth surfaces (Foschino et al., 2003; Stone and Zottola, 1985, Wang et al., 2009). Therefore, to maximize *E. coli* O157:H7 attachment, stainless steel coupons (2 × 5 × 0.08 cm, type 304, #2b finish) abraded with glass beads in previous studies were used (Stopforth et al., 2002, 2003a,b). Coupons were cleaned and sterilized before use in the study, as follows: manual scrubbing with a Scotch-Brite® Heavy Duty sponge (3M, St. Paul, MN) in warm soapy water, rinsing five times with sterile distilled water, soaking in acetone (1 h; Fisher Scientific, Fair Lawn, NJ), soaking in warm soapy water (30 min), rinsing five times with sterile distilled water, and autoclaving (Stopforth et al., 2002, 2003a,b).

Inoculation and storage of stainless steel coupons

To examine the attachment and growth of *E. coli* O157:H7 from either a dry or hydrated initial level of inoculation and to select a method of coupon inoculation for subsequent studies, stainless steel coupons were inoculated using two methods: (i) placing 0.1 ml of the 1:100 diluted composite inoculum directly on the coupon, spreading it over the entire

surface with a sterile bent glass rod, and allowing to dry at room temperature in a biological safety cabinet (Class II Type A/B3, NuAire Inc., Fernbrook, IL) or (ii) submerging the coupon in 40 ml of the 1:10,000 diluted *E. coli* O157:H7 inoculum inside a 100 × 15 mm petri dish (Fisher Scientific, Houston, TX). In both cases, cells were allowed to attach to the surface for 10 min. Inoculation methods i and ii represented initial environmental hydration levels of dry and wet, respectively. Since the initial state of pathogen cells (planktonic or sessile) has been shown to affect subsequent attachment to stainless steel (Rivas et al., 2007a,b), the unattached cells were removed so that all subsequent bacterial populations originated from the attached cell phenotype. Unattached cells were rinsed off of the coupons by placing each coupon in a 100 × 15 mm petri dish (Fisher Scientific) containing 10 ml sterile-distilled water, removing the coupon from the water and placing in another 10 ml sterile-distilled water. Both methods i and ii were designed to obtain similar levels of viable pathogen cells before incubation in substrate. After inoculation, coupons were placed in 150 × 15 mm petri dishes containing 45 ml of dTSB. Flowing fluids have been shown to affect the amount of attachment of organic materials to surfaces (Sharma et al., 1992). Therefore, to test the effects of fluid flow on bacterial attachment and growth, coupons were incubated at 15ºC for up to 4 days either statically or with agitation (60 rpm, Lab Rotator, Lab-Line Instruments, Inc., Melrose Park, IL) to simulate fluid flow during storage. For the experiment that examined the effects of natural flora present in NSBGW and fluid flow on the attachment and growth of rifampicin-resistant *E. coli* O157:H7, the stainless steel coupons were inoculated using method ii. Coupons were placed in 150 × 15 mm petri dishes containing 45 ml of dTSB,

NSBGW or FSBGW and incubated at 15°C for up to 4 days either statically or with agitation.

Sanitizer challenge

The efficacy of sanitizers against rifampicin-resistant *E. coli* O157:H7 in dry or hydrated mature biofilms was examined by inoculating stainless steel coupons using method ii, and incubating in dTSB or NSBGW. On day-4, coupons were dried (30 min, 25°C) or directly placed in water or the manufacturer's recommended concentrations of peroxyacetic acid (114 ppm)/octanoic acid (89 ppm) mixture (PAOA; 0.26%; Vortexx, Ecolab Inc., St. Paul, MN), quaternary ammonium compound (QUAT; 300 ppm; Oasis®146, Eco Lab Inc.), or sodium hypochlorite (SH; 200 ppm; XY-12®, Eco Lab Inc.) for either 1 or 10 min (Rossoni and Gaylarde, 2000; Sharma and Anand, 2002) and sampled for viable total bacterial and pathogen population.

Microbial analysis

At each sampling interval one coupon from each treatment was rinsed twice, by submerging it in 10 ml of sterile distilled water to remove unattached cells, and then it was placed in an 85 ml centrifuge tube (Oakridge, Nalgene, Nalge Nunc International, Rochester, NY) containing 40 ml of maximum recovery diluent (MRD; 0.1% peptone and 0.85% NaCl). Tubes were vortexed at a low setting (1,000 rpm; Digital Vortex Mixer, Fisher Scientific) for 30 s to remove loosely attached cells. Coupons were then removed, placed in a new 85 ml centrifuge tube containing 40 ml of MRD plus 10 glass beads (4 mm; Kimble Chase - Kimble Glass, Vineland, NJ), and were vortexed at a high

setting (3,000 rpm) for 2 min to remove firmly attached cells (Skandamis et al., 2009; Stopforth et al. 2002, 2003a,b). When coupons were exposed to sanitizers, Dey-Engley Neutralizing Broth (Difco) instead of MRD was added to the tubes to inactivate any residual sanitizing solutions on the coupons (Minei et al., 2008).

Loosely and firmly attached *E. coli* O157:H7 populations originating from strains ATCC 43889, ATCC 43894, ATCC 51658, ATCC 43895/ISEHGFP, and EO-139 were enumerated by plating appropriately diluted (0.1% buffered peptone water, Difco) samples onto tryptic soy agar (TSA; Accumedia, Lansing, MI). Loosely and firmly attached rifampicin-resistant *E. coli* O157:H7 strains ATCC 51657, ATCC 51658, ATCC 43895/ISEHGFP, F284, and F469 and total bacterial populations were enumerated by plating diluted samples on TSA + rifampicin and TSA, respectively. Similarly, planktonic populations of *E. coli* O157:H7 in each of the substrates were enumerated. All plating media were incubated at 35ºC for 48 h. To calculate the total bacterial and *E. coli* O157:H7 populations on a stainless steel coupon the following equation was used:

Total cell count CFU/cm^2 = Loosely attached (CFU/cm^2) + Firmly attached (CFU/cm^2).

To calculate strength of bacterial attachment (Sr) to stainless steel coupons the following equation was used (Firstenberg-Eden et al., 1978):

Sr = log [total cell counts CFU/cm^2] – log [loosely attached cell counts CFU/cm^2]

A Sr value further from 0 would indicate a more firmly attached population of bacteria.

Statistical Analysis

Experiments were repeated two times with three coupons analyzed per repeat at each sampling time. A complete factorial design, including interactions, of the main factors for each experiment ([inoculation method: pipette inoculums onto coupon, submerging coupon in inoculum] × [fluid flow: fluid flow, static] × [sampling time: 0, 0.25, 0.5, 1, 2 and 4 days]), ([substrate: dTSB, NSBGW, FSBGW] × [fluid flow: fluid flow, static] × [sampling time: 0, 1, 2, 4 days]), or ([substrate: dTSB, NSBGW] × [pre sanitizer challenge level of biofilm hydration: dried 30 min, not dried] × [sanitizer challenge: water, PAOA, QUAT, SH] × [sanitizer exposure time: 1 min, 10 min]) was analyzed in the PROC Mixed procedure of SAS (v9.2, SAS Institute, Cary, NC) for the dependant variables *E. coli* O157:H7 and total bacterial Sr, attached cell count, and planktonic cell counts. Mean separation of significant interactions of main effects were performed using an *F*-protected pairwise t-test comparisons of least squares means with significance set at the $P<0.05$.

RESULTS AND DISCUSSION

Attachment and growth of *E. coli* O157:H7 from initially dried or hydrated inocula

E. coli O157:H7 allowed to air dry onto stainless steel had a higher ($P<0.05$) initial (day-0) strength of attachment (0.18) than cells kept hydrated throughout inoculation (0.04; Figure 3.1). However, this difference disappeared ($P\geq0.05$) after the dried cells were re-hydrated in substrate during incubation (Figure 3.1). Bradford et al. (1997) observed that less *E. coli* O157:H7 was transferred to beef from dried than from wet pathogen inoculated surfaces, also indicating a stronger attachment of *E. coli* O157:H7 to dry than

wet surfaces. This observation may be pathogen specific as Rodriguez and McLandsborough (2007) observed that drying weakened *Listeria monocytogenes* cell-to-cell and cell-to-surface interactions, thus decreasing the pathogen's strength of attachment to stainless steel and increasing the ease of removal. As for *E. coli* O157:H7, the data suggest that optimal physical removal would occur before the stainless steel surfaces were allowed to dry and that cells remaining on stainless steel surfaces after insufficient cleaning would attach more firmly as the surface dried during storage.

Attached cells of *E. coli* O157:H7 on stainless steel coupons increased from initial levels of approximately 3 log CFU/cm^2 on day-0 to between 6.7 and 7.3 log CFU/cm^2 on day-4 (Figure 3.2), which is similar to findings by Ryu et al. (2004a) who observed maximal growth of 7 to 8 log CFU/cm^2. Interestingly, Dewanti and Wong (1995) and Hood and Zottola (1997) observed maximal growth of approximately 5 log CFU/cm^2 on stainless steel between 22-25ºC with agitation (70-90 rpm) or without, respectively. In this study *E. coli* O157:H7 was stored at 15ºC and reached maximal attached populations by day-4; however, Dewanti and Wong (1995), Hood and Zottola (1997), and Ryu et al. (2004a) observed maximal populations at approximately 2-days of storage at higher temperatures (22-25, 23, and 37ºC, respectively). Attached *E. coli* O157:H7 was able to grow on stainless steel surfaces, indicating that stainless steel surfaces that have been exposed to *E. coli* O157:H7 may be a source of contamination if the pathogen is not removed during cleaning and the surfaces are present in environments of temperatures that allow its growth.

E. coli O157:H7 and natural flora attachment and growth on stainless steel

The initial experiment that characterized the Sr and growth of *E. coli* O157:H7 on stainless steel was performed in dTSB and contained no meat residues or natural flora from NSBGW. The goal of the subsequent experiment was to introduce and examine the effect of natural flora and meat residues originating from NSBGW on the Sr and growth of *E. coli* O157:H7 on stainless steel. Therefore, rifampicin-resistant strains of *E. coli* O157:H7 were used for the selective differentiation of the inoculua from natural flora introduced by the meat residues.

The Sr of *E. coli* O157:H7 in NSBGW increased from 0.05 ± 0.09 on day-0 to 0.32 ± 0.09 on day-2 then down to 0.22 ± 0.09 on day-4 (Figure 3.3). No significant increases in Sr were observed in dTSB or FSBGW during the study (Figure 3.3). Under a fluid flow, *E. coli* O157:H7 Sr values on coupons incubated in NSBGW were higher (0.23 ± 0.07) than those incubated statically (0.13 ± 0.07). In dTSB and FSBGW, no increases ($P \geq 0.05$) in *E. coli* O157:H7 Sr from levels observed during static incubation due to fluid flow was observed (0.03±0.07 vs. 0.06±0.07 and 0.05±0.07 vs. 0.05±0.07, respectively). Only NSBGW contained meat residues and natural flora, thus suggesting that these two factors contributed to the increasing attachment of pathogens to surfaces during storage and in a fluid flow (Bremer et al., 2001; Dickson and Koohmaraie, 1989; McEldowney and Fletcher, 1987; Rivas et al., 2007a,b).

Attached cells of *E. coli* O157:H7 on coupons increased, from initial levels of approximately 3 log CFU/cm^2 on day-0 to ≥ 5.6 log CFU/cm^2 on day-4, in substrates containing no natural flora (dTSB and FSBGW; Figure 3.4). This observed growth of *E. coli* O157:H7 on stainless steel was similar to previously published data (Hood and

Zottola, 1997; and Ryu et. al., 2004a). However, attached cells of *E. coli* O157:H7 in non-sterile NSBGW decreased from initial levels of 3.2 log CFU/cm^2 on day-0 to ≤ 1.8 log CFU/cm^2 on day-4 (Figure 3.4). Simultaneously, the competitive flora populations on stainless steel coupons incubated in NSBGW increased (P<0.05) from 3.3 ± 0.4 log CFU/cm^2 on day-0 to 6.1 ± 0.4 log CFU/cm^2 on day-4 (Figure 3.5). Previous studies in our laboratory (Samelis et al., 2001a, 2001b, 2002; Stopforth et. al., 2003a) and by others (Minei et al., 2008; Norwood and Gilmore, 2001) also have shown the inability of *E. coli* O157:H7 populations to increase in the presence of other competing flora. Additionally, Samelis et al. (2001a, 2002) indicated that natural flora in combination with a low storage temperature (10ºC) acted synergistically to inhibit *E. coli* O157:H7 growth. In combination, the aforementioned data indicate that the natural flora of the NSBGW had an inhibitory effect (P<0.05) on the growth and attachment of *E. coli* O157:H7 on stainless steel surfaces. Interestingly in this study, natural flora in the NSBGW was able to out-compete and dominate over *E. coli* O157:H7; however, the presence of natural flora and flowing fluids appeared to have increased the Sr of the pathogen on stainless steel surfaces.

In NSBGW, attached *E. coli* O157:H7 populations were 1.2 log CFU/cm^2 lower on coupons stored under a fluid flow than statically (Figure 3.4). Our results support the previously published data (Leclercq-Perlat and Lalande, 1994; Sharma et al., 1992; Wang et al., 2007) suggesting that flowing fluids may remove or inhibit reattachment of *E. coli* O157:H7 on stainless steel surfaces; however, under the conditions of this study, fluid flow was insufficient to entirely remove pathogen cells from stainless steel coupons in all samples.

Planktonic *E. coli* O157:H7 populations in NSBGW ranged from 2.7 ± 0.7 to 3.2 ± 0.3 log CFU/ml during the 4-day experiment (Figure 3.6). However, the total bacterial concentrations in NSBGW increased ($P<0.05$) from 4.4 ± 0.9 to greater than 8.5 log CFU/ml (Figure 3.7). As the experiment progressed, from day-0 to day-4, the relative pathogen populations in comparison to total bacterial populations decreased in NSBGW (Figures 3.6 and 3.7), thus decreasing the relative amount of pathogen cells available for reattachment and subsequent growth on stainless steel coupons which may have contributed to the decrease in the attached *E. coli* O157:H7 populations observed.

The substrates dTSB, FSBGW, and NSBGW had similar ($P\geq0.05$) pH values on day-0, but during incubation there was an increase ($P<0.05$) in the pH of all substrates. In this study, the difference in pH values of NSBGW (7.70 ± 0.32) and dTSB and FSBGW (6.62 ± 0.32 and 6.59 ± 0.32, respectively) was most pronounced on day-4 and the highest pH values were associated with the natural flora present in the NSBGW (Figure 3.8). Also, the differences in pH due to the presence and growth of natural flora may have been a contributing factor to the observed differences found in Sr and growth of *E. coli* O157:H7 between the substrates (Samelis et al., 2001a).

Effects of sanitizers on *E. coli* O157:H7 associated contamination

A sustained reduction (4.3 log CFU/cm^2) in attached *E. coli* O157:H7 populations in conjunction with natural flora from NSBGW on stainless steel coupons to below the detection limit was observed after 1 min of exposure to PAOA (Figure 3.9). Reductions in pathogen populations in a biofilm containing natural flora of 4.0 and 3.5 log CFU/cm^2 were observed on stainless steel coupons challenged with SH and QUAT for 1 min,

respectively, and to the detection level (0.6 log CFU/cm^2) by 10 min of sanitizer exposure (Figure 3.9). Similar to the reports of Stopforth et al. (2002, 2003b), in this study, peroxyacetic acid was more effective than QUAT and SH in reducing pathogen populations on stainless steel surfaces. However, Rossoni and Gaylarde (2000) and Somers and Wong (2004) observed that chlorine was more effective than peroxyacetic acid in reducing attached bacterial populations on stainless steel. Rossoni and Gaylarde (2000) challenged bacterial cells that were given only 1 h to attach to stainless steel whereas in this study mature (4-day old) biofilms were exposed to the sanitizers. It should also be noted that different sanitizer formulations were used in the two studies. Additionally, Somers and Wong (2004) challenged *L. monocytogenes* with PAOA and SH suggesting that the efficacy of each sanitizer may be pathogen specific. As for *E. coli* O157:H7, in this study, all three tested sanitizers were able to decrease pathogen populations on stainless steel by more than 99.9% (>3 log CFU/cm^2).

Previously air dried coupons had lower (P<0.05) *E. coli* O157:H7 populations (1.2 log CFU/cm2; Figure 3.10) than coupons that were kept hydrated before challenge with water containing no sanitizer (Figure 3.10). Other studies have shown that drying of biofilms decreased attached viable bacterial populations (Kusumaningrum et al., 2003; Skandamis et al., 2009; Somers and Wong, 2004). Also, greater reductions (P<0.05) in pathogen populations were observed on coupons that were first dried for 30 min compared to those that were kept hydrated before QUAT challenge (Figure 3.10). The efficacy of PAOA and SH was not increased by drying the stainless steel coupons containing bacteria. However, greater than 3 log CFU/cm^2 reductions were observed by all sanitizing treatments regardless of drying of the coupons before sanitizer challenge.

These data also suggest that during periods of inactivity, drying of stainless steel surfaces will reduce but not eliminate *E. coli* O157:H7, if present. Also, pre-operational application of PAOA, QUAT, or SH to dry rather than hydrated stainless steel was the best method for reducing *E. coli* O157:H7 populations.

All sanitizers decreased ($P<0.05$) total bacterial populations after 1 min of exposure by more than 3 log CFU/cm^2. However, they failed to reduce counts to below the detection limit (0.6 log CFU/cm^2), even after 10 min (Figure 3.11) indicating that the sanitizers were not able to penetrate throughout the biofilm and the biofilm provided a non-specific, physical barrier (Kim et al., 2007; Gilbert et al., 2003; Ryu and Beuchat, 2005; Somers and Wong, 2004; Taormina and Beauchat, 2002). Also, Sharma and Anand (2002) observed reductions between 1 and 2 log CFU/cm^2 of bacterial populations on stainless steel which were lower that the reductions observed in this study. The biofilm formed in this study was composed of the *E. coli* O157:H7 inoculum and natural flora from the NSBGW, while the biofilm used by Sharma and Anand (2002) was from pasteurization lines in a dairy plant. It has been observed that the efficacy of sanitizers is dependent on the composition of the surface and bacteria within the biofilm (Lalla and Dingle, 2004; Rossoni and Gaylarde, 2000) which may explain the differences in observed reductions between this study and the study of Sharma and Anand (2002), suggesting that sanitization programs should be designed specifically for each plant for maximum efficacy against its natural flora.

The average pH values of water, PAOA, QUAT and SH solutions were 7.66 ± 0.69, 3.49 ± 0.18, 6.71 ± 0.66, and 9.86 ± 0.24, respectively. It is interesting to note that the two most effective sanitizers were associated with the lowest and greatest pH values

(PAOA and SH; respectively) indicating a pH effect on *E. coli* O157:H7 inactivation. Samelis et al. (2002) found that acid tolerance of *E. coli* O157:H7 may be pH inducible and observed that previous acid adaptation of *E. coli* O157:H7 increased the pathogen's ability to survive when exposed to subsequent acid challenge. Conversely, the same study (Samelis et al., 2002) indicated greater reductions due to acid challenge in non-acid-adapted as compared to acid-adapted *E. coli* O157:H7 populations. In the present study the growth of natural flora was associated with an increase ($P<0.05$) in the pH of NSBGW (Figure 3.8) which may contribute to increasing *E. coli* O157:H7 susceptibility to PAOA challenge, since the susceptibility to acid (acid tolerance response) is pH dependant or inducible by alkaline conditions (Samelis et al., 2002).

CONCLUSIONS

The data suggest that under the conditions of these studies, *E. coli* O157:H7 allowed to dry maybe more difficult to remove from stainless steel surfaces since it exhibited a stronger strength of attachment on dried compared to hydrated surfaces. This observation supports the importance of cleaning stainless steel surfaces for optimal removal of contaminating pathogen cells before they are allowed to dry. There was sufficient nutrient content in water residues collected during washing of a meat grinder that allowed proliferation, to maximal levels by day-4, of *E. coli* O157:H7 on stainless steel. This underlines the need for thorough washing of surfaces to remove nutrients. However, growth of *E. coli* O157:H7 may be limited if there is competitive microflora present in the substrate. Even though the competitive microflora may inhibit growth of *E. coli* O157:H7, it allowed increased strength of *E. coli* O157:H7 attachment to stainless steel

potentially making removal more difficult. Flowing fluids reduced attachment and growth of *E. coli* O157:H7 on stainless steel surfaces but increased the strength of pathogen attachment to surfaces. A Peroxyacetic acid/octanoic acid mixture, quaternary ammonium compound, and sodium hypochlorite were effective in reducing pathogen populations to below the detection limit when applied to *E. coli* O157:H7 on dried surfaces. Therefore, these sanitizers may be appropriate for use pre-operationally, as an extra precaution, on surfaces that were previously cleaned and dried. However, POAO and SH gave greater reductions on hydrated surfaces containing natural flora and organic materials suggesting that these two sanitizers might be preferred for used on surfaces during operation or post-operational cleaning.

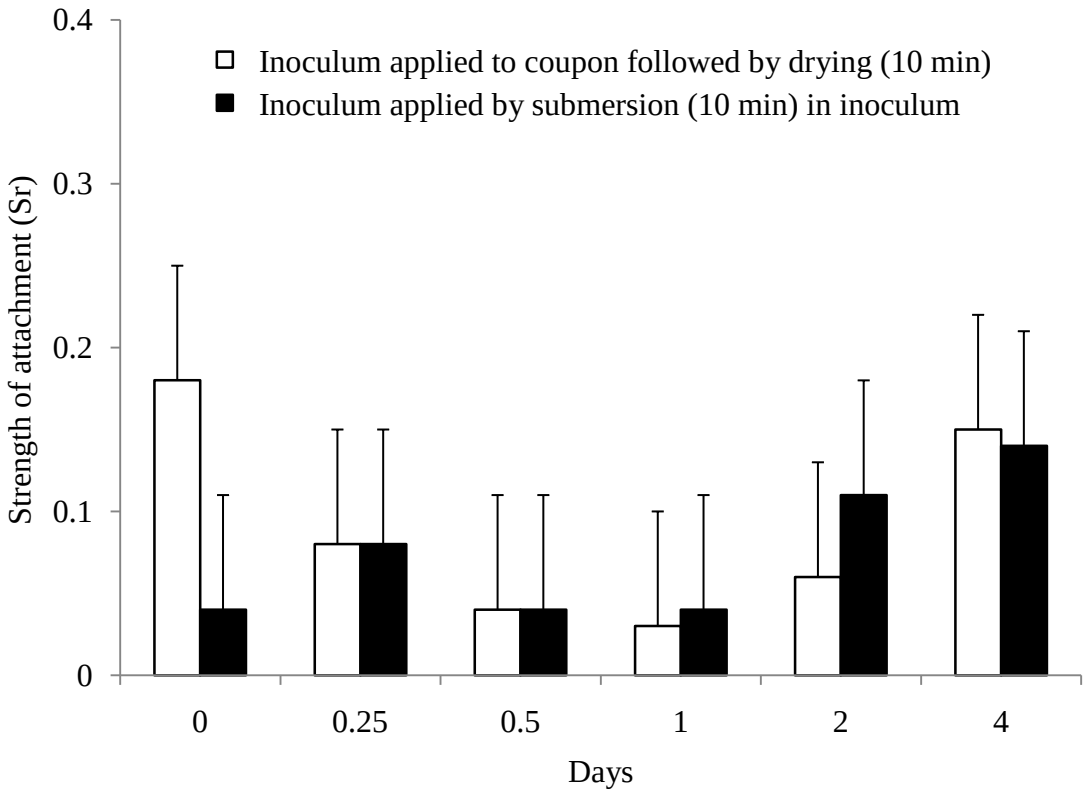

Figure 3.1 (Data in appendix Table 1). Effects of initial (day-0) hydration level during inoculation on mean strength of attachment (Sr ± standard deviation) of *Escherichia coli* O157:H7 on stainless steel coupons stored for 4 days at 15°C in 10-fold diluted tryptic soy broth.

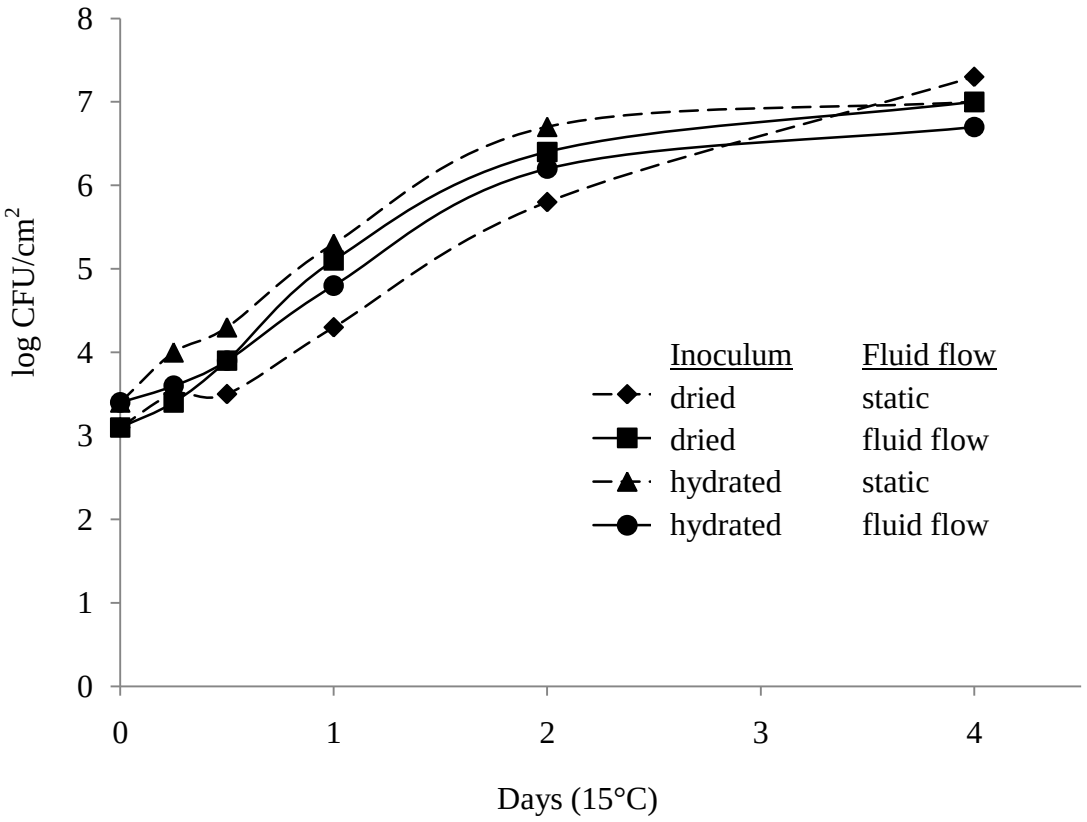

Figure 3.2 (Data in Appendix Table 1). Effect of hydration during inoculation and fluid flow during storage and on extent of attachment and growth of *Escherichia coli* O157:H7 (log CFU/cm^2 ± standard deviation). Stainless steel coupons were inoculated by placing the inoculum (0.1 ml) directly on the coupon and then spreading it over the entire surface with a sterile bent glass rod and dried for 10 min (dried) or submerged in a liquid suspension (40 ml) of inoculum for 10 min (hydrated) and stored for 4 days at 15°C under static and agitation conditions (60 rpm) to simulate a fluid flow in tryptic soy broth diluted 10-fold with sterile distilled water.

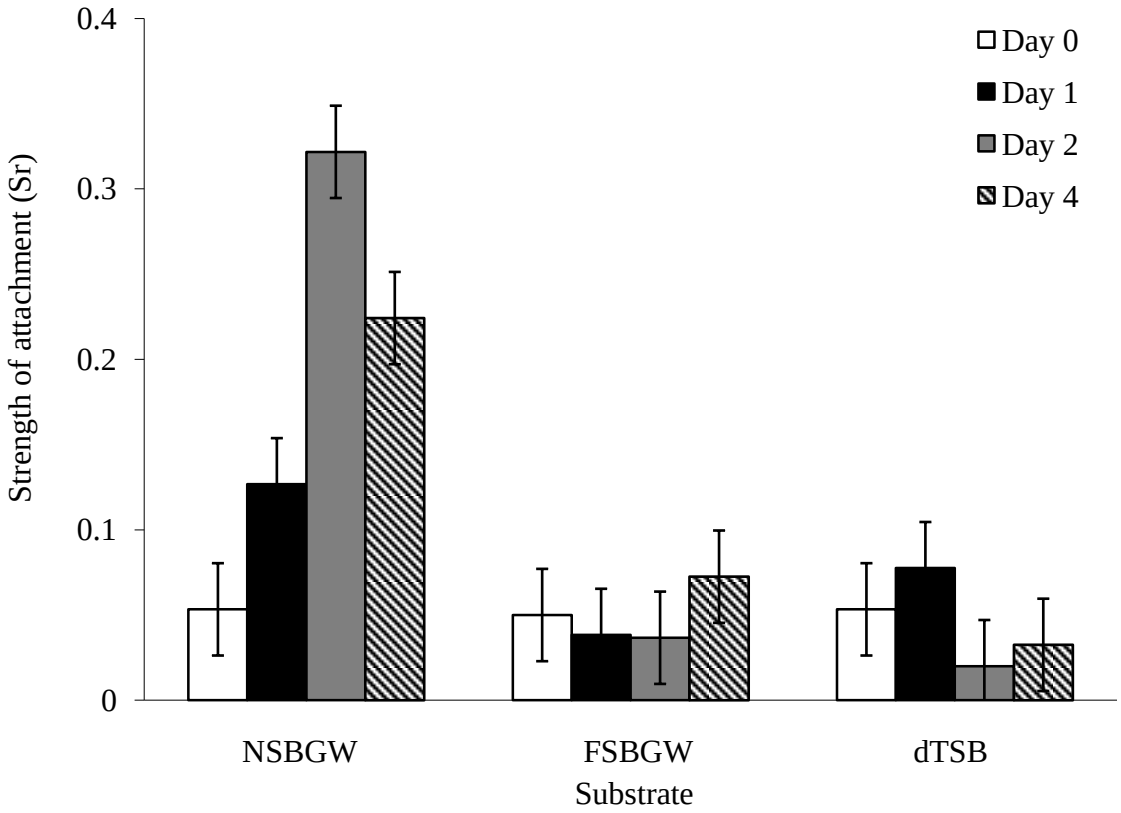

Figure 3.3 (Data in Appendix Table 2). Effect of non-sterile beef-grinder washings (NSBGW), filter-sterilized beef-grinder washings containing meat residues and natural flora, and 10-fold diluted tryptic soy broth on mean strength of attachment (Sr ± standard deviation) of *Escherichia coli* O157:H7 on stainless steel coupons stored for 4 days at 15°C

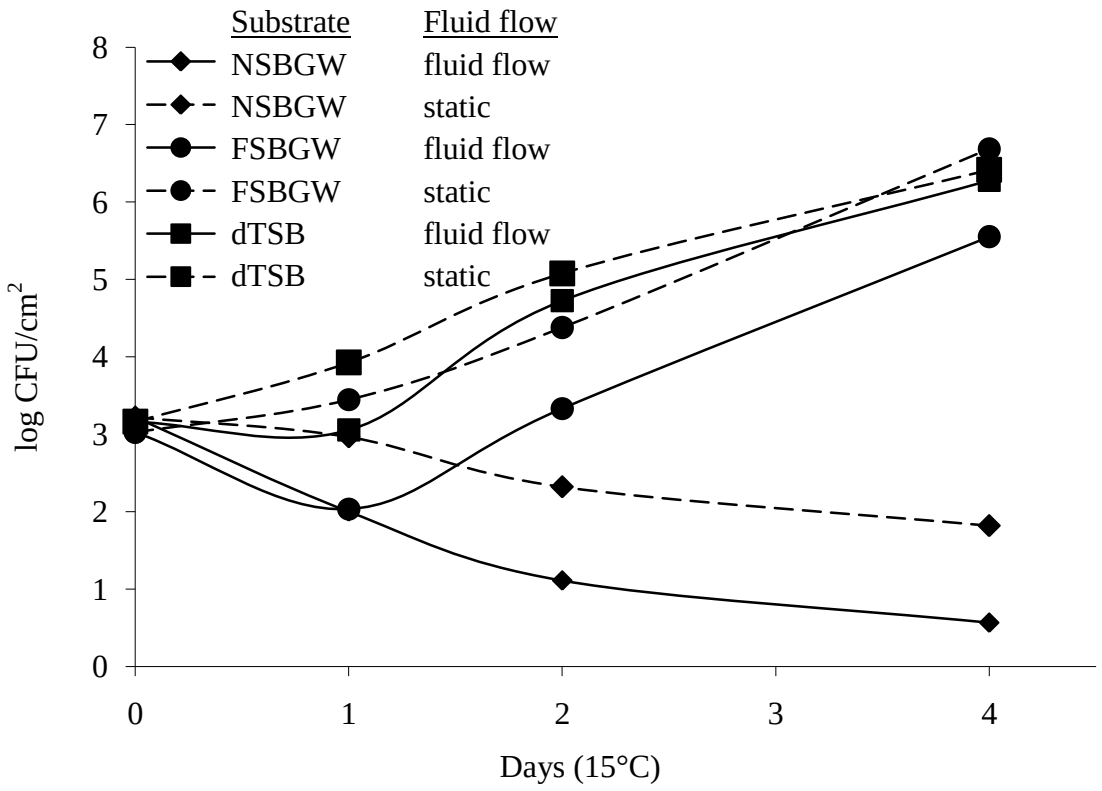

Figure 3.4 (Data in Appendix Table 2). Effect of substrate on the extent of attachment and growth of *Escherichia coli* O157:H7 (log CFU/cm^2 ± standard deviation) on stainless steel coupons incubated in non-sterile beef-grinder washings (NSBGW), filter-sterilized beef-grinder washings (FSBGW), and 10-fold diluted tryptic soy broth (dTSB) and stored for 4 days at 15°C under static or fluid flow (60 rpm) conditions.

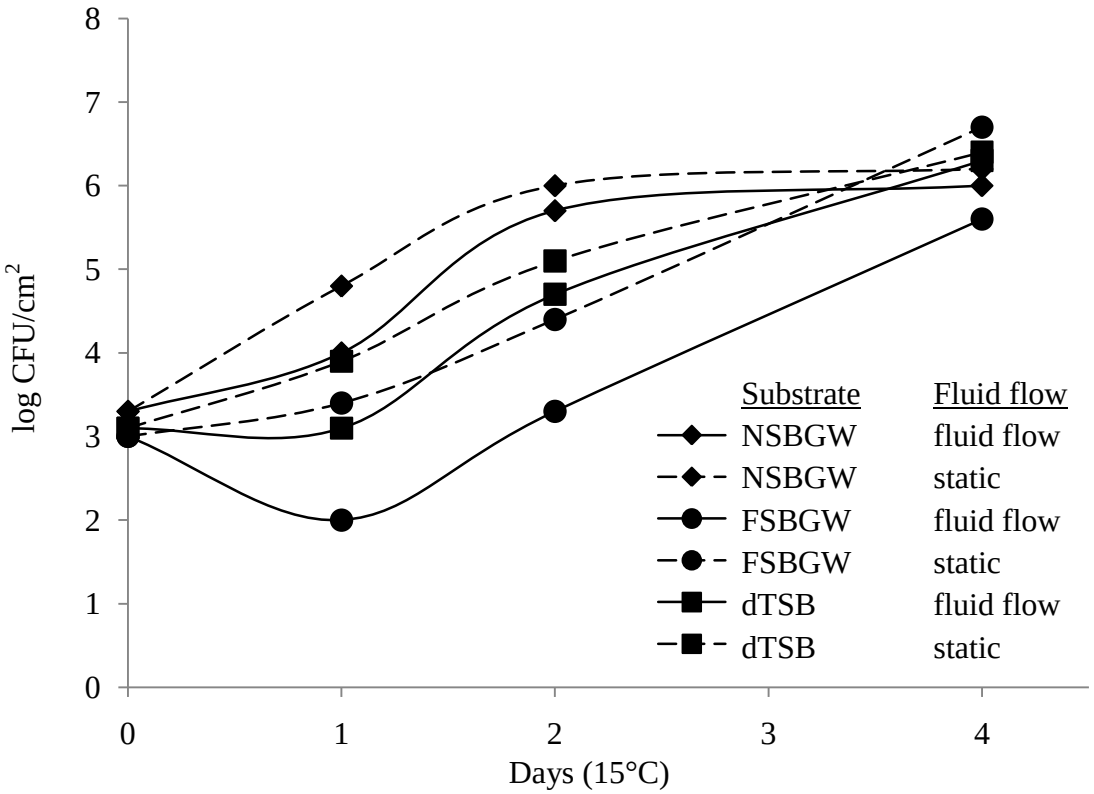

Figure 3.5 (Data in Appendix Table 3) . Effect of substrate during incubation on the extent of attached total bacterial counts (log CFU/cm^2 ± standard deviation) on stainless steel coupons inoculated (3 log CFU/cm^2) with *Escherichia coli* O157:H7and incubated in non-sterile beef-grinder washings (NSBGW), filter-sterilized beef-grinder washings (FSBGW), and 10-fold diluted tryptic soy broth (dTSB) and stored for 4 days at 15°C under static or fluid flow (60 rpm) conditions.

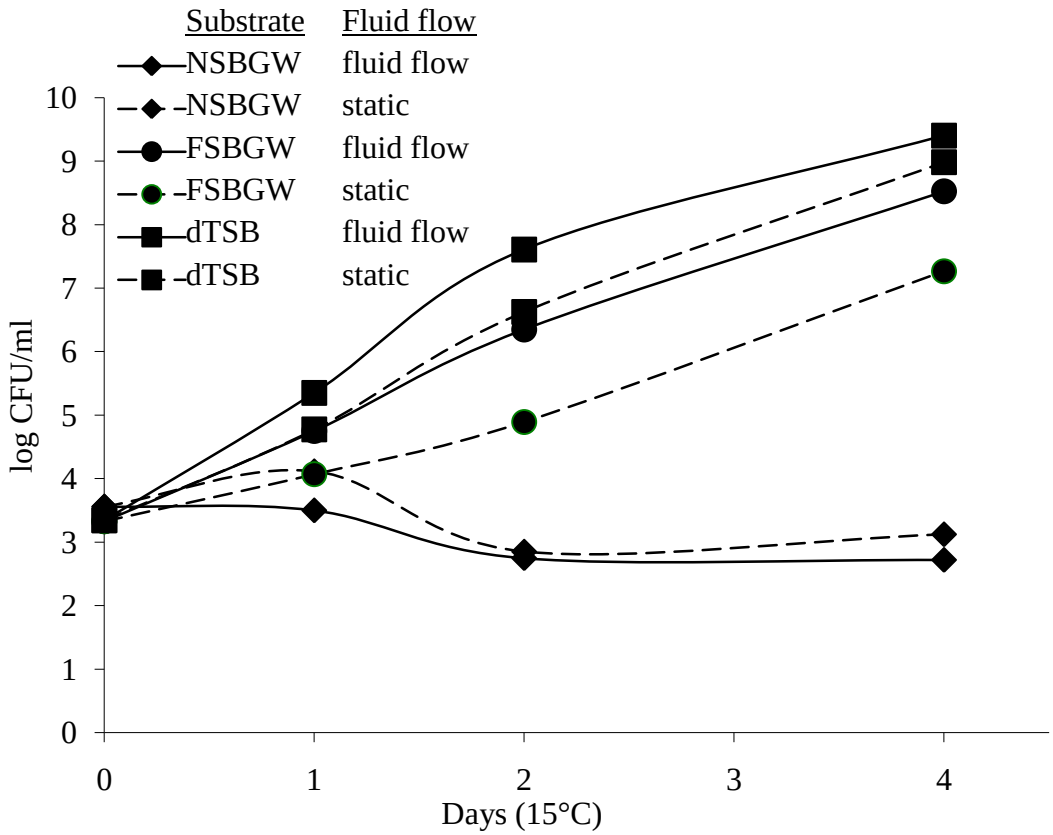

Figure 3.6 (Data in Appendix Table 4). Effects of substrates and fluid flow during storage on the extent of planktonic *Escherichia coli* O157:H7 (log CFU/ml ± standard deviation) recovered in non-sterile beef-grinder washings (NSBGW), filter-sterilized beef-grinder washings (FSBGW), and 10-fold diluted tryptic soy broth (dTSB) stored for 4 days at 15°C under static or fluid (60 rpm) flow conditions.

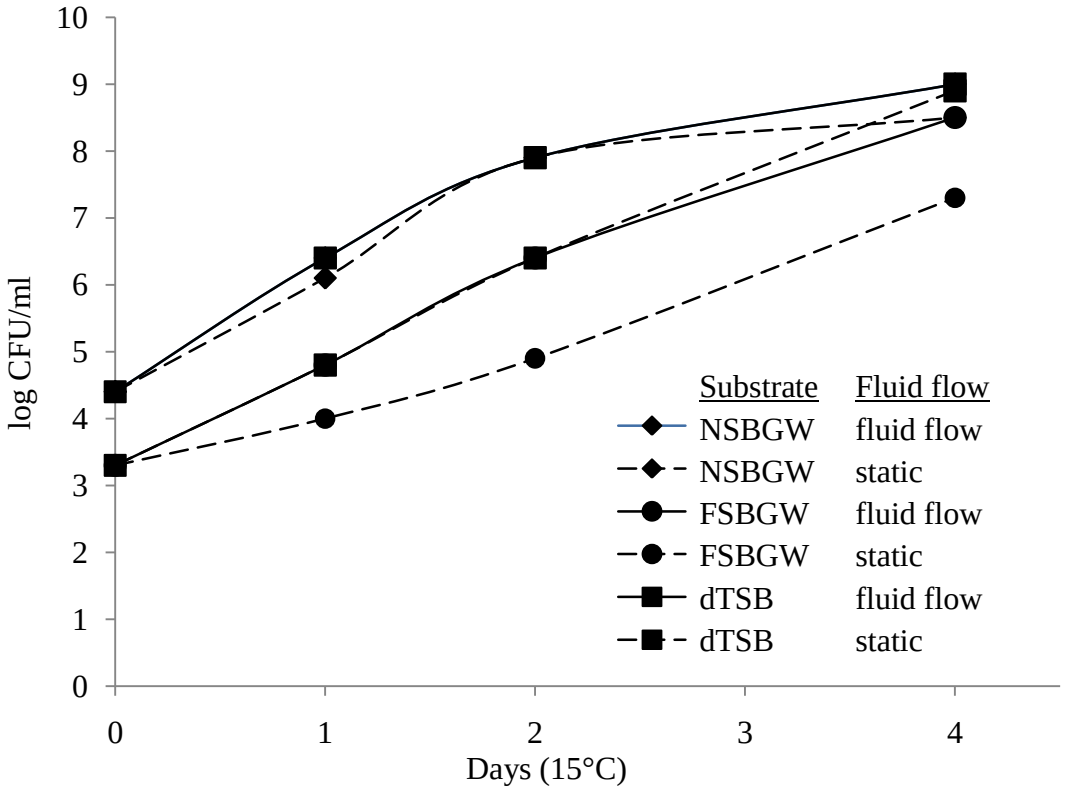

Figure 3.7 (Data in Appendix Table 5). Effects of substrates and fluid flow during storage on the extent of planktonic total bacterial counts (log CFU/ml ± standard deviation) recovered in non-sterile beef-grinder washings (NSBGW), filter-sterilized beef-grinder washings (FSBGW), and 10-fold diluted tryptic soy broth (dTSB) stored for 4 days at 15°C under static or fluid (60 rpm) flow conditions

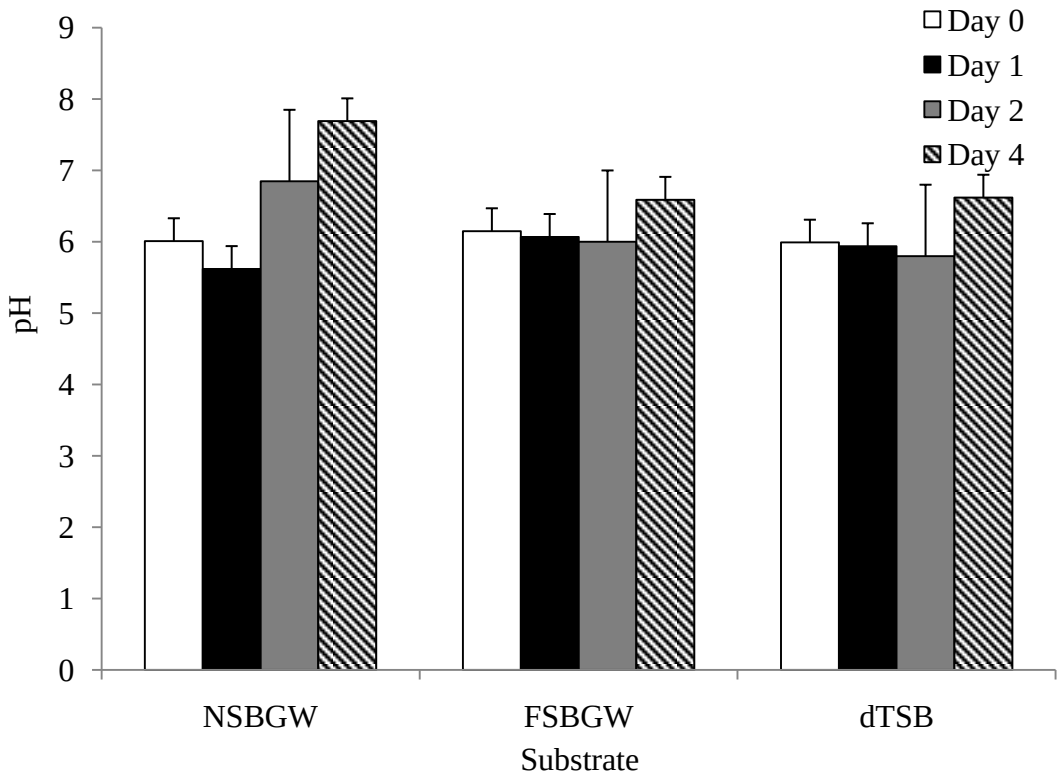

Figure 3.8 (Data in Appendix Table 6). The pH values (pH ± standard deviation) of non-sterile beef-grinder washings (NSBGW), filter-sterilized beef-grinder washings (FSBGW), and 10-fold diluted tryptic soy broth stored for 4 days at15°C.

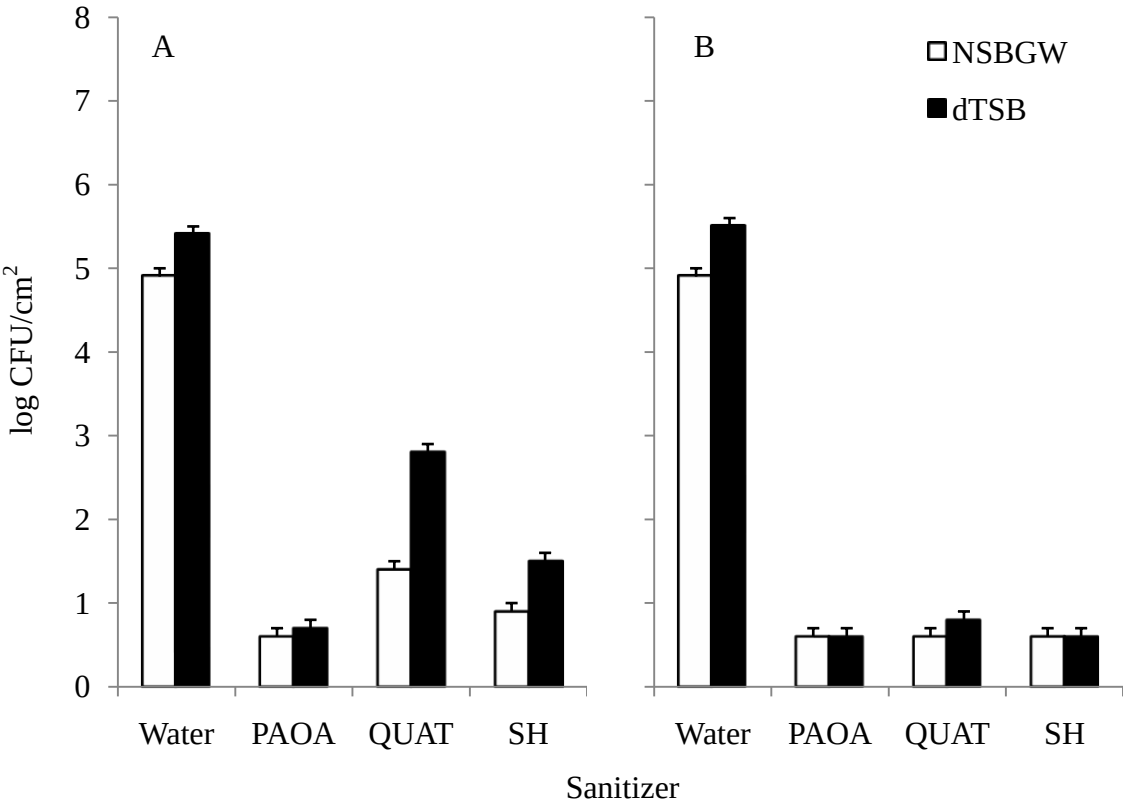

Figure 3.9 (Data in Appendix Table 7). *Escherichia coli* O157:H7 populations (log CFU/cm^2 ± standard deviation) on stainless steel coupons (2×5×0.1 cm) incubated in non-sterile beef-grinder washings (NSBGR) and 10-fold diluted tryptic soy broth (dTSB) for 4 days at 15°C then exposed to water or the manufacturer's maximum recommended concentration of peroxyacetic acid/octanoic acid mixture (PAOA), quaternary compound (Quat), or sodium hypochlorite (SH) of sanitizer for 1 (A) or 10 min (B).

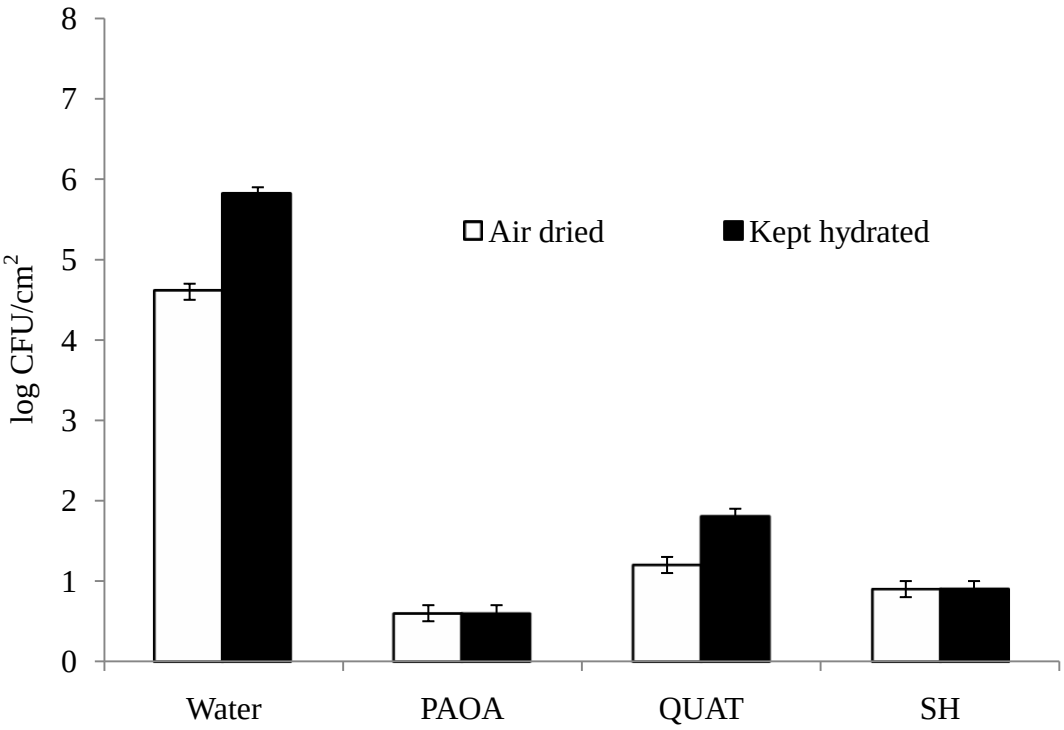

Figure 3.10 (Data in Appendix Table 8). Effect of hydration before sanitizer challenge on extent of attached *Escherichia coli* O157:H7 populations (log CFU/cm^2 ± standard deviation) on stainless steel coupons that were dried for 30 min (Air dried) or kept hydrated (Kept hydrated) followed by exposure to water or the manufacturer's maximum recommended concentration of peroxyacetic acid/octanoic acid mixture (PAOA), quaternary compound (Quat), or sodium hypochlorite (SH).

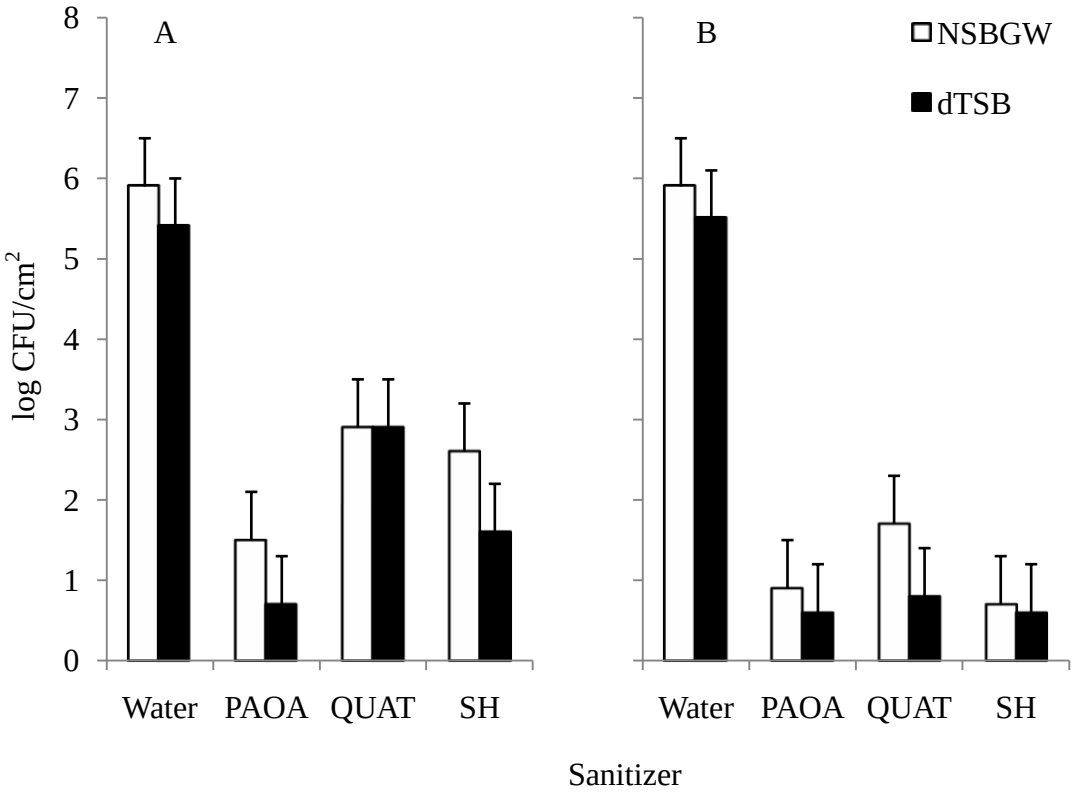

Figure 3.11 (Data in Appendix Table 9). Total bacterial populations populations (log CFU/cm^2 ± standard deviation) on stainless steel coupons (2×5×0.1 cm) incubated in non-sterile beef-grinder washings (NSBGR) and 10-fold diluted tryptic soy broth (dTSB) for 4 days at 15°C then exposed to water or the manufacturer's maximum recommended concentration of peroxyacetic acid/octanoic acid mixture (PAOA), quaternary compound (Quat), or sodium hypochlorite (SH) of sanitizer for 1 (A) or 10 min (B).

CHAPTER 4

Evaluation of effects of antimicrobials, natural flora, and meat residues on *Escherichia coli* O157:H7 survival in model meat product brines

ABSTRACT

Illness has been associated with consumption of *Escherichia coli* O157:H7-contaminated non-intact beef products. Moisture enhancement of contaminated meat cuts with brine injection may lead to entrapment of *E. coli* O157:H7 within the tissue. This study evaluated survival of *E. coli* O157:H7 in brines containing antimicrobials as follows: sodium chloride (NaCl; 5.5%) + sodium tripolyphosphate (STP; 2.75%), NaCl + sodium pyrophosphate (SPP; 2.75%), NaCl + STP + postassium lactate (PL; 22%), NaCl + STP + sodium diacetate (SD; 1.65%), NaCl + STP + PL + SD, NaCl + STP + lactic acid (3.3%), NaCl + STP + acetic acid (3.3%), NaCl + STP + citric acid (3.3%), NaCl + STP + nisin (0.165%) + EDTA (200 mM), NaCl + STP + pediocin (11,000 AU/ml) + EDTA, NaCl + STP + sodium metasilicate (2.2%), NaCl + STP + cetylpyridinium chloride (CPC; 5.5%), or NaCl + STP + hops beta acids (0.0055%). The brines were formulated in sterile distilled water at concentrations used to enhance meat to 110% upon injection (two replications/ three samples each) with or without a 3% unsterile meat residues. The solutions were inoculated (3.5 log CFU/ml) with rifampicin-resistant *E. coli* O157:H7 (8-

strain composite), stored (48 h) at 4 or 15°C, and analyzed periodically. Immediate and continued reductions of *E. coli* O157:H7 populations were observed in brines containing CPC or sodium metasilicate. In the presence of natural flora and meat residues, pathogen levels decreased to below the detection limit (1.3 log CFU/ml) in brines containing nisin+EDTA at 4°C and lactic or acetic acid at 4 and 15°C. The natural flora increased by 1.2-3.0 log CFU/ml in the control (no ingredients) and single treatments of NaCl, STP and SPP at 15°C, and in the control and NaCl treatment by 0.5-1.2 log CFU/ml at 4°C. CPC and sodium metasilicate would best reduce the probability of product cross-contamination through pathogen transfer in contaminated re-circulated brine injection.

INTRODUCTION

Non-intact beef products are defined by the United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS) as: ground beef; beef that has been moisture enhanced through brine injection; mechanically tenderized beef by needling, cubing, Frenching, or pounding devices; and, beef that has been reconstituted into formed entrees (USDA-FSIS, 2009b). Also, all beef manufacturing trimmings and raw beef patty components that are to be ground or reformed are included in the non-intact definition, and are tested for the presence of *E. coli* O157:H7 (USDA-FSIS, 2009b). Non-intact beef products are considered as adulterated if they are found to be contaminated with *Escherichia coli* O157:H7 (USDA-FSIS, 1999). Furthermore, the USDA-FSIS recommends that establishments that produce moisture enhanced beef products should

address *E. coli* O157:H7 as a potential hazard in their hazard analysis critical control point (HACCP) plan (USDA-FSIS, 2009b).

Moisture-enhanced meat products are not required to be labeled as such in the United States. Therefore, they may be considered intact and consequently be cooked to rare or medium-rare temperatures which may be insufficient to inactivate internalized pathogens (Sofos et al., 2008). Indeed, consumption of *E. coli* O157:H7-contaminated moisture-enhanced products has been associated with *E. coli* O157:H7 outbreaks since 2003 that resulted in identified illnesses (CFP, 2008; Laine et al., 2005; USDA-FSIS, 2005, 2007).

Even though brine enhancement improves the quality of the meat (Vote et al., 2000), brine injection may introduce pathogens to the interior of the moisture enhanced product (Bohaychuk and Greer, 2003). There are two proposed mechanisms by which bacteria can be introduced into meat during moisture enhancement by brine injection. First, the injection needles may come in contact with the bacteria on the surface of the meat and push cells into the muscle (Wicklund et al., 2007). Secondly, the brine may become contaminated with bacteria, introduced through contaminated meat (Heller et al., 2007), and the cells may then be injected into the meat with the brine (Greer et al., 2004).

During moisture enhancement, bacteria may accumulate in the brine (Gill and McGinnis, 2004; Greer et al., 2004) and the bacterial load of brines may be directly associated with the amount of contamination on the product being moisture enhanced (Gill and McGinnis, 2004; Gill et al. 2005; Greer et al., 2004). Gill and McGinnis (2004) demonstrated that even though *E. coli* was undetectable on individual samples destined for moisture enhancement, the bacteria accumulated in the re-circulated brine to a level of

2.7 log CFU/15 ml after 60 min of processing. In the same study, aerobic bacteria, coliforms, and *Listeria* spp. in brines increased from undetectable levels at the start of moisture enhancement to 5.5, 4.5, and 2.9 log CFU/15 ml; respectively, after 45 min of re-circulation. Thus, if injected fluids are recycled, bacteria from contaminated cuts of meat may be introduced into other cuts, and the greater the microbial population of the brine the higher the amount on incoming product and the more likely that contaminating bacteria will be introduced into the deep tissue of the meat through moisture enhancement (Bohaychuk and Greer, 2003; USDA-FSIS, 2002).

The concentration of ingredients used in brines is dependent on the amount of moisture to be added to the product. Since the brine is diluted into the meat, the ingredients of the brine should be at higher concentrations than their target concentration in the product. For example, if the end target concentration of NaCl in a final product is to be 0.5% and the product is to be enhanced by 10% of its initial (green) weight, then the concentration of NaCl in the brine should be 5.5% [0.5%(final concentration of ingredient) × 110% (final weight moisture enhanced product) / 10% (enhancement weight of brine)]. Since there are different concentrations of ingredients in the brine and the product, brining ingredients may have differing effects on the microbial populations of the brine and product (Barbut, 2002; Feiner, 2006).

Wicklund et al. (2005) evaluated the effects of brine ingredients on the survival of *E. coli* K12 after 1 min exposure to 4, 50, 60, and 70ºC. It was observed that the initial microbial load and temperature of the brine had a significant effect ($P<0.05$) on the reduction of *E. coli* K12 populations; in general, the higher the initial microbial load and temperature the greater the reductions of *E. coli* K12 populations. It was also observed in

the same study (Wicklund et al. 2005) that addition of sodium lactate and diacetate increased the reductions of *E. coli* K12 at each temperature.

Various agents have been tested for antimicrobial activity in solutions without salt and phosphate. Weber et al. (2004) found that 20 min of *E. coli* O157:H7 exposure to 3% lactic acid (pH 2.4) without salt and phosphate resulted in no viable cells from an intial 6 log CFU/ml concentration; however, injured cells were recovered in a resuscitation broth. In the same study, when 6 log CFU/ml of *E. coli* O157:H7 were exposed to a 0.6%, pH 12.1, solution of sodium metasilicate for 5 to 10 s and no viable pathogen was recovered after treatment. Given that lactic acid and sodium metasilicate have been shown to decrease bacterial populations, it would be interesting to evaluate their effects on pathogen populations in a salt-phosphate brining solution.

In general, data concerning the survival of *E. coli* O157:H7 in brines and its subsequent control through addition of antimicrobial ingredients are limited (Pfeiffer et al., 2008; Sofos et al., 2008). Therefore, the objectives of this study were to evaluate survival of *E. coli* O157:H7 in model meat brines containing antimicrobials, natural flora, and meat residues at temperatures associated with processing and storage of non-intact meat products. *E. coli* O157:H7 survival was evaluated in model fresh (no natural flora or meat residues) and re-circulated (natural flora and meat residues from a meat homogenate) brines. The effects of natural flora and meat residues on the survival of *E. coli* O157:H7 in brines without antimicrobial ingredients could be accounted for by comparing the results from the two brines.

MATERIALS AND METHODS

E. coli O157:H7 strains

The inoculum was comprised of eight rifampicin-resistant *E. coli* O157:H7 strains [ATCC 43888, ATCC 43895, ATCC 43895/ISEHGFP (Noah et al., 2005), and cattle feces isolates C1-057, C1-072, C1-109, C1-154, and C1-158 (Carlson et al., 2009)]. Rifampicin-resistant cultures of each strain were developed (Kaspar and Tamplin, 1993) to selectively differentiate the inoculum from the natural flora of brines. The strains were activated and subcultured in 10 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) supplemented with 100µg/ml rifampicin (35°C, 24±2 h; Sigma-Aldrich Inc., St. Louis, MO). Each strain (10 ml) was harvested individually by centrifugation (Eppendorf model 5810 R, Brinkman Instruments Inc., Westbury, NY; 4,629×g , 15 min, 4°C), washed with 10 ml phosphate buffered saline (PBS; pH 7.4; 0.2 g KH_2PO_4, 1.5 g $NaHPO_4 \cdot H_2O$, 8.0 g of NaCl, and 0.2 g of KCL in 1 L sterile distilled water), re-centrifuged, and resuspended in PBS to obtain a concentration of 4.5 log CFU/ml.

Preparation and inoculation of brines

Brine samples (50 ml) were prepared in 85-ml oakridge tubes (Nalgene, Nalge Nunc, Rochester, NY). Sterile stock solutions of sodium chloride (27.5%) and sodium tripolyphosphate (13.5%) were used in the preparation of the brines when applicable. The concentrations of the ingredients in the brines were based on the assumption that meat would be enhanced to 110% over initial (green) weight (i.e., 10% pump rate). The control and treatment formulations tested included: control (inoculated water containing no

ingredients), sodium chloride (NaCl; 5.5%; Fisher Scientific, Fair Lawn, NJ), sodium tripolyphosphate (STP; 2.75%; kindly provided by BK Giulini Corporation, Semi Valley, CA), sodium pyrophosphate (SPP; 2.75%; kindly provided by BK Giulini Corporation), NaCl (5.5%) + STP (2.75%), NaCl (5.5%) + SPP (2.75%), NaCl (5.5%) + STP (2.75%) + potassium lactate (PL; 22%; kindly provided PURAC America Inc., Lincolnshire, IL), NaCl (5.5%) + STP (2.75%) + sodium diacetate (SD; 1.65%; Niacet Corporation, Niagara Falls, NY), NaCl (5.5%) + STP (2.75%) + PL (22%) + SD (1.65%), NaCl (5.5%) + STP (2.75%) + lactic acid (3.3%; kindly provided by PURAC America Inc.), NaCl (5.5%) + STP (2.75%) + acetic acid (3.3%; EMD Chemicals Inc., Gibbstown, NJ), NaCl (5.5%) + STP (2.75%) + citric acid (3.3%; Fisher Scientific), NaCl (5.5%) + STP (2.75%) + nisin (0.0165%; added as Nisaplin® at 0.66%; kindly provided Danisco USA Inc., New Century, KS) + EDTA (200 mM, Fisher Scientific), NaCl (5.5%) + STP (2.75%) + pediocin (11,000 AU/ml; added as ALTA™ 2341 5.5%; kindly provided by Kerry Bio Sciences, Rochester, NY) + EDTA (200 mM), NaCl (5.5%) + STP (2.75%) + sodium metasilicate (added as AvGard® 2.2%; kindly provided by Danisco USA Inc.), NaCl (5.5%) + STP (2.75%) + cetylpyridinium chloride (CPC; 5.5%; added as Cecure® at 9.2%; Safe Foods Corporation, North Little Rock, AR), and NaCl (5.5%) + STP (2.75%) + hops beta acids (0.0055%; kindly provided by S.S. Steiner Inc., New York, NY). Except for pediocin, all antimicrobial ingredients are approved by the USDA-FSIS for use in or on meat products, but, not specifically for use in injection brines (USDA-FSIS, 2009a).

To evaluate the effect of natural flora and meat residues on *E. coli* O157:H7 survival in model re-circulated brines, brines were also formulated to include 3% meat

residues prepared by homogenizing (2 min; Masticator; IUL Instruments, Barcelona, Spain) 250 g of ground beef with 250 ml of sterile distilled water in a filter bag (Whirl-Pak, Nasco, Modesto, CA). The filtered portion of the homogenate was mixed with the brining ingredients to give a final meat residue concentration in the brining solution of 3%. These brines contained an uninoculated water control containing no ingredients to evaluate survival of natural flora associated with the meat residues.

The brines were inoculated with 1.5 ml of the inoculum added to 48.5 ml of brine, to achieve a target inoculation level of 3.5 log CFU/ml. Immediately after inoculation, a 2-ml subsample was taken for bacterial enumeration and pH measurement. Brines were incubated statically at 4°C or 15°C to cover conditions found in processing environments.

Microbiological analysis

Brines containing no meat residues were analyzed at 0, 4, 8, and 24 h of storage at both storage temperatures. Due to the unknown effects of natural flora on pathogen survival, brines containing 3% meat residues were also analyzed at 48 h; an additional 12 h sample was analyzed at 15°C since natural flora and pathogen growth was expected at this abusive temperature. At each sampling interval 2 ml of brining solution was removed and analyzed for *E. coli* O157:H7 and total microbial populations and a pH value (Denver Instruments, Arvada, CO). Bacterial enumeration was performed by spiral plating (Autoplate 4000, Spiral Biotech, Inc.; Bethesda, MD) duplicate samples on tryptic soy agar (TSA; Accumedia, Lansing, MI) plus 100 µg/ml rifampicin and TSA, respectively. The plates of both media were incubated at 35°C for 48 h, following which, colonies

were enumerated (Colony Image Analysis CASBA^{TM} 4 scanner and software system; Spiral Biotech, Inc.). The detection limit of the analysis was 1.3 log CFU/ml.

Statistical analysis

The experiments were repeated two times at each storage temperature with three samples analyzed at each sampling interval. For each sampling-time by brine-formulation sampling interval duplicate cell counts were averaged and converted to log CFU/ml. Data were analyzed in a two factor ANOVA (sampling time×brine formulation) using PROC Mixed in SAS (version 9.2, 2009). Means were separated using and *F*-protected pairwise *t*-test with a significance level of alpha=0.05.

RESULTS AND DISCUSSION

Microbial survival and growth in brines without meat residues

Similar ($P \geq 0.05$) *E. coli* O157:H7 counts were recovered on TSA and TSA + rifampicin media from brines that did not contain meat residues when incubated at 4 and 15ºC (Tables 4.1 - 4.4). This suggested that counts of TSA were those of the rifampicin-resistant *E. coli* O157:H7 inoculum.

No immediate decreases in *E. coli* O157:H7 populations were found in brines without meat residues, including inoculated water control, NaCl, STP or SPP and the combinations of NaCl + STP and NaCl + SPP. In addition, *E. coli* O157:H7 counts did not increase during storage at 4 and 15ºC (Tables 4.1 and 4.2). In a related study, Wicklund et al. (2005) observed a less than 1 log CFU/ml decrease in brines at 4ºC.

Therefore, addition of antimicrobials may be examined as a potential option to reduce *E. coli* O157:H7, if present, in brines (Pfeiffer et al., 2008; Sofos et al., 2008). If effective, antimicrobial ingredients that immediately reduce bacterial populations may reduce the spread of contaminating bacteria through re-circulated brines.

Immediate and sustained reductions in *E. coli* O157:H7 populations to below the detection limit (1.3 log CFU/ml) were observed in brines containing CPC and sodium metasilicate at either storage temperature (Tables 4.1 and 4.2). Thus, both CPC and sodium metasilicate were effective in reducing (up to a 2.5 log reduction) *E. coli* O157:H7 populations in the presence of salt (5.5%) and phosphate (2.75%) in brines. CPC has been shown to reduce *E. coli* O157:H7 inoculated on beef tissues (Cutter et al., 2000; Lim and Mustapha, 2004; Stopforth et al, 2004), and sodium metasilicate has been shown to be effective in reducing *E. coli* O157:H7 populations on beef hides (Carlson et al., 2007a , 2007b) or in an aqueous solution (Weber et al., 2004). None of the other antimicrobials tested (PL, SD, LA, AA, CA, nisin + EDTA, pediocin + EDTA, and HBA) caused immediate (0 h) reductions in pathogen populations to below the detection limit (1.3 log CFU/ml) in the model brines formulated without meat residues (Tables 4.1 and 4.2). However, pathogen counts decreased to below the detection limit (1.3 log CFU/ml) by 8 and 4 h at 4 and 15°C, respectively, in brines containing lactic acid (Tables 4.1 and 4.2). As previously indicated, Weber et al. (2004) found no viable pathogen populations after 20 min of exposure to an aqueous solution of 3% lactic acid containing no salt and phosphate at 20ºC. The results of Weber et al. (2004) in conjunction with the data of the present study support the findings of Wicklund et al. (2005) who observed that lactic acid was more effective in reducing bacterial populations at higher temperature.

Neither acetic, citric, nor hops beta acids were as effective as lactic acid in reducing *E. coli* O157:H7 populations during brine storage. Brines containing citric acid had pathogen populations below the detection limit (1.3 log CFU/ml) by 24 and 8 h at 4 and 15°C, respectively (Tables 4.1 and 4.2). Acetic and hops beta acids were only able to decrease pathogen populations to below the detection limit (1.3 log CFU/ml) by 24 h at 15°C (Table 4.2). These data suggest that the effectiveness of acetic, citric, hops beta, and lactic acids in reducing *E. coli* O157:H7 populations in brine solutions are dependent on time and temperature. Thus, if brines with these antimicrobials are used, they may not be usedful for the immediate control of *E. coli* O157:H7.

Consistent with other published data (Wicklund et al., 2007), *E. coli* O157:H7 populations did not change ($P \geq 0.05$) in model fresh brines containing NaCl + STP with or without added potassium lactate and sodium diacetate throughout the experiment (Tables 4.1 and 4.2). This indicated that potassium lactate and sodium diacetate, at the concentrations used, were ineffective in reducing pathogen populations.

A 0.7 log CFU/ml decrease in *E. coli* O157:H7 was observed at 0 h and reached 2.0 log CFU/ml by 24 h in brine samples containing nisin plus EDTA when stored at 15 °C (Table 4.2). The bacteriocins nisin and pediocin are reported to be more effective against gram-negative organisms, such as *E. coli* O157:H7, when cells have been stressed and are metabolically active (Boziaris et al., 1998; Kalchayanand et al., 1992; Osmanagaoglu, 2005). Inhibition of pathogen growth and metabolic activity (previously discussed) associated at 15 °C by salt and phosphate may have decreased the efficacy of nisin plus EDTA against the pathogen (Osmanagoaoglu, 2005). As reported by Stevens et al. (1991) nisin plus EDTA in the absence of salt and phosphate caused reductions of 6.9

log CFU/ml in *E. coli* O157:H7 populations after 60 min of exposure. Therefore, our results suggest that nisin plus EDTA may be more appropriate for use in brines that may be stored at abusive temperatures which would allow pathogen growth.

Microbial survival and growth in model brines containing natural flora and meat residues

Throughout storage at 4 or 15°C no rifampicin-resistant bacteria were detected in the uninoculated water control containing no ingredients (Tables 4.5 and 4.6), suggesting that the recorded counts of *E. coli* O157:H7 from brines containing meat residues on TSA plus rifampicin media were from the inoculum. The amount of natural flora from the 3% meat residues were approximately 4 log CFU/ml (Tables 4.7 and 4.8). Compared to the uninoclated and inoculated water controls containing no ingredients, no immediate (day-0) decreases ($P<0.05$) in *E. coli* O157:H7 or total bacterial populations were observed in treatments of NaCl, STP, SPP, NaCl + STP, and NaCl + SPP at 4 or 15°C (Tables 4.5, 4.6, 4.7, and 4.8). Greer et al. (2004) and Wicklund et al. (2005) also observed that salt and phosphate brines had little (0.5 log CFU/ml decrease) or no immediate effect on bacterial counts at 4ºC (Greer et al., 2004; and Wicklund et al., 2005).

E. coli O157:H7 populations remained relatively constant in brines containing no antimicrobial ingredients and 3% meat residues (Tables 4.5 and 4.6). However, growth of total bacterial populations was observed in control solutions containing no ingredients or only SPP at 4°C by 0.5-0.9 log CFU/ml (Table 4.7) and 15°C by 2.7 – 3.0 log CFU/ml (Table 4.8). Also, at 15°C growth of 1.2 and 2.2 log CFU/ml in total bacterial populations was observed at 48 h in solutions containing only NaCl and STP, respectively. In general,

these data suggest that, the combinations of NaCl + STP and NaCl + SPP were sufficient to control *E. coli* O157:H7 and natural flora growth in brines, while individually NaCl, STP and SPP were able to inhibit the growth of *E. coli* O157:H7, but not natural flora, at 15ºC. Since growth of *E. coli* O157:H7 and natural flora was not observed in brines containing NaCl, STP, or SPP (Tables 4.6 and 4.8), a competitive inhibition of the pathogen by the natural flora of the meat residues may exist. Competitive inhibition of *E. coli* O157:H7 by natural flora was also observed in broth by Duffy et al. (1999), in carcass decontamination fluids by Samelis et al. (2001 , 2002), and on meat by Brashears and Durre (1999), Bredholt et al. (1999), Nissen et al. (2001), and Vold et al. (2000).

CPC and sodium metasilicate, in the presence of natural flora and meat residues, were slightly less effective in reducing pathogen populations; however, these treatments were still able to cause immediate *E. coli* O157:H7 reductions of greater than 1.4 and 2.5 log CFU/ml at 4 and 15°C, respectively (Tables 4.5 and 4.6). Furthermore, CPC and sodium metasilicate reduced *E. coli* O157:H7 counts to or below the detection limit (1.3 log CFU/ml) in brines by the first sampling period (0 h) at 15°C or the second sampling period (4 h) at 4°C (Tables 4.5 and 4.6). Similarly, immediate reductions in total bacterial populations were observed in brines containing CPC and sodium metasilicate and reductions to or below the detection limit (1.3 log CFU/ml) were observed by 4 h (Tables 4.7 and 4.8). It appears that meat residues may buffer the activity of CPC and sodium metasilicate against *E. coli* O157:H7 (Pohlman et al., 2002, 2005); however, CPC and sodium metasilicate were still able to cause immediate decreases in *E. coli* O157:H7 counts in modeled re-circulated brines containing 3% meat residues.

In the presence of natural flora and meat residues, *E. coli* O157:H7 counts were decreased to below the detection limit (1.3 log CFU/ml) by 8 h at 4°C in brines containing nisin plus ETDA (Table 4.5). However, without natural flora and meat residues, nisin plus EDTA was unable to lower E. coli O157:H7 populations to below the detection limit (1.3 log CFU/ml) indicating that the natural flora was associated with an increase in pathogen cell sensitivity to nisin (Hanlin et al., 1993; Schillinger, 1999; and Schillinger et al., 2001). Interestingly, nisin plus EDTA was less effective in reducing E. coli O157:H7 populations at 15ºC and against the total natural flora at both temperatures (Tables 4.5-4.8).

In general, a quicker decrease in pathogen populations to below the detection limit (1.3 log CFU/ml) was observed in brines containing acetic and lactic acid with (Tables 4.5 and 4.6) than without 3% meat residues (Tables 4.1 and 4.2) at both temperatures. Conversely, citric acid was more effective in reducing *E. coli* O157:H7 populations in model fresh brines (Tables 4.1 and 4.2) than in brines containing meat residues (Tables 4.5 and 4.6). Samelis et al. (2002) indicated that natural flora or meat residues could stress pathogen cells thus making them more susceptible to organic acid (lactic and acetic) challenge. It is also interesting to note that citric acid is a larger molecule (192.13 g/mol) than acetic and lactic acid (60.05 and 90.08 g/mol, respectively), and therefore, at the same 3.3 % concentration, based on weight in brines, there are less citric acid molecules (0.17 mM) than acetic and lactic acid molecules (0.55 and 0.36 mM) available to decrease pathogen populations within the brines, which may explain why citric acid was less effective in reducing *E. coli* O157:H7 population. These

data suggest that the efficacy of organic acids in the presence of natural flora and meat residues in decreasing pathogen populations may be acid specific.

Brine pH

The initial (day-0) pH tended to be higher in control solutions containing inoculated water and no ingredients than in those with 3% meat residues (7.19 and 8.41 vs. 5.90 and 5.94, respectively; Tables 4.9 and 4.10). Addition of NaCl + STP or NaCl + SPP reduced this difference as pH values of brines without and with meat residues ranged from 6.40 to 7.03 and 6.25 to 7.91, respectively (Tables 4.9 and 4.10). The observed pH values of brines containing NaCL and STP or SPP were lower than those of salt and phosphate brines formulated by others which ranged between 8.34 and 9.1 (Gill et al., 2009; Wicklund et al., 2005, 2006, 2007). Lower NaCl (2.0 to 3.0%) and higher phosphate (3.0 to 5.0%) concentrations were used by Gill et al. (2009) and Wicklund et al. (2005, 2006, 2007) than those used in this study (5.5% and 2.75%, respectively), and this may explain the discrepancy between the observed pH values. Also, the observed pH of brines containing NaCl + STP ranged from 6.96 to 7.91 and tended to be higher than that of those containing NaCl + SPP, which ranged from 6.04 to 6.53 (Tables 4.9 and 4.10) confirming that the form of phosphate may affect the pH of brines (Trout and Schmidt, 1984).

The addition of CPC did not alter the pH of the NaCl + STP brine solution (Tables 4.9 and 4.10). In general, when injected into muscle samples with brines, phosphate increases the pH of the muscle and its water holding capacity (Hedrick et al., 1994). Since the water holding capacity of the meat is pH dependent (Hedrick et al.,

1994), antimicrobials that alter the pH of brines should also alter the water holding capacity of injected meat product. Antimicrobials that do not alter the pH of brines, such as CPC, may be preferred to those that do alter the pH.

A few antimicrobials were able to appreciably alter the pH of salt and phosphate brines, and included acetic, citric, and lactic acids which reduced the pH to values of 3-4 and sodium metsilicate which increased the pH to approximately 12 (Tables 4.9 and 4.10). The low and high pH values associated with addition of organic acids and sodium metasilicate, respectively, were associated with decreases in pathogen populations (Tables 4.1 - 4.4). Weber et al. (2004) observed that solutions (no salt or phosphate) of lactic acid (3.0%) and sodium metasilicate had pH values of 2.4 and 12.1 and were also associated with decreases in pathogen populations. It appears that acetic, citric and lactic acids and sodium metasilicate may be able to overcome the buffering capabilities of the phosphate used in the formulated brines.

There was no storage time by brine solution effect on pH values of brines that did not contain meat residues stored at 4 or 15ºC, and of brines that contained meat residues stored at 4ºC (Table 4.9). For brines containing meat residues and stored at 15ºC, uninoculated and inoculated water containing no ingredients were the only solutions with significant ($P<0.05$) increases in pH value (5.92 – 6.61 and 5.94 - 6.73; respectively; Table 4.10) during storage. The increases of pH were associated with a 2.6 log CFU/ml increase in bacterial populations (Table 4.8) indicating that the metabolic activity of the natural flora of the meat residues was sufficient to increase pH of uninoculated and inoculated water controls.

CONCLUSIONS

Under the conditions of this study, the results indicated that natural flora introduced through meat residues multiplied in re-circulated model brines at temperatures of 15ºC. The amounts of salt and phosphate included in the model fresh and re-circulated brines were sufficient to control *E. coli* O157:H7 growth for up to 24 h, even at 15ºC. However, *E. coli* O157:H7 was able to remain viable in brines containing only salt and phosphate for at least 48 h at 4 and 15ºC, thus presenting the possibility of viable pathogen transfer through contaminated brines. Antimicrobials, such as cetylpyridinium chloride, sodium metasilicate, citric acid, lactic acid, hops beta acid, nisin plus EDTA, and pediocin plus EDTA added to brines were an effective means of reducing *E. coli* O157:H7 and total bacterial populations in brines. In this study, cetylpyridinium chloride and sodium metasilicate elicited immediate reductions and ultimately reduced *E. coli* O157:H7 populations to below detectable levels at 4 and 15ºC without and with the presence of natural flora and meat residues, thus decreasing the chance of pathogen spread through brines. The decreases in *E. coli* O157:H7 in brines by added acetic acid, citric acid, lactic acid, hops beta acid, nisin plus EDTA, and pediocin plus EDTA were less effective in reducing *E. coli* O157:H7 populations than cetylpyridinum chloride and sodium metasilicate time and temperature dependent, thus, even though they reduced pathogen populations, pathogen remained viable and able to be spread through the brines to subsequent injected meat product. Irrespective of the presence of meat residues, the addition of acetic acid, citric acid, or lactic acid decreased, while sodium metasilicate increased, the pH of brines, which may possibly effect the organoleptic quality and water holding capacity of the injected meat product. Further research is needed to examine the

physiochemical and olfactory effects of antimicrobials, in brines, in moisture-enhanced non-intact products.

Table 4.1. Mean *Escherichia coli* O157:H7 populations (log CFU/ml ± standard deviation) recovered on tryptic soy agar + rifampicin (100µl/ml) from brine solutions stored at 4°C.

	Time (h)			
Formulation[a]	0	4	8	24
Control (inoculated water; no ingredients)	3.7±0.2 aA	3.5±0.5 aA	3.5±0.1 aA	3.8±0.1 aA
NaCl	3.8±0.1 aA	3.6±0.1 aA	3.5±0.2 aA	3.8±0.0 aA
STP	3.8±0.0 aA	3.5±0.1 aA	3.5±0.2 aA	3.8±0.1 aA
SPP	3.7±0.2 aA	3.5±0.1 aA	3.5±0.1 aA	3.8±0.1 aA
NaCl + STP	3.7±0.1 aA	3.5±0.1 aA	3.5±0.1 aA	3.7±0.1 aA
NaCl + SPP	3.6±0.1 aA	3.5±0.1 aA	3.5±0.2 aA	3.7±0.0 aA
NaCl + STP + PL	3.7±0.2 aA	3.6±0.2 aA	3.5±0.2 aA	3.7±0.1 aA
NaCl + STP + SD	3.7±0.2 aA	3.5±0.1 aA	3.5±0.1 aA	3.7±0.2 aA
NaCl + STP + PL + SD	3.8±0.2 aA	3.6±0.4 aA	3.6±0.2 aA	3.7±0.2 aA
NaCl + STP + LA	3.7±0.1 aA	2.2±0.6 cB	<1.3 dC	<1.3 dC
NaCl + STP + AA	3.6±0.1 aA	3.4±0.6 abA	3.0±0.9 bB	2.6±0.7 cC
NaCl + STP + CA	3.6±0.1 aA	3.1±0.9 bB	2.0±1.0 cC	<1.3 dD
NaCl + STP + nisin + EDTA	3.5±0.2 aA	3.2±0.2 bB	3.1±0.1 bB	3.0±0.2 bB
NaCl + STP + pediocin + EDTA	3.5±0.3 aA	3.5±0.1 aA	3.5±0.1 aA	3.3±0.1 bA
NaCl + STP + sodium metasilicate	<1.3 bA	<1.3 dA	<1.3 dA	<1.3 dA
NaCl + STP + CPC	<1.3 bA	<1.3 dA	<1.3 dA	<1.3 dA
NaCl + STP + HBA	3.5±0.1 aA	3.2±0.1 bAB	3.0±0.4 bB	3.1±0.3 bB

[a]All solutions formulated in sterile distilled water.

Means with different lowercase letters within the same column are different ($P<0.05$).

Means with different uppercase letters within the same row are different ($P<0.05$).

NaCl: sodium chloride, 5.5%; STP: sodium tripolyphosphate, 2.75%; SPP: sodium pyrophosphate, 2.75%; PL: potassium lactate, 22%; SD: sodium diacetate, 1.65%; LA: lactic acid, 3.3%; AA: acetic acid, 3.3%; CA: citric acid, 3.3%; nisin: 0.66%; EDTA: 200 mM; pediocin 5.5%; sodium metasilicate: 2.2%; CPC: cetylpyridinium chloride, 5.5%; HBA: hops beta acids, 0.0055%.

Table 4.2. Mean *Escherichia coli* O157:H7 populations (log CFU/ml ± standard deviation) recovered on tryptic soy agar + rifampicin (100µl/ml) from brine solutions stored at 15°C.

	Time (h)			
Formulation[a]	0	4	8	24
Control (inoculated water; no ingredients)	3.8±0.2 aA	3.6±0.2 aAB	3.5±0.1 abB	3.6±0.1 abAB
NaCl	3.6±0.1 aA	3.6±0.1 aA	3.5±0.0 abAB	3.4±0.1 cB
STP	3.6±0.1 aA	3.6±0.2 aA	3.4±0.2 bcA	3.6±0.1 abA
SPP	3.6±0.2 aA	3.7±0.2 aA	3.5±0.1 abA	3.6±0.1 abA
NaCl + STP	3.6±0.1 aA	3.5±0.0 aA	3.6±0.1 abA	3.5±0.2 abcA
NaCl + SPP	3.6±0.2 aA	3.6±0.1 aA	3.6±0.1 abA	3.6±0.1 abA
NaCl + STP + PL	3.7±0.1 aA	3.7±0.1 aA	3.6±0.1 abA	3.5±0.2 abcA
NaCl + STP + SD	3.6±0.2 aA	3.6±0.1 aA	3.6±0.1 abA	3.5±0.2 bcA
NaCl + STP + PL + SD	3.6±0.2 aA	3.7±0.1 aA	3.7±0.1 aA	3.7±0.1 aA
NaCl + STP + LA	3.6±0.0 aA	<1.3 eB	<1.3f B	<1.3 fB
NaCl + STP + AA	3.6±0.1 aA	2.9±0.6 bB	1.6±0.3 eC	<1.3 fD
NaCl + STP + CA	3.6±0.1 aA	2.1±0.8 dB	<1.3 fC	<1.3 fC
NaCl + STP + nisin + EDTA	3.1±0.3 bA	2.3±0.6 cB	2.1±0.4 dB	1.8±0.3 eC
NaCl + STP + pediocin + EDTA	3.6±0.3 aA	3.5±0.1 aAB	3.3±0.2 cB	2.5±0.2 dC
NaCl + STP + sodium metasilicate	<1.3 cA	<1.3 eA	<1.3 fA	<1.3 fA
NaCl + STP + CPC	<1.3 cA	<1.3 eA	<1.3 fA	<1.3 fA
NaCl + STP + HBA	3.4±0.2 bA	2.3±0.4 cB	1.5±0.2 eC	1.3±0.0 fD

[a]All solutions formulated in sterile distilled water.

Means with different lowercase letters within the same column are different ($P<0.05$).

Means with different uppercase letters within the same row are different ($P<0.05$).

NaCl: sodium chloride, 5.5%; STP: sodium tripolyphosphate, 2.75%; SPP: sodium pyrophosphate, 2.75%; PL: potassium lactate, 22%; SD: sodium diacetate, 1.65%; LA: lactic acid, 3.3%; AA: acetic acid, 3.3%; CA: citric acid, 3.3%; nisin: 0.66%; EDTA: 200 mM; pediocin 5.5%; sodium metasilicate: 2.2%; CPC: cetylpyridinium chloride, 5.5%; HBA: hops beta acids, 0.0055%.

Table 4.3. Mean total bacterial populations (log CFU/ml ± standard deviation) recovered on tryptic soy agar from brine solutions stored at 4°C.

	Time (h)			
Formulation[a]	0	4	8	24
Control (inoculated water; no ingredients)	4.0±0.1 aA	3.8±0.4 abA	4.0±0.0 aA	4.0±0.0 aA
NaCl	4.0±0.1 aA	4.0±0.0 aA	4.0±0.1 aA	4.0±0.0 aA
STP	4.1±0.1 aA	4.0±0.0 aA	4.0±0.1 aA	4.0±0.0 aA
SPP	4.0±0.1 aA	4.0±0.0 abA	4.0±0.1 aA	3.9±0.1 aA
NaCl + STP	4.0±0.1 aA	3.9±0.0 abA	3.9±0.1 aA	3.9±0.1 aA
NaCl + SPP	4.0±0.0 aA	4.0±0.0 abA	3.9±0.1 aA	3.9±0.1 aA
NaCl + STP + PL	3.9±0.1 aA	3.9±0.1 abA	3.9±0.1 aA	3.8±0.1 aA
NaCl + STP + SD	4.0±0.1 aA	4.0±0.0 abA	3.9±0.1 aA	3.9±0.1 aA
NaCl + STP + PL + SD	3.9±0.1 aA	3.8±0.2 abA	3.7±0.2 abA	3.8±0.2 aA
NaCl + STP + LA	3.9±0.0 aA	2.7±0.7 eB	<1.3 eC	<1.3 eC
NaCl + STP + AA	3.8±0.3 aA	3.6±0.5 bcA	3.2±0.9 cBD	2.8±0.7 dC
NaCl + STP + CA	3.9±0.1 aA	3.2±0.9 dB	2.3±0.8 dC	<1.3 eD
NaCl + STP + Nisaplin + EDTA	3.7±0.1 aA	3.2±0.2 dB	3.1±0.3 cB	3.0±0.3 cB
NaCl + STP + ALTA 2341 + EDTA	3.8±0.3 aA	3.8±0.1 abA	3.6±0.2 bA	3.3±0.1 bB
NaCl + STP + Avgard XP	<1.3 bA	<1.3 fA	<1.3 eA	<1.3 eA
NaCl + STP + CPC	<1.3 bA	<1.3 fA	<1.3 eA	<1.3 eA
NaCl + STP + HBA	3.7±0.1 aA	3.4±0.1 dB	3.1±0.3 cC	3.1±0.2 bcC

[a]All solutions formulated in sterile distilled water.

Means with different lowercase letters within the same column are different ($P<0.05$).

Means with different uppercase letters within the same row are different ($P<0.05$).

NaCl: sodium chloride, 5.5%; STP: sodium tripolyphosphate, 2.75%; SPP: sodium pyrophosphate, 2.75%; PL: potassium lactate, 22%; SD: sodium diacetate, 1.65%; LA: lactic acid, 3.3%; AA: acetic acid, 3.3%; CA: citric acid, 3.3%; nisin: 0.66%; EDTA: 200 mM; pediocin 5.5%; sodium metasilicate: 2.2%; CPC: cetylpyridinium chloride, 5.5%; HBA: hops beta acids, 0.0055%.

Table 4.4. Mean total bacterial populations (log CFU/ml ± standard deviation) recovered on tryptic soy agar from brine solutions stored at 15°C.

	Time (h)			
Formulation[a]	0	4	8	24
Control (inoculated water; no ingredients)	3.9±0.0 aA	4.0±0.1 aA	3.9±0.1 aA	3.9±0.0 aA
NaCl	3.9±0.0 aA	3.9±0.3 aA	3.7±0.1 aA	3.5±0.2 cB
STP	4.0±0.1 aA	4.0±0.1 aA	3.7±0.1 aB	3.8±0.1 abAB
SPP	4.0±0.2 aA	4.0±0.2 aA	4.0±0.1 aA	3.9±0.1 aA
NaCl + STP	3.9±0.1 aA	3.8±0.1 abAB	3.7±0.2 aAB	3.6±0.1 bcB
NaCl + SPP	3.9±0.0 aA	3.9±0.0 aA	3.7±0.1 aAB	3.6±0.1 bcA
NaCl + STP + PL	3.9±0.1 aA	3.9±0.1 aA	3.7±0.1 aAB	3.6±0.2 bcB
NaCl + STP + SD	3.9±0.0 aA	3.8±0.1 abAB	3.7±0.1 aAB	3.6±0.2 bcB
NaCl + STP + PL + SD	3.8±0.3 aAB	4.0±0.4 aA	3.8±0.1 aAB	3.7±0.0 abB
NaCl + STP + LA	3.8±0.1 aA	<1.3 fB	<1.3 eB	<1.3 fB
NaCl + STP + AA	3.9±0.0 aA	3.0±0.5 cB	1.8±0.4 cC	<1.3 fD
NaCl + STP + CA	3.9±0.1 aA	2.1±0.9 eB	<1.3 eC	<1.3 fC
NaCl + STP + Nisaplin + EDTA	3.0±0.3 cA	2.5±0.5 dB	2.1±0.3 cC	1.9±0.3 eC
NaCl + STP + ALTA 2341 + EDTA	3.8±0.3 aA	3.6±0.1 bAB	3.4±0.1 bB	2.8±0.2 dC
NaCl + STP + Avgard XP	<1.3 dA	<1.3 fA	<1.3 eA	<1.3 fA
NaCl + STP + CPC	<1.3 dA	<1.3 fA	<1.3 eA	<1.3 fA
NaCl + STP + HBA	3.4±0.2 bA	2.4±0.3 dB	1.5±0.3 dC	1.4±0.2 fC

[a]All solutions formulated in sterile distilled water.

Means with different lowercase letters within the same column are different ($P<0.05$).

Means with different uppercase letters within the same row are different ($P<0.05$).

NaCl: sodium chloride, 5.5%; STP: sodium tripolyphosphate, 2.75%; SPP: sodium pyrophosphate, 2.75%; PL: potassium lactate, 22%; SD: sodium diacetate, 1.65%; LA: lactic acid, 3.3%; AA: acetic acid, 3.3%; CA: citric acid, 3.3%; nisin: 0.66%; EDTA: 200 mM; pediocin 5.5%; sodium metasilicate: 2.2%; CPC: cetylpyridinium chloride, 5.5%; HBA: hops beta acids, 0.0055%.

Table 4.5 (Graph in Appendix Figure 4 and 5). Mean *Escherichia coli* O157:H7 populations (log CFU/ml ± standard deviation) recovered on tryptic soy agar + rifampicin (100 µg/ml) from brines prepared with 3% meat residues and stored at 4°C.

	Time (h)				
Formulation[a]	0	4	8	24	48
Control (uninoculated water; no ingredients)	<1.3 dA	<1.3 eA	<1.3dA	<1.3 dA	<1.3 cA
Control (inoculated water; no ingredients)	3.4±0.3 bB	4.0±0.1 aA	3.8±0.0abA	4.0±0.2 abA	3.8±0.1 aA
NaCl	3.8±0.1 aAB	4.0±0.1 aA	3.6±0.4abB	4.1±0.0 aA	3.8±0.1 aAB
STP	3.8±0.1 aA	3.9±0.1 abA	3.9±0.1aA	4.0±0.0 abA	3.8±0.0 aA
SPP	3.8±0.0 aA	4.0±0.0 aA	3.8±0.1abA	4.0±0.1 abA	3.8±0.0 aA
NaCl + STP	3.8±0.0 aB	4.1±0.1 aA	3.9±0.1aAB	3.8±0.1 abB	3.7±0.0 aB
NaCl + SPP	3.7±0.0 aB	4.1±0.1 aA	3.9±0.1aAB	3.8±0.2 abB	3.8±0.0 aB
NaCl + STP + PL	3.6±0.0 abA	3.9±0.0 abA	3.9±0.1aA	3.9±0.2 abA	3.8±0.0 aA
NaCl + STP + SD	3.8±0.1 aA	4.0±0.0 aA	3.9±0.0aA	4.0±0.1 abA	3.8±0.0 aA
NaCl + STP + PL + SD	3.7±0.1 aA	3.7±0.3 bcA	3.8±0.0aA	3.9±0.0 abA	3.8±0.0 aA
NaCl + STP + LA	3.7±0.1 abA	<1.3 eB	<1.3dB	<1.3 dB	<1.3 cB
NaCl + STP + AA	3.6±0.2 abA	3.6±0.1 cA	2.0±0.4cB	1.5±0.4 eC	1.3±0.1 cD
NaCl + STP + CA	3.8±0.0 aAB	3.9±0.0 abA	3.6±0.1abB	3.2±0.0 cC	3.1±0.1 bC
NaCl + STP + nisin + EDTA	3.5±0.0 abA	3.2±0.1 dB	<1.3dC	<1.3 dC	<1.3 cC
NaCl + STP + pediocin + EDTA	3.7±0.1 aA	3.6±0.0 cA	3.7±0.1abA	3.7±0.1 bA	3.1±0.1 bB
NaCl + STP + sodium metasilicate	2.0±0.0 cA	<1.3 eB	2.0±1.3cA	<1.3 dB	<1.3 cB
NaCl + STP + CPC	1.9±0.0 cA	<1.3 eB	<1.3dB	<1.3 dB	<1.3 cB
NaCl + STP + HBA	3.8±0.1 aA	3.8±0.0 abcA	3.5±0.3bAB	3.3±0.1 cB	3.3±0.0 bB

[a]All solutions formulated in sterile distilled water.

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

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

NaCl: sodium chloride, 5.5%; STP: sodium tripolyphosphate, 2.75%; SPP: sodium pyrophosphate, 2.75%; PL: potassium lactate, 22%; SD: sodium diacetate, 1.65%; LA: lactic acid, 3.3%; AA: acetic acid, 3.3%; CA: citric acid, 3.3%; nisin: 0.66%; EDTA: 200 mM; pediocin 5.5%; sodium metasilicate: 2.2%; CPC: cetylpyridinium chloride, 5.5%; HBA: hops beta acids, 0.0055%.

Table 4.6 (Graph in Appendix Figure 4 and 5). Mean *Escherichia coli* O157:H7 populations (log CFU/ml ± standard deviation) recovered on tryptic soy agar + rifampicin (100 µg/ml) from brines prepared with 3% meat residues and stored at 15°C.

	Time (h)					
Formulation[a]	0	4	8	12	24	48
Control (uninoculated water; no ingredients)	<1.3 cA	<1.3 fA	<1.3 eA	<1.3 eA	<1.3 iA	<1.3 fA
Control (inoculated water; no ingredients)	3.8±0.1 abBC	3.6±0. 1 abC	3.7±0.1 abBC	3.9±0.3 aB	4.1±0.3 aA	4.1±0.2 aA
NaCl	3.8±0.0 abA	3.7±0.1 abA	3.7±0.1 abA	3.8±0.2 abA	3.8±0.3 bA	3.7±0.1b cA
STP	3.7±0.1 abA	3.7±0.1 abA	3.7±0.0 abA	3.7±0.2 abA	3.7±0.1 bcA	3.6±0.1b cA
SPP	3.7±0.0 abA	3.7±0.1 abA	3.6±0.1 abA	3.6±0.1 bA	3.7±0.2 bcA	3.7±.0b cA
NaCl + STP	3.7±0.0 abA	3.5±0.1 abcA	3.5±0.0 abA	3.2±0.4 cB	3.1±0.5 fgB	3.5±0.1 cA
NaCl + SPP	3.7±0.0 abA	3.6±0.1 abcAB	3.6±0.0 abAB	3.6±0.1 bAB	3.4±0.3 deC	3.7±0.2 bcA
NaCl + STP + PL	3.8±0.0 abA	3.8±0.1 aA	3.8±0.1 aA	3.7±0.2 abA	3.7±0.1 bcA	3.7±0.1 bcA
NaCl + STP + SD	3.7±0.1 abA	3.7±0.0 abA	3.7±0.1 abA	3.4±0.4 cB	3.6±0.1 bcA	3.8±0.2 bA
NaCl + STP + PL + SD	3.9±0.1 aA	3.8±0.0 aA	3.7±0.0 abAB	3.6±0.1 bBC	3.5±0.1 cdeC	3.7±0.2 bcAB
NaCl + STP + LA	3.6±0.1 bA	1.5±0. 7 eB	<1.3 eC	<1.3 eC	<1.3 iC	<1.3 fC
NaCl + STP + AA	3.7±0.1 abA	3.0±0.3 dB	1.5±0.3 dC	1.3±0.0 eD	<1.3 iD	<1.3 fD
NaCl + STP + CA	3.7±0.1 abA	3.6±0. 1 abcA	3.5±0.2 bAB	3.4±0.1 cBC	3.3±0.2 efC	2.8±0.5 dD
NaCl + STP + nisin + EDTA	3.6±0.1 bA	3.4±0.1 cA	3.0±0.3 cB	2.9±0.3 dB	2.6±0.6 hC	1.9±0.6 eD
NaCl + STP + pediocin + EDTA	3.7±0.1 abA	3.5±0.2 bcA	3.2±0.2 cB	2.9±0.3 dC	2.6±0.8 hD	1.8±0.3 eE
NaCl + STP + sodium metasilicate	<1.3 cA	<1.3 fA	<1.3 eA	<1.3 eA	<1.3i A	<1.3 fA
NaCl + STP + CPC	1.3±0.1 cA	<1.3 fA	<1.3 eA	<1.3 eA	<1.3 iA	<1.3 fA
NaCl + STP + HBA	3.6±0.1 bA	3.4±0.2 cAB	3.2±0.1 cBC	3.2±0.3 cBC	3.0±0.2 gCD	2.8±0.3 dD

[a]All solutions formulated in sterile distilled water.

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

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

NaCl: sodium chloride, 5.5%; STP: sodium tripolyphosphate, 2.75%; SPP: sodium pyrophosphate, 2.75%; PL: potassium lactate, 22%; SD: sodium diacetate, 1.65%; LA: lactic acid, 3.3%; AA: acetic acid, 3.3%; CA: citric acid, 3.3%; nisin: 0.66%; EDTA: 200 mM; pediocin 5.5%; sodium metasilicate: 2.2%; CPC: cetylpyridinium chloride, 5.5%; HBA: hops beta acids, 0.0055%.

Table 4.7 (Partial graph in Appendix Figure 5). Mean total bacterial populations (log CFU/ml ± standard deviationl) recovered on tryptic soy agar from brines prepared with 3% meat residues and stored at 4°C.

	Time (h)				
Formulation[a]	0	4	8	24	48
Control (uninoculated water; no ingredients)	4.1±0.3 aC	4.3±0.2 aC	4.4±0.2 abBC	4.6±0.1 aB	5.0±0.2 aA
Control (inoculated water; no ingredients)	4.2±0.3 aD	4.4±0.2 aCD	4.6±0.0 aC	4.6±0.2 aAC	4.9±0.4 aA
NaCl	4.2±0.3 aA	4.3±0.1 aA	4.4±0.2 abA	4.4±0.0 abA	4.4±0.0 bA
STP	4.3±0.3 aA	4.3±0.1 aA	4.5±0.1 abA	4.4±0.1 abA	4.4±0.1 bA
SPP	4.4±0.1 aB	4.4±0.1 aB	4.4±0.0 abC	4.6±0.1 aB	4.9±0.4 aA
NaCl + STP	4.4±0.1 aA	4.4±0.2 aA	4.3±0.1 abAC	4.2±0.1 bAB	4.1±0.1 cB
NaCl + SPP	4.4±0.1 aA	4.4±0.2 aA	4.3±0.1 abA	4.3±0.1 abA	4.3±0.1 bcA
NaCl + STP + PL	4.2±0.1 aA	4.2±0.0 aA	4.2±0.1 bA	4.1±0.1 bA	4.1±0.1 cA
NaCl + STP + SD	4.4±0.1 aA	4.3±0.2 aA	4.3±0.1 abA	4.3±0.1 abA	4.2±0.0 bcA
NaCl + STP + PL + SD	4.3±0.1 aA	4.1±0.1 aA	4.3±0.1 abA	4.2±0.1 bA	4.1±0.1 cA
NaCl + STP + LA	4.1±0.0 bA	<1.3 eB	<1.3 fB	<1.3 eB	<1.3 gB
NaCl + STP + AA	4.1±0.1 aA	3.7±0.3 bB	2.8±0.4 dC	1.9±0.8 eD	1.6±0.3 fE
NaCl + STP + CA	4.2±0.1 aA	3.4±0.5 bB	3.0±0.7 dC	2.5±0.9 fD	2.3±1.0 eD
NaCl + STP + nisin + EDTA	3.7±0.5 bA	3.0±0.5 cB	2.4±0.2 eC	1.9±0.9 eD	1.4±0.3 fgE
NaCl + STP + pediocin + EDTA	4.1±0.1 aA	3.8±0.2 bB	3.6±0.1 cBC	3.2±0.0 eC	3.3±0.2 dC
NaCl + STP + sodium metasilicate	<1.3 dA	<1.3 eA	<1.3 fA	<1.3 dA	<1.3 gA
NaCl + STP + CPC	1.7±0.5 cA	<1.3 eB	<1.3f B	<1.3 dB	<1.3 gB
NaCl + STP + HBA	4.3±0.1 aA	4.1±0.2 aAB	3.8±0.1 cBC	3.7±0.2 dC	3.6±0.2 dC

[a]All solutions formulated in sterile distilled water.

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

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

NaCl: sodium chloride, 5.5%; STP: sodium tripolyphosphate, 2.75%; SPP: sodium pyrophosphate, 2.75%; PL: potassium lactate, 22%; SD: sodium diacetate, 1.65%; LA: lactic acid, 3.3%; AA: acetic acid, 3.3%; CA: citric acid, 3.3%; nisin: 0.66%; EDTA: 200 mM; pediocin 5.5%; sodium metasilicate: 2.2%; CPC: cetylpyridinium chloride, 5.5%; HBA: hops beta acids, 0.0055%.

Table 4.8 (Partial graph in Appendix Figure 5). Mean total bacterial populations (log CFU/ml ± standard deviation) recovered on tryptic soy agar from brines prepared with 3% meat residues and stored at 15°C.

	Time (h)					
Formulation[a]	0	4	8	12	24	48
Control (uninoculated water; no ingredients)	4.4±0.1 aD	4.5±0.1 aD	4.6±0.2 aD	5.3±0.1 aC	6.4±0.1 aB	7.1±0.2 aA
Control (inoculated water; no ingredients)	4.5±.01 aD	4.5±0.0 aD	4.6±0.2 aD	5.3±0.1 aC	6.5±0.1 aB	7.1±0.1 aA
NaCl	4.5±0.1 aB	4.5±0.1 aB	4.5±0.2 aB	4.6±0.2 bcA	4.7±0.1 dB	5.7±0.6 dA
STP	4.4±0.1 aD	4.5±0.1 aCD	4.6±0.2 aCD	4.7±0.1 bC	5.4±0.1 cB	6.6±0.3 cA
SPP	4.4±0.1 aD	4.5±0.1 aCD	4.6±0.1 aCD	4.7±0.1 bC	5.8±0.1 bB	7.4±0.3 bA
NaCl + STP	4.4±0.1 aA	4.5±0.1 aA	4.4±0.2 aA	4.3±0.2 dAB	4.3±0.2 eAB	4.2±0.2 eB
NaCl + SPP	4.4±0.1 aAB	4.5±0.1 aA	4.6±0.2 aA	4.4±0.2 cdAB	4.3±0.2 eB	4.3±0.2 eB
NaCl + STP + PL	4.4±0.1 aA	4.4±0.2 aA	4.3±0.3 aA	4.3±0.3 dA	4.3±0.3 eA	4.3±0.2 eA
NaCl + STP + SD	4.4±0.0 aA	4.5±0.1 aA	4.5±0.2 aA	4.4±0.3 cdA	4.4±0.1 eA	4.4±0.2 eA
NaCl + STP + PL + SD	4.4±0.2 aAB	4.5±0.2 aA	4.4±0.2 aAB	4.3±0.2 dAB	4.2±0.2 eB	4.2±0.2 eB
NaCl + STP + LA	4.2±0.1 bcA	1.6±0.7 dB	1.4±0.3 fBC	<1.3 gC	<1.3 hC	<1.3 hC
NaCl + STP + AA	4.3±0.2 abcA	3.4±0.1 cB	2.5±0.2 eC	1.4±0.3 gD	<1.3 hD	<1.3 hD
NaCl + STP + CA	4.4±0.1 abA	4.0±0.4 bB	3.8±0.3 bB	3.6±0.1 eC	3.3±0.2 fD	3.1±0.1 fE
NaCl + STP + nisin + EDTA	3.9±0.1 cA	3.5±0.2 cB	3.3±0.1 dC	3.3±0.1 fC	2.9±0.4 gD	2.5±0.8 gE
NaCl + STP + pediocin + EDTA	4.1±0.1 cA	3.8±0.1 bB	3.6±0.1 cC	3.5±0.1 efCD	3.4±0.1 fCD	3.3±0.1 fD
NaCl + STP + sodium metasilicate	2.0±0.7 dA	1.3±0.1 eB	<1.3 fB	<1.3 gB	1.3±0.1 hB	<1.3 hB
NaCl + STP + CPC	1.3±0.1 fA	<1.3 eA	<1.3 fA	<1.3 gA	<1.3 hA	<1.3 hA
NaCl + STP + HBA	4.3±0.0 abcA	3.8±0.1 bB	3.6±0.1 cBC	3.5±0.1 efCD	3.4±0.0 fDE	3.2±0.0 hE

[a]All solutions formulated in sterile distilled water.

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

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

NaCl: sodium chloride, 5.5%; STP: sodium tripolyphosphate, 2.75%; SPP: sodium pyrophosphate, 2.75%; PL: potassium lactate, 22%; SD: sodium diacetate, 1.65%; LA: lactic acid, 3.3%; AA: acetic acid, 3.3%; CA: citric acid, 3.3%; nisin: 0.66%; EDTA: 200 mM; pediocin 5.5%; sodium metasilicate: 2.2%; CPC: cetylpyridinium chloride, 5.5%; HBA: hops beta acids, 0.0055%.

Table 4.9. Mean pH values (pH ± standard deviation) of brines[a].

	Meat residues (%)		
	0		3
	Storage temperature (°C)		Storage temperature (°C)
Formulation[b]	4	15	4
Control (uninoculated water; no ingredients)[c]	-	-	5.89±0.38 g
Control (inoculated water; no ingredients)	7.91±0.62 b	8.45±1.01 b	5.90±0.38 g
NaCl	5.66±0.14 i	5.80±0.37 g	5.54±0.05 h
STP	7.63±0.08 c	7.74±0.12 c	8.79±0.48 b
SPP	6.57±0.06 f	6.58±0.05 e	7.22±0.14 e
NaCl + STP	6.86±0.26 e	7.03±0.09 d	7.91±0.55 c
NaCl + SPP	6.40±0.37 g	6.53±0.48 e	6.41±0.07 f
NaCl + STP + PL	7.01±0.17 d	7.13±0.06 d	7.60±0.28 d
NaCl + STP + SD	5.14±0.57 j	5.59±0.08 h	5.44±0.05 i
NaCl + STP + PL + SD	6.07±0.10 h	6.09±0.06 f	5.95±0.05 g
NaCl + STP + LA	3.35±0.12 l	3.36±0.11 j	3.28±0.09 l
NaCl + STP + AA	3.95±0.10 h	3.98±0.08 i	3.93±0.06 k
NaCl + STP + CA	3.25±0.18 l	3.27±0.19 j	3.11±0.22 l
NaCl + STP + nisin + EDTA	6.13±0.06 h	6.14±0.10 f	4.53±1.01 j
NaCl + STP + pediocin + EDTA	6.09±0.04 h	6.10±0.06 f	4.66±1.10 j
NaCl + STP + sodium metasilicate	12.44±0.37 a	12.13±0.27 a	12.38±0.21 a
NaCl + STP + CPC	6.95±0.16 de	7.00±0.12 d	7.87±0.55 c
NaCl + STP + HBA	6.95±0.08 de	7.02±0.08 d	7.91±0.53 c

[a]There was no sampling-time×brine-formulation interaction and no significant ($F \geq 0.05$) main effect for sampling time suggesting pH was similar throughout the study for each brine formulation.

[b]All solutions formulated in sterile distilled water.

[c]pH was observed in uninoculated water containing no ingredients with 3% meat residues due to the natural flora introduced by the meat residues.

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

NaCl: sodium chloride, 5.5%; STP: sodium tripolyphosphate, 2.75%; SPP: sodium pyrophosphate, 2.75%; PL: potassium lactate, 22%; SD: sodium diacetate, 1.65%; LA: lactic acid, 3.3%; AA: acetic acid, 3.3%; CA: citric acid, 3.3%; nisin: 0.66%; EDTA: 200 mM; pediocin 5.5%; sodium metasilicate: 2.2%; CPC: cetylpyridinium chloride, 5.5%; HBA: hops beta acids, 0.0055%.

Table 4.10. Mean pH values (pH ± standard deviation) of brines prepared with 3% meat residues and stored at 15°C[a].

Formulation[b]	Time (h)					
	0	4	8	12	24	48
Control (uninoculated water; no ingredients)	5.92±0.60 gCD	6.20±0.58 fB	5.84±0.22 efD	5.59±0.18 efE	6.13±0.31 deBC	6.61±0.28 fA
Control (inoculated water; no ingredients)	5.94±0.64 gCD	6.21±0.44 fB	5.98±0.35 deCD	5.79±0.08 cD	6.06±0.24 deBC	6.73±0.27 eA
NaCl	5.43±0.04 hA	5.45±0.10 iA	5.39±0.05 gA	5.29±0.06 gA	5.44±0.04 gA	5.31±0.11 jA
STP	8.05±0.18 bA	8.00±0.15 bA	8.00±0.16 bA	7.85±0.18 bA	8.02±0.18 bA	7.96±0.14 bA
SPP	6.96±0.27 eA	6.87±0.23 eA	6.91±0.30 cA	6.76±0.27 cA	6.95±0.26 cA	6.95±0.31 deA
NaCl + STP	7.19±0.25 cA	7.13±0.21 dAB	7.11±0.21 cAB	6.96±0.27 cB	7.15±0.26 cAB	7.18±0.18 cAB
NaCl + SPP	6.25±0.06 fA	6.20±0.07 fA	6.19±0.06 dA	6.04±0.02 dA	6.21±0.04 dA	6.23±0.11 gA
NaCl + STP + PL	7.22±0.19 cA	7.11±0.15 dAB	7.10±0.14 cAB	6.94±0.22 cB	7.15±0.20 cAB	7.17±0.12 cdA
NaCl + STP + SD	5.44±0.07 hA	5.42±0.08 iA	5.42±0.06 gA	5.23±0.15 gA	5.39±0.10 gA	5.41±0.03 jA
NaCl + STP + PL + SD	6.00±0.08 dA	5.93±0.06 gAB	5.93±0.06 efAB	5.75±0.12 efB	5.94±0.09 efAB	5.97±0.03 hAB
NaCl + STP + LA	3.26±0.07 jA	3.30±0.01 kA	3.34±0.09 iA	3.17±0.13 iA	3.24±0.06 jA	3.21±0.08 lA
NaCl + STP + AA	3.99±0.04 iA	3.98±0.10 jA	4.01±0.07 hA	3.83±0.14 hA	3.93±0.09 iA	3.91±0.08 kA
NaCl + STP + CA	3.20±0.08 jA	3.28±0.03 kAB	3.32±0.20 iA	2.94±0.25 jC	3.03±0.20 jBC	3.01±0.17 lBC
NaCl + STP + nisin + EDTA	5.78±0.10 gA	5.72±0.09 ghA	5.72±0.08 fA	5.56±0.14 fA	5.73±0.11 fA	5.71±0.12 iA
NaCl + STP + pediocin + EDTA	5.75±0.08 gA	5.70±0.09 hA	5.71±0.07 fA	5.54±0.14 fA	5.72±0.11 fA	5.70±0.12 iA
NaCl + STP + sodium metasilicate	12.18±0.20 aA	12.16±0.25 aA	12.06±0.27 bA	12.09±0.10 aA	12.12±0.19 aA	12.07±0.24 aA
NaCl + STP + CPC	7.15±0.23 cA	7.16±0.35 cA	7.08±0.20 cAB	6.91±0.26 cB	7.12±0.23 cAB	7.06±0.24 cdAB
NaCl + STP + HBA	7.21±0.23 cA	7.13±0.19 dAB	7.08±0.20 cAB	6.93±0.34 cB	7.15±0.24 cAB	7.14±0.23 cdAB

[a]A significant (F<0.05) sampling-time×brine-formulation interaction was observed in brines prepared with 3% meat residues and stored at 15°C, therefore, individual means for each interaction are presented and compared statistically.
[b]All solutions formulated in sterile distilled water.
Mean values with different lower case letters in the same column are significantly different (P<0.05).
NaCl: sodium chloride, 5.5%; STP: sodium tripolyphosphate, 2.75%; SPP: sodium pyrophosphate, 2.75%; PL: potassium lactate, 22%; SD: sodium diacetate, 1.65%; LA: lactic acid, 3.3%; AA: acetic acid, 3.3%; CA: citric acid, 3.3%; nisin: 0.66%; EDTA: 200 mM; pediocin 5.5%; sodium metasilicate: 2.2%; CPC: cetylpyridinium chloride, 5.5%; HBA: hops beta acids, 0.0055%.

CHAPTER 5

Inactivation of *Escherichia coli* O157:H7 inoculated at different depths into non-intact beef under-cooked by pan-broiling or roasting from different starting cooking temperatures

ABSTRACT

Escherichia coli O157:H7 transferred into internal muscle tissue during blade tenderization or brine enhancement is a potential cause of foodborne illness if the meat is undercooked, and current pathogen lethality guidelines only indicate time and temperature as parameters to ensure the safe consumption of beef products. This study evaluated thermal inactivation of *E. coli* O157:H7 at different depths in blade tenderized non-intact steaks stored at cold (4°C) or frozen (-20°C) temperatures and cooked by pan-broiling or roasting to a sub-lethal temperature (60°C) directly from a frozen or thawed state. Fresh beef (*semitendinosus*; 3% fat) was cut into 0.3 or 0.6 cm slices. Eight 0.3 or four 0.6 cm, or two 0.6 cm slices were stacked on top of each other to form either 2.4 or 1.2 cm thick steaks. All steaks were blade tenderized (4.8 blades/cm^2) using a Supertendermatic™ hand-held tenderizer. Then steaks were inoculated with rifampicin-resistant *E. coli* O157:H7 (8 strains; 3.7 log CFU/cm2) on the surface (0.0 cm) or between the slices and vacuum-packaged, and stored at 4 (2.4 cm thick) or -20°C (2.4 and

1.2 cm thick) for 5 days. Steaks were cooked at 149°C, directly from the frozen state (-20°C) or after thawing to 4 or 25°C, by pan-broiling or roasting, to a geometric center temperature of 60°C. Longer (P<0.05) cooking times to reach a geometric center temperature of 60°C were observed for 2.4 than 1.2 cm thick steaks and steaks that were cooked directly from the frozen state. Higher internal temperatures were observed during cooking by pan-broiling than roasting at 0.3 (88.0±7.3 vs 63.4±4.8°C), 0.6 (73.7±8.9 vs 59.6±10.0°C) and 0.9 (64.5±4.8 vs 60.5±2.1°C) cm depths of inoculation. Surviving pathogen populations increased (P<0.05) from 0.3±0.6 log CFU/cm^2 at 0.0 cm to 2.5±0.6 log CFU/cm^2 at 1.2 cm depth of inoculation in steaks cooked by pan-broiling. For samples cooked by roasting, although a similar increasing trend in pathogen populations was observed, thermal destruction of *E. coli* O157:H7 was less and differences in pathogen populations were not significant (P≥0.05) for each depth of inoculation. Greater surviving (P<0.05) populations of *E. coli* O157:H7 were recovered from thinner (1.2 cm) than thicker (2.4 cm) steaks cooked to the same internal temperature. The method of thawing did not affect (P≥0.05) extent of the thermal inactivation. These data indicate that pan-broiling and 2.4 cm thick steaks are preferred to roasting and 1.2 cm steaks when undercooking non-intact steaks suggesting that cooking method and non-intact steak thickness should be considered in lethality guidelines developed to ensure the safe consumption of non-intact meat products.

INTRODUCTION

Blade tenderizing and moisture enhancing through brine injection of beef subprimals before portioning increase the perceived tenderness of beef, thus, increasing the value of tougher muscles (Bidner et al., 1985; George-Evins et al., 2004). The difference between blade tenderization and moisture enhancement is that a brine solution is injected in the latter process. Both processes involve mechanical tenderization in which needles or blades pierce into the deep tissues of the intact cut of beef and physically separate the meat fibers rendering the product non-intact. After mechanical tenderization, whole muscle cuts may be portioned into non-intact steaks. These non-intact steaks may appear intact and consequently be cooked to rare and medium-rare temperatures, which are insufficient to inactivate internalized pathogens (Sofos et al., 2008). It has been estimated that between 25 and 53% of consumers prefer to consume their beef cooked to an endpoint internal temperature of medium-rare or rare (Cox et al., 1997; Schmidt et al., 2002; USDA-FSIS, 2002a), indicating that a substantial portion of non-intact meat products may be under-cooked.

Pathogen cells, such as those of *E. coli* O157:H7, pathogens may be transfered to the interior of blade cuts by blade tenderization (BIFSCO, 2006; Gill et al., 2005; Hajmeer et al., 2000; Krizner, 1999; Lambert et al., 2001; Luchansky et al., 2008, 2009; Phebus et al., 2000; Sporing, 1999; Thippareddi, 2000). However, in order for bacteria to be transfered by mechanical tenderization into meat, they must first be present on the surface of the meat. Ransom et al. (2002) and Warren et al. (2003) observed a low prevalence of pathogens on meat that was to be tenderized. Heller et al. (2007) examined

1,014 uninoculated beef subprimals and found the prevalence of *E. coli* O157:H7 to be 0.2%. In fact, the USDA-FSIS (2002a) estimated that 0.02% of non-intact steaks have at most 1 cell of *E. coli* O157:H7 (USDA-FSIS, 2002a). This prevalence rate on product translates to an estimated 1 illness per 14.2 million servings of non-intact steaks (USDA-FSIS, 2002a).

There are conflicting reports, even by the same authors, that describe the amount of bacteria transfer into the interior of the muscle in relation to the concentration of bacterial cells on the meat surface during mechanical tenderization. It has been reported that the amount of internalized bacteria due to blade tenderization was not proportional to the amount of surface bacteria (Gill and McGinnis, 2004; Gill et al., 2005a; Gill et al., 2005b). However, others have observed the opposite and concluded that the translocation of bacteria from the surface to the interior of beef cuts during blade tenderization was dependent on the amount of bacteria on the surface of the muscle (Gill and McGinnis, 2005a; Hajmeer et al.; 2000, Luchansky et al. 2008b; Sporing 1999). For example, higher levels of surface bacteria would be correlated with higher amounts of bacteria transfered into the muscle. This discrepancy in the data suggests that there are factors, other that the surface population of bacteria, contributing to the amount of bacterial cells transfered into the interior of the meat due to mechanical tenderization. Factors mentioned in the aforementioned papers that may influence the amount of transfered bacteria during mechanical tenderization, but not necessarily studied, include product storage time post inoculation, storage temperature post inoculation, shape of the tenderization blades or needles, depth of penetration, size of subprimal being blade tenderized, and the species and growth of the bacteria on meat that is tenderized.

Blade tenderization transfers *E. coli* O157:H7 cells primarily to the topmost 1 cm; however, it also transfers pathogen cells to deep tissues (Luchansky et al., 2008a,b, 2009). Sporing (1999) reported that the amount of *E. coli* O157:H7 contamination transfered to the interior of meat decreased with increasing depth of penetration; specifically, a 0.5 log decrease in *E. coli* O157:H7 populations for each 1 cm penetration by tenderizing blades or needles. This was later supported by Luchansky et al. (2009) who characterized the percent translocation of bacteria to be 33.00, 3.04, 3.09, 0.44, 0.10, and 0.82% at 0-1, 1-2, 2-3, 3-4, 4-6, and 6-8 cm of depths; respectively. These data indicate that the meat cleans the contamination off the blades as they penetrate.

In the limited published reports, there are conflicting data regarding the thermal inactivation of *E. coli* O157:H7 after ground beef was cooked from the frozen or thawed state (Jackson et al., 1995; Juneja et al., 1997; Sage and Ingham, 1998). In ground beef patties cooked from the frozen state (-18°C), Jackson et al. (1996) observed that *E. coli* O157:H7 was more heat resistant than those cooked from refrigerated storage (3°C). Also, Jackson et al. (1996) observed that thawing (holding patties at 21 or 30°C prior to cooking) increased the pathogen's heat sensitivity. Conversely, Juneja et al. (1997) observed similar thermal inactivation of *E. coli* O157:H7 in refrigerated and frozen ground beef samples after cooking, and Sage and Ingham (1998) observed that "no thawing method had consistently and significantly greater lethality". Juneja et al. (1997) further explained that the observed difference between their and data presented by Jackson et al. (1996) may be attributed to differences in sample size, heating rates, and storage time between the two papers. Since a majority of the data was a collected in ground beef and that data is inconsistent, there is a need to examine the effects of storage

temperature and method of thawing on the thermal inactivation of *E. coli* O157:H7 in non-intact steaks.

Lethality of pathogens during cooking is a function of cooking temperature, method of cooking, time of cooking and thickness of meat (USDA-FSIS, 2002b). Heat transfer to the meat during cooking may also affect the thermal inactivation of pathogens. Radiation, conduction, and convection are forms of heat transfer used to cook meat products (Gisslen, 2010). Radiation is the transfer of heat through waves of energy. The excitation of molecules within the meat by the waves of radiation causes the production of heat. Conduction is the direct transfer of heat energy from one surface to another, whereas convection is the transfer of heat through a gas or a liquid. It is important to note that radiation, conduction, and convection are methods of heat transfer to the meat surface, while the heat transfer through the meat is by conduction. Given this, the more energy transferred to the surface of the meat during cooking the faster the conduction through the meat.

The cooking method, and hence the method of heat transfer, have been suggested to affect the thermal inactivation of internalized pathogens. To assure the safe consumption of non-intact products the endpoint temperature for broiling in a conventional oven (radiation), which provides a more uniform even heat for cooking (Sporing, 1999), is 60°C, whereas for grilling (radiation or convection) and pan-broiling (conduction), which provide a more variable heat transfer (USDA, 2002c), is 65°C (Luchansky and Call, 2008a; NACMCF, 2002; Phebus et al., 2000; USDA, 2002a). The data suggest that cooking by radiation heat transfer would allow for the safe consumption of non-intact steaks cooked to a rare (60°C) or medium-rare (63°C) endpoint temperature.

However, when cooking by convection or conduction, a higher endpoint cooking temperature (≥65°C) may be needed to assure the safe consumption of a non-intact steak.

Cooking by pan-broiling requires that the meat be turned in order for both sides of the meat to come in contact with the heating source. The time interval used to turn the non-intact steaks may affect the amount of thermal inactivation of internalized pathogens. Rhee et al. (2003) observed faster cooking times for ground beef patties that were turned every 30 s (6.6 min) as opposed to a single turn (10.9 min). Upon microbiological analysis of the patties after cooking to an internal temperature of 71.1°C, when a lower concentration of inoculum (3-4 log CFU/g) was used, only ground beef patties cooked by pan-broiling with a single turn at an internal temperature of 41°C contained recoverable *E. coli* O157:H7 cells (2 of 9 samples). There were no recoverable pathogen cells from ground beef patties cooked by pan-broiling with turning every 30 s. These data suggest that often turning of meat products while pan-broiling is preferred to best inactivate any internalized pathogens (Rhee et al., 2003).

The objective of this study was to evaluate thermal inactivation of *E. coli* O157:H7 at different depths of non-intact steaks cooked by pan-broiling and roasting to a 60°C internal temperature from either the frozen or thawed state.

MATERIAL AND METHODS

E. coli O157:H7 strains and inoculum preparation

Eight rifampicin-resistant *E. coli* O157:H7 strains [ATCC 43888, ATCC 43895, ATCC 43895/ISEHGFP (Noah et al., 2005), C1-057, C1-072, C1-109, C1-154, and C1-158]

were used for an inoculum. Strains C1-057, C1-072, C1-109, C1-154, and C1-158 were isolated from cattle feces in a previous study (Carlson et al., 2009). For each strain, rifampicin-resistant derivatives were isolated using the method described by Kaspar and Tamplin (1993). The strains were activated and subcultured (35°C, 24±2 h) in 10 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) supplemented with 100µg/ml rifampicin (Sigma-Aldrich Inc., St. Louis, MO). Each strain (10 ml) was harvested individually by centrifugation (Eppendorf model 5810 R, Brinkman Instruments Inc., Westbury, NY; 4,629×g , 15 min, 4°C), washed to remove residual rifampicin with 10 ml phosphate buffered saline (PBS; pH 7.4; 0.2 g KH_2PO_4, 1.5 g $NaHPO_4 \cdot H_2O$, 8.0 g of NaCl, and 0.2 g of KCL in 1 L sterile distilled water), centrifuged, and suspended in and diluted with PBS to obtain a concentration of 6.5 log CFU/ml.

Preparation of non-intact steaks, inoculation, and storage of samples

The first study consisted of experiments that evaluated the thermal inactivation of *E. coli* O157:H7 inoculated at different depths (0.0, 0.3, 0.6, 0.9, and 1.2 cm) in 2.4 cm thick non-intact steaks after storage at 4°C (Table 5.1). Steaks were prepared from beef eye of round (s*emitendinosus*, NAMP 171C) purchased from a local packing plant and stored at -20°C in the Meat Science Laboratory, Center for Meat Safety & Quality, Department of Animal Sciences at Colorado State University for no longer than 2 weeks. Whole muscles were tempered at 4°C for 48 h, trimmed of external fat, connective tissue and silver skin (epimysium) and placed at -20°C for 1 h to achieve a crust freeze to facilitate slicing. The meat was sliced (Hobart 2712 12" Semi Automatic Slicer; Hobart Mfg. Co., Troy, OH) perpendicular to the muscle fibers (longitudinal axis) into 0.3 cm slices. Eight slices were

then stacked on top of each other to form a 2.4 cm thick non-intact steak, which was then tenderized using a Supertendermatic™ handheld tenderizer (Jaccard Corporation, Orchard Park, New York). Steaks were transferred to the Pathogen Reduction Laboratory, Center for Meat Safety & Quality, Department of Animal Sciences at Colorado State University, for inoculation, incubation, cooking and analysis. Steaks were inoculated with rifampicin-resistant *E. coli* O157:H7 on each surface (0.0 cm) or between the slices at each depth of 0.3, 0.6, 0.9, or 1.2 cm to achieve an initial inoculation level of 3.7 log CFU/cm^2. Since there were two 0.0, 0.3, 0.6, and 0.9 cm and only one 1.2 cm (middle) depths of inoculation for each steak, either 200 µl was spread over (0.0 cm) the surface or between (0.3, 0.6, or 0.9 cm) the slices using a sterile bent glass rod or 400 µl of inoculua at 1.2 cm. Steaks were individually placed into vacuum bags (15.2 by 21.6 cm, 3 mil standard barrier, nylon-polyethylene vacuum pouch, water vapor and oxygen transmission rates of 9.3 g/m^2/24 h [97% relative humidity] and 54.3 cm^3/m^2/24 h [21°C, 0% relative humidity], respectively; Koch, Kansas City, MO), vacuum packaged (Hollymatic, Corp., Countryside, IL) and stored at 4°C for 5 days before cooking.

Experiments of the second study evaluated the effects of non-intact steak thickness and method of thawing including initial cooking temperature on the thermal inactivation of *E. coli* O157:H7 (Table 5.1). Steaks were prepared as above with the following minor changes. Beef eye of round muscles were sliced into 0.6 cm slices and 1.2 and 2.4 cm thick steaks were formed by placing two or four slices on top of each other, respectfully. The 1.2 and 2.4 cm thick steaks were tenderized, inoculated on the surface or between slices and vacuum packaged as previously mentioned, and stored at -20°C for 5 days. On day 5 steaks were either cooked directly from the frozen state or

thawed at 4 or 25°C. For steaks that were cooked directly from the frozen state, sterile thermocouples (Type-K, Pico Technology Limited, Cambridgeshire, United Kingdom) were placed in the geometric center and at the internal (0.6 or 1.2 cm, depending on the steak thickness) depth of inoculation before freezing and vacuum packaging in order to be able to record temperatures while steaks were in the solid frozen state. A preliminary experiment (data not shown) was performed to identify the amount of time needed for steaks to be thawed at 4 or 25°C in order to reach a geometric temperature of 0 to 4°C and 20 to 25°C, respectively. Consequently, before cooking, non-intact beef steaks that were 2.4 cm thick were thawed at 4 or 25°C for 5.5 and 7.0 hr, respectively, and 1.2 cm thick steaks were thawed 4 or 25°C for 3.0 and 5.0 hr, respectively.

Cooking

On day-5 of storage, packaged steaks and bag purge were weighed before cooking. Except for steaks that were cooked directly from the frozen state (explained above), a sterile thermocouple (Type-K, Pico Technology Limited) was inserted at the geometric center (0.6 or 1.2 cm for 1.2 and 2.4 cm thick steaks, respectively) of each steak and, if applicable, at the internal depth of inoculation. Steaks were cooked by (i) pan-broiling (Toastmaster Cool-Touch Griddle, Toastmaster Inc., Columbia, MI) or (ii) roasting (Magic Chef, Maytag Corp., Newton, IA) at 149°C until a geometric center steak temperature of 60°C was reached. The steaks that were pan-broiled were turned every 2 min (AMSA, 1995) during cooking to assure that all depths of the non-intact steak at least obtained the desired endpoint temperature of 60°C (Thippareddi et al., 2000). Temperatures of the geometric center and, if applicable, depth of internal inoculation were continuously monitored and recorded at 10 s intervals during cooking using the

PicoLog data acquisition software (Pico Technology Limited). After cooking samples were reweighed to determine cooking loss and immediately analyzed for total bacterial and *E. coli* O157:H7 populations.

Microbiological analyses

Immediately after cooking, a 1.61cm^2×2.4 cm core was excised from the center of each steak, placed in a 24-oz bag (Whirl-Pak, Nasco, Modesto, CA) and weighed. Ten ml of maximum recovery diluent (MRD; 0.85% NaCl and 0.1% peptone) were added to each bag and the samples were homogenized by pummeling in a Masticator (IUL Instruments, Barcelona, Spain) for 2 min. Ten-fold serial dilutions of the homogenate were made with 0.1% buffered peptone water (BPW; Difco). Appropriate dilutions were then spread-plated, in duplicate, on tryptic soy agar (TSA; Acubedia, Lansing, MI) containing 0.1% pyruvate (Fisher Scientific, Fair Lawn, NY) (TSAP) and TSAP containing 100 µg/ml rifampicin for the enumeration of total bacterial and *E. coli* O157:H7 populations, respectively. Plates were incubated at 35°C for 48 h before colony enumeration. The bacterial detection limit using this method was 0.3 log CFU/cm^2.

Statistical analysis

Experiments were repeated twice with three samples analyzed in each replicate. Bacterial counts were converted to log CFU/cm^2 before statistical analysis. For each cooking method (pan-broiling and roasting), cell counts, storage loss (purge in bag), pre- and post-cooking weights, cooking time, and core weight at each depth of inoculation (0.0, 0.3, 0.6, 0.9 and 1.2 cm) were analyzed as a one-way ANOVA using PROC MIXED of SAS.

Based on significance in the ANOVA (F<0.0.5), means were separated using a pairwise t-test comparison with significance set at the $P < 0.05$.

There is a significant variation in cooking times when cooking non-intact steaks (Shen et al., 2010). To account for this variation, time-temperature curves for each level of inoculation were normalized to the average time it took to reach the endpoint temperature for each cooking method. Each time point were multiplied by the average time to reach endpoint temperature for all steaks within cooking method divided by the observed time to reach endpoint temperature of each steak (average time to reach endpoint temperature/observed time to reach endpoint temperature). For each level of inoculation, all normalized cooking curves were analyzed using PROC REG in SAS for a best fit equation.

RESULTS AND DISCUSSION

E. coli O157:H7 populations of non-intact steaks

The thickness of steaks affected the extent of thermal inactivation of *E. coli* O157:H7. In general greater (P<0.05) populations were recovered after cooking from 1.2 than 2.4 cm thick steaks (Figure 5.1). Sporing (1999) also reported a greater recovery of *E. coli* O157:H7 from thinner blade tenderized steaks (1.3 cm) compared to thicker ones (3.2) after cooking on an electric skillet (pan-broiling) to an internal temperature of 60°C. Similarly, Shen et al. (2010) reported that thicker (4.0 cm) comminuted non-intact steaks had greater inactivation of *E. coli* O157:H7 than thinner (1.5 cm) steaks when cooked to a geometric center temperature of 65°C. Therefore, it appears that the amount of

recoverable pathogen cells after cooking is dependent on the thickness of the non-intact steaks.

The amount of recovered *E. coli* O157:H7 from either on or in non-intact steaks was dependant on the depth of inoculation. Recovered surface inoculated *E. coli* O157:H7 populations on non-intact steaks that were stored at 4°C and cooked by pan-broiling and roasting were 0.3±0.6 and 1.5±1.0 log CFU/cm^2, respectively (Figure 5.2). Even though the surfaces of the non-intact steaks were exposed to the cooking temperature of 149°C, protective enclaves/structures could have formed on the non-intact steaks as they cooked, thus providing a protective environment and shielding the pathogen from the heat of cooking (Thippareddi et al., 2000; USDA-FSIS, 2002a; USDA-FSIS, 2002b). Mechanical tenderization may have been the source of the niches as Gill et al. (2008) isolated inoclated *Listeria innocua* from in between and within damaged muscle fibers separated by tenderizing blades. Also, it has been proposed that an evaporative phenomenon occurs that would allow for the survival of surface bacteria (Blankenship, 1978; Blankenship and Craven, 1982; Blankenship et al., 1980; Harrison and Carpenter, 1989) which may dry the surface and lower the water activity and immediate temperature at the surface of the steak. The combination of low water activity and evaporative heat loss may allow for the survival of pathogens during cooking. Regardless of the reasoning, surface *E. coli* O157:H7 contamination was not eliminated by cooking non-intact steaks to a 60°C internal temperature.

In general, from the surface (0.0 cm) to the geometric center (1.2 cm) there was a trend of increasing numbers of recovered *E. coli* O157:H7 cells (Figures 5.1 and 5.2). However, probably due to the large variability observed, this trend was not significant

($P \geq 0.05$) for steaks that were cooked by roasting (Figures 5.1 and 5.2). To my knowledge these are the first data that have analyzed the thermal inactivation of *E. coli* O157:H7 inoculated at the same concentration at different depths into non-intact steaks; therefore, differences in recovered pathogen population can be attributed to the depth of the inoculation. The aforementioned data suggest that the extent of thermal inactivation of *E. coli* O157:H7 is dependent on the depth of the pathogen within the steak with greater theremal inactivation being observed the closer the inoculum is to the surface of the cut.

Non-intact steaks stored at 4 (2.4 cm) and -20°C (2.4 and 1.2 cm) were inoculated with 3.9±0.1and 4.0±0.3 log CFU/cm^2, respectively. There was no observed difference ($P \geq 0.05$) in *E. coli* O157:H7 populations in non-intact beef steaks from the initial inoculation levels after storage at 4 or -20°C (3.7±0.2 and 3.8±0.4for log CFU/cm^2, respectively) for 5 days before cooking, which concurs with other published data that indicate *E. coli* O157:H7 populations remain unchanged in ground beef (Ansay et al., 1999; Barkocy-Gallagher et al., 2002; Black, 2010; Jackson et al, 1996; Sage and Ingham, 1998) and beef trimmings (Dykes, 2000, 2006) during freezing. Regardless of temperature (4 or -20°C), *E. coli* O157:H7 populations within non-intact steaks were unaffected by a 5-day storage.

The method of thawing before cooking did not have a significant effect ($P \geq 0.05$) on *E. coli* O157:H7 populations, which was expected for steaks that were cooked from the frozen state or thawed at 4°C (Sage and Ingham, 1998). Interestingly, to simulate thawing on a kitchen countertop, 1.2 and 2.4 cm thick non-intact beef steaks were also thawed for 3.0 and 5.0 hr, respectively, at the abusive temperature of 25°C which was insufficient to allow pathogen growth on or in non-intact beef steaks. Ingham et al.

(2005) reported similar results in ground beef and suggested that thawing portions > 453 g at ≤ 22°C for ≤ 9h was not a "particularly hazardous" practice. According to our results, as long as non-intact steaks are cooked directly after thawing, the method of thawing doesn't seem to affect the thermal inactivation of pathogen during cooking.

The method of cooking had a significant (P<0.05) effect on inactivated *E. coli* O157:H7 populations. In general, greater (P<0.05) *E. coli* O157:H7 populations were recovered from non-intact steaks that were cooked to a geometric center temperature of 60°C by roasting compared to pan-broiling (Figures 5.1 and 5.2) suggesting that cooking by pan-broiling may better inactivate internalized pathogens. Similarly, Gill and McGinnis (2004) suggested that cooking non-intact blade-tenderized steaks to a target medium-rare temperature (61°C) by pan-broiling may be adequate to assure the microbial safety of meat if the initial intact product is of "good microbial condition." However, it should be noted that Gill and McGinnis (2004) removed steaks from the cooking apparatus at an internal temperature of 61°C and monitored the further rise in temperature (63 to 68°C) before sampling where as in this study samples were collected immediately when the endpoint temperature of 60°C was reached. When cooking blade tenderized steaks to 60°C, pan-broiling is preferred to roasting to assure safe consumption.

Purge formation

Tenderized, inoculated, and vacuum packaged non-intact beef steaks that were 1.2 and 2.4 cm thick averaged 92.8±13.2 and 183±25.6 g. By weight (g), 2.4 cm thick steaks produced more (P<0.05) purge than 1.2 cm thick steaks (Table 5.2). However, by percentage (weight of purge (g)/ weight of steak (g)), similar (P≥0.05) purge volume was

observed in both 1.2 and 2.4 cm thick non-intact steaks at 25°C than at 4°C (Table 5.3). Purge loss for steaks stored at 4°C for 5 days and for those thawed at 4°C after frozen storage were comparable. Also, at each thickness non-intact steaks thawed at 25°C produced the most amount of purge containing *E. coli* O157:H7 available for the potential spread of contamination throughout the steak and cooking environment (Table 5.2 and 5.3). It appears that final endpoint thawing temperature is a major determinant of the volume of purge formation; therefore, to best decrease the amount of purge available for the spread of contamination thawing or storage of non-intact steaks at 4°C would be preferred.

E. coli O157:H7 populations in purge

Interestingly, the *E. coli* O157:H7 populations in the purge for all blade tenderized non-intact steaks averaged 4.2±0.5 log CFU/ml and were not affected ($P \geq 0.05$) by steak thickness, depth of inoculation, and method of thawing (Figure 5.3), which indicates that blade tenderization along with purge formation may provide a means of pathogen spread throughout non-intact steaks. Even without tenderization, bacteria have been shown to be able to migrate into meat (Elmossalami and Wassef, 1971; Gill and Penney, 1977, 1982; Johnston et al., 1978; Maxcy, 1981; Sikes and Maxcy, 1980; Thomas, 1966). Specifically, Johnston et al. (1978) observed that *Salmonella* was able to migrate from the surface of muscle into the interior without assistance from a physical process (e.g. blade tenderization) and, in comparison, roasts that were blade tenderized contained 1 log higher level of *Salmonella* Newport than non-tenderized roasts. In addition to the natural

tendency for bacteria to migrate, blade tenderization and the formation of purge provides additional means of microbial cell dispersion in a contaminated meat product.

Cooking temperatures, time, and losses and final pH of cooked non-intact steaks

Similar ($P \geq 0.05$) starting cooking temperatures were obtained for both thicknesses of non-intact steaks thawed at 4°C and 25°C (Table 5.4). It is important to the analysis of the data that the starting cooking temperatures are similar because any observed differences between 1.2 and 2.4 cm thick non-intact steaks in cooking or pathogen data can then be attributed to the thickness of the steak and not to the starting cooking temperature. Considering this, thickness of non-intact steak and method of thawing had a significant ($P<0.05$) effect on cooking times. Faster ($P<0.05$) cooking times were observed for 1.2 cm compared to 2.4 thick non-intact steaks and steaks thawed and 25°C as opposed to 4°C or cooked directly from the frozen state (Table 5.5; Figures 5.4 and 5.5).

Higher ($P<0.05$) temperatures were observed at depths that were closer to the surface, and hence heating apparatus, of the non-intact steaks (Table 5.6). Also, more variation was explained by the normalized heating curves (i.e. higer observed R^2 value) the closer the temperature was recorded to the geometric center of the non-intact steak (Table 5.7), suggesting a more uniform (less unexplained variation) cooking in the middle of the steak as opposed to the external edges. Given that temperature differences exist at different depths of non-intact steaks during cooking and that there is greater unexplained variation in temperatures the closer to the heating element, thermocouples inserted into the geometric center of the steak to monitor the endpoint temperature do not

indicate the temperatures in other parts of the steak as suggested by Thippareddi et al. (2000). Similarly a single temperature probe may not reflect the temperature of the whole steak; therefore, cooking guidelines that only recommend observing a single internal temperature do not assure the safety of the whole steak but rather the portion of the meat that is in contact with the temperature monitoring device.

At 0.3 cm depth the modeled temperature profile fluctuated with every 2 min steak turning interval when cooking by pan-broiling, and fluctuations diminished the closer the temperature was recorded to the geometric center of the steaks (Figure 5.6). There were little fluctuations in the model temperature profiles of non-intact steaks that were cooked by roasting (Figure 5.7) and over 90% of the variation ($R^2 \geq 0.90$) could be explained by the modeled heating curves at all internal depths of non-intact steaks that were cooked by roasting (Table 5.7). In comparison, the data suggests a more uniform and less variable heat transfer associated with cooking by roasting than by pan-broiling. Observed temperature at 0.3, 0.6, and 0.9 cm depths were greater ($P<0.05$) in steaks cooked by pan-broiling than by roasting (Figures 5.6 and 5.7). In fact, for non-intact steaks that were cooked by pan-broiling the maximum observed temperatures at 0.3 and 0.6 cm depths of inoculation were above 71.1°C (Figure 5.6), a recommended final cooking temperature to assure the safe consumption of cooked ground beef (Rhee, 2003; USDA-ERS, 2002). However, internal temperatures of non-intact steaks cooked by roasting were not observed to be above 63.4±4.8°C. Therefore, cooking guideline to assure the safe consumption of meat products should take into account the method of cooking because different internal temperatures were observed when cooking to the same geometric endpoint temperature with different procedures.

Cooking times and loses and final pH values were comparable between non-intact steaks that were stored at 4°C for 5 days and -20°C for 5 days folled by thawing at 4°C (Tables 5.8, 5.9, and 5.10). Greater (P<0.05) cooking losses were observed for non-intact steaks that were cooked by pan-broiling than roasting (Table 5.8). The greatest cooking losses were observed for both 1.2 and 2.4 cm thick steaks that were cooked from the frozen state (Table 5.8). The method of thawing or cooking or non-intact steak thickness did not have a significant effect on the pH of the final cooked steaks and pH averages ranged from 5.67±0.07 to 5.74±0.05 (Table 5.9).

CONCLUSIONS

There is a significant uncontrolled variability due to uncontrolled confounding variables in experiments that characterize thermal inactivation of *E. coli* O157:H7 in non-intact steaks (Sporing 1999; USDA-FSIS, 2002a). In this study, there was a large source of variation as indicated by the reported standard deviations. Others have also reported a significant uncontrolled variability in recovered *E. coli* O157:H7 populations in cooked meat due to uncontrolled confounding variables in cooking experiments (Sporing, 1999; USDA-FSIS, 2002a). Given that there are unidentified variables that contribute to large variations in these experiments; it may be very difficult to make cooking recommendations based on the single parameter of endpoint temperature to consistently ensure the safe consumption of meat after cooking. More cooking parameters may need to be considered and controlled in order to ensure safer consumption of cooked meat.

Even though both apparatuses used in this study were set at the same cooking temperature and steaks were cooked to the same geometric endpoint temperature, the

amount of heat energy transferred into the non-intact steaks was different as indicated by the differing internal, non-geometric center temperature observed between the two cooking methods. There was an inverse relationship between recovered *E. coli* O157:H7 populations and the maximum observed temperature at each depth of inoculation, indicating that greater pathogen populations would be associated with lower internal temperatures. There were higher temperatures and greater pathogen reductions in steaks cooked by pan-broiling than roasting, therefore the method of cooking may be a determinant of the extent of thermal inactivation of internalized pathogen cells in addition to the endpoint temperature.

Ultimately, from these experiments, it is apparent that *E. coli* O157:H7 introduced into fresh beef remains viable during either frozen (-20°C) or cold storage (4°C) and, in most cases, populations remain unchanged. Also, thawing steaks to an internal temperature of 0 to 4°C at 4°C or of 20 to 25°C at 25°C did not have an effect on pathogen populations; therefore, the method of thawing may not increase the risk associated with the consumption of non-intact meat products as long as the cuts are cooked immediately when the desired thawing temperature is reached. In general after cooking, greater pathogen inactivation and non-geometric center temperatures were observed in non-intact steaks that were pan-broiled as compared to those that were roasted. Therefore, since difference were observed between pan-broiling and roasting, cooking method is a good candidate to be included in lethality guidelines that are designed to ensure the safe preparation and consumption of beef products, and pan-broiling is preferred to roasting when cooking non-intact beef to medium-rare temperatures.

TABLES AND FIGURES

Table 5.1. Description of treatments used in each of the two performed studies.

	Levels of treatments	
Treatment	Study 1	Study 2
Steak thickness (cm)	2.4	1.2, 2.4
Storage	5 days at 4°C	5 days at -20°C
Method of thawing	None	None, 4°C, 25°C
Cooking method	Pan-broiling, roasting	Pan-broiling, roasting
Depth of inoculation (cm)	0.0, 0.3, 0.6, 0.9, 1.2	0.0, 0.6 (1.2 cm thick steaks) 0.0, 0.6, 1.2 (2.4 cm thick steak)

Table 5.2. Mean purge loss (g ± SD) of non-intact beef steaks after thawing and before cooking.

	Starting cooking temperature (°C)		
Thickness (cm)	Frozen(-20°C)[a]	Thawed at 4°C [b]	Thawed at 25°C[c]
1.2	-0.3±1.4 aC	3.3±1.5 bB	8.1±5.0 bA
2.4	0.2±0.9 aC	8.1±2.3 aB	15.6±4.7 aA

[a]Non-intact steaks were cooked from the frozen state.

[b]1.2 and 2.4 cm steaks were thawed for 5.5 and 7.0 hr, respectively.

[c]1.2 and 2.4 cm steaks were thawed for 3.0 and 5.0 hr, respectively.

Means with different lowercase letters within the same column are different ($P<0.05$).

Means with different uppercase letters within the same row are different ($P<0.05$).

Table 5.3. Mean purge loss (% ± SD) of non-intact beef steaks after thawing and before cooking.

	Starting cooking temperature (°C)		
Thickness (cm)	Frozen(-20°C)[a]	Thawed at 4°C [b]	Thawed at 25°C[c]
1.2	0.0±1.4 aC	3.4±1.7 aB	9.7±6.0 aA
2.4	0.0±0.0 aC	4.4±1.3 aB	9.6±2.7 aA

[a]Non-intact steaks were cooked from the frozen state.

[b]1.2 and 2.4 cm steaks were thawed for 5.5 and 7.0 hr, respectively.

[c]1.2 and 2.4 cm steaks were thawed for 3.0 and 5.0 hr, respectively.

Means with different lowercase letters within the same column are different ($P<0.05$).

Means with different uppercase letters within the same row are different ($P<0.05$).

Table 5.4. Mean geometric center temperature (°C ± SD) directly before cooking by pan-broiling or roasting of thawed 1.2 and 2.4 think non-intact beef steaks cooked to a geometric center temperature of 60°C.

	Starting cooking temperature (°C)		
Thickness (cm)	Frozen(-20°C)[a]	Thawed at 4°C [b]	Thawed at 25°C[c]
1.2	-5.4±2.5 aC	4.1±3.1 aB	24.0±3.6 aA
2.4	-8.1±3.1 bC	2.7±3.4 aB	22.6±1.8 aA

[a]Non-intact steaks were cooked from the frozen state.

[b]1.2 and 2.4 cm steaks were thawed for 5.5 and 7.0 hr, respectively.

[c]1.2 and 2.4 cm steaks were thawed for 3.0 and 5.0 hr, respectively.

Means with different lowercase letters within the same column are different ($P<0.05$).

Means with different uppercase letters within the same row are different ($P<0.05$).

Table 5.5. Mean cooking times (min ± SD) of 1.2 and 2.4 cm thick non-intact beef steaks cooked to a geometric center temperature of 60°C by pan-broiling or roasting.

		Starting cooking temperature (°C)		
Cooking method	Thickness (cm)	Frozen(-20°C)[a]	Thawed at 4°C [b]	Thawed at 25°C[c]
Pan-broiling	1.2	5.6±0.8 dA	3.7±0.5 dA	3.6±0.4 dA
	2.4	15.5±4.1 cA	10.4±2.5 cB	9.3±0.6 cB
Roasting	1.2	22.8±3.5 bA	17.4±3.2 bB	13.6±1.6 bB
	2.4	45.0±6.7 aA	32.5±6.3 aB	26.9±3.7 aC

[a]Non-intact steaks were cooked from the frozen state.

[b]1.2 and 2.4 cm steaks were thawed for 5.5 and 7.0 hr, respectively.

[c]1.2 and 2.4 cm steaks were thawed for 3.0 and 5.0 hr, respectively.

Means with different lowercase letters within the same column are different ($P<0.05$).

Means with different uppercase letters within the same row are different ($P<0.05$).

Table 5.6. Mean maximum observed temperatures (°C ± SD) at different depths of non-intact steaks during cooking by pan-broiling or roasting.

	Cooking method	
Depth (cm)	Pan-Broiling	Roasting
0.3	88.0±7.3 aA	63.4±4.8 aB
0.6	73.7±8.9 bA	61.3±2.0 abB
0.9	64.5±6.5 cA	60.5±2.0 bB

Means with different lowercase letters within the same column are different ($P<0.05$).
Means with different uppercase letters within the same row are different ($P<0.05$).

Table 5.7. Selection statistics for models chosen to predict the time and temperature relationship at each depth when cooking 2.4 cm non-intact steaks that were stored at 4°C for 5 days before cooking.

Cooking method	Depth	MSE	Adj R^2
Roasting	3	4.69	0.910
	6	3.96	0.933
	9	2.79	0.969
	12	1.06	0.995
Pan-broiling	3	10.22	0.585
	6	10.61	0.677
	9	4.82	0.921
	12	1.54	0.992

Table 5.8. Mean cooking losses (% ± SD) of 1.2 and 2.4 cm thick non-intact beef steaks after 5 days of storage at -20°C, thawing, and cooking to a geometric center temperature of 60°C by pan-broiling or roasting.

		Starting cooking temperature (°C)		
Cooking method	Thickness (cm)	Frozen(-20°C)[a]	Thawed at 4°C [b]	Thawed at 25°C[c]
Pan-broiling	1.2	35.6±3.8 aA	26.7±1.8 bB	26.4±2.8 aB
	2.4	34.8±3.0 aA	32.3±1.8 aAB	29.2±1.2 aB
Roasting	1.2	31.9±3.4 aA	21.1±2.5 cB	18.2±2.1 bB
	2.4	33.5±3.6 aA	24.1±2.5 bcB	22.2±1.2 bB

[a]Non-intact steaks were cooked from the frozen state.

[b]1.2 and 2.4 cm steaks were thawed for 5.5 and 7.0 hr, respectively.

[c]1.2 and 2.4 cm steaks were thawed for 3.0 and 5.0 hr, respectively.

Means with different lowercase letters within the same column are different ($P<0.05$).

Means with different uppercase letters within the same row are different ($P<0.05$).

Table 5.9. Mean pH values[a] (pH ± SD) of thawed 1.2 and 2.4 cm thick non-intact beef steaks cooked to a geometric center temperature of 60°C by pan-broiling or roasting.

		Starting cooking temperature (°C)		
Cooking method	Thickness (cm)	Frozen(-20°C)[a]	Thawed at 4°C [b]	Thawed at 25°C[c]
Pan-broiling	1.2	5.72±0.04	5.74±0.05	5.71±0.04
	2.4	5.71±0.04	5.72±0.05	5.67±0.07
Roasting	1.2	5.59±0.06	5.70±0.04	5.72±0.03
	2.4	5.64±0.07	5.67±0.04	5.71±0.04

[a]There were no observed significant differences between pH values.

[b]Non-intact steaks were cooked from the frozen state.

[c]1.2 and 2.4 cm steaks were thawed for 5.5 and 7.0 hr, respectively.

[d]1.2 and 2.4 cm steaks were thawed for 3.0 and 5.0 hr, respectively.

Means with different lowercase letters within the same column are different ($P<0.05$).

Means with different uppercase letters within the same row are different ($P<0.05$).

Table 5.10. Cooking data (mean ± SD) of non-intact steaks stored at 4°C for 5 days and cooked to an internal temperature of 60°C.

Cooking Method	Cooking Loss (%)	Core (g)	pH	Cooking Time (min)
Roasting	14.2	8.3±0.9	5.77±0.07	26.2±4.3
Pan-broiling	18.7	9.1±0.9	5.93±0.10	11.5±1.2

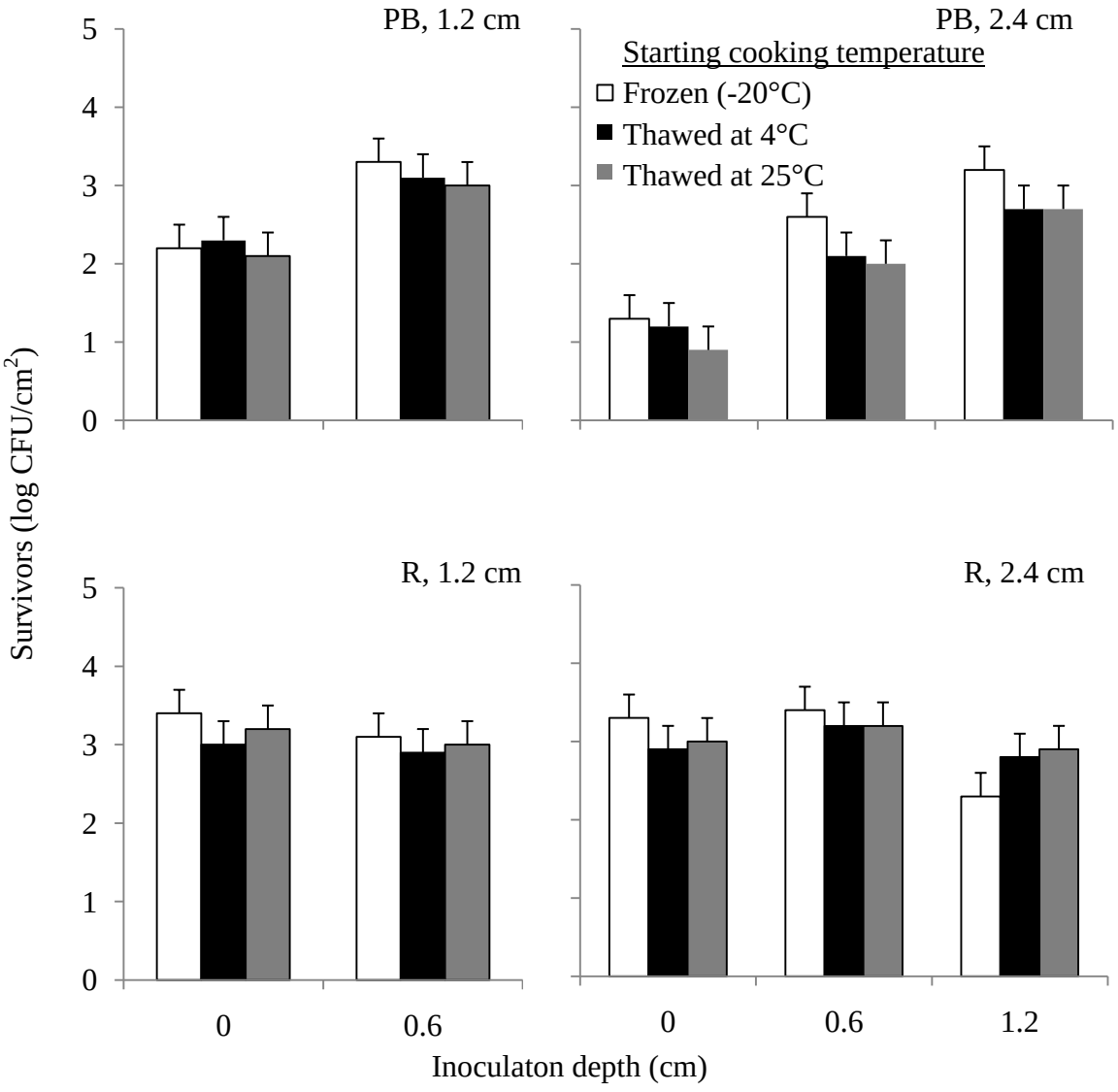

Figure 5.1 (Data in Appendix Table 10). Mean *E. coli* O157:H7 populations (log CFU/cm^2±SD) from 1.2 and 2.4 cm thick non-intact steaks cooked from the frozen state (-20°C) or after thawing at 4 and 25°C to a geometric center temperature of 60°C by pan-broiling (PB) or roasting (R) after storage at -20°C for 5 days. Individual steaks were inoculated with 3.7 log CFU/cm^2 at single depth of inoculation.

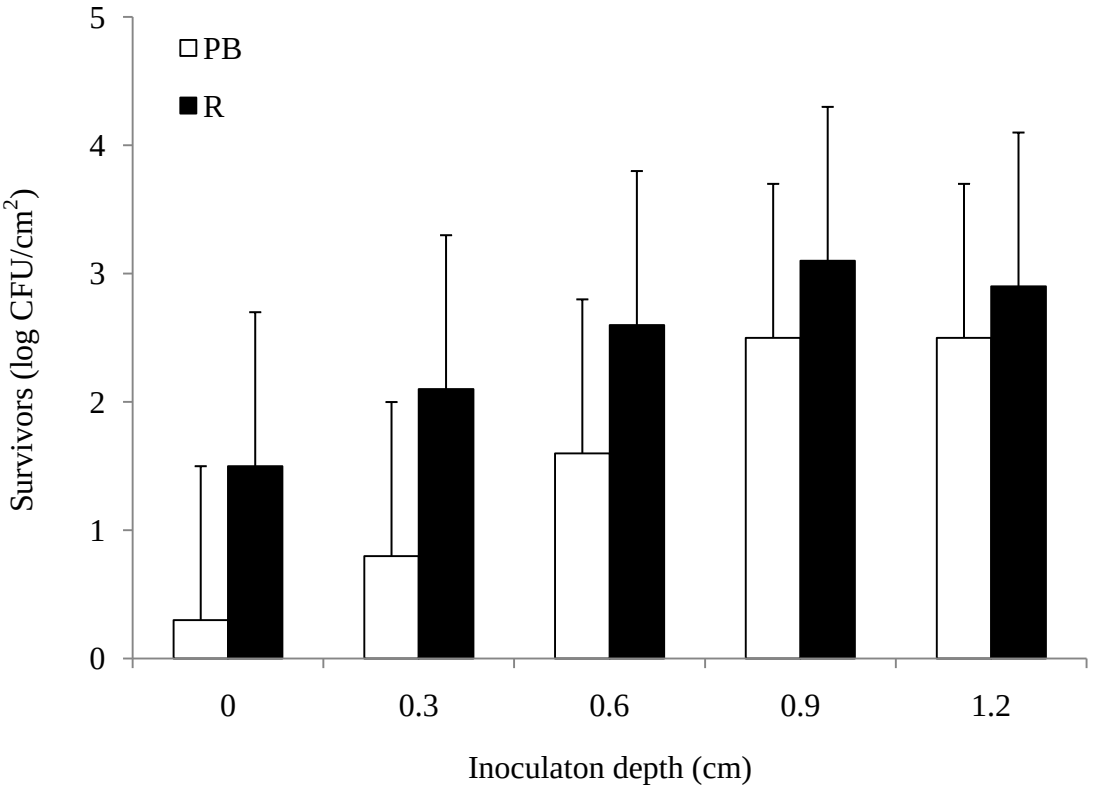

Figure 5.2 (Data in Appendix Table 11). Mean *E. coli* O157:H7 populations (log CFU/cm^2±SD) at different depths of 2.4 cm non-intact steaks after cooking at 149°C to a geometric center temperature of 60°C by pan-broiling (PB) and roasting (R) after storage at 4°C for 5 days. Individual steaks were inoculated with 3.5 log CFU/cm^2 at a single depth of inoculation.

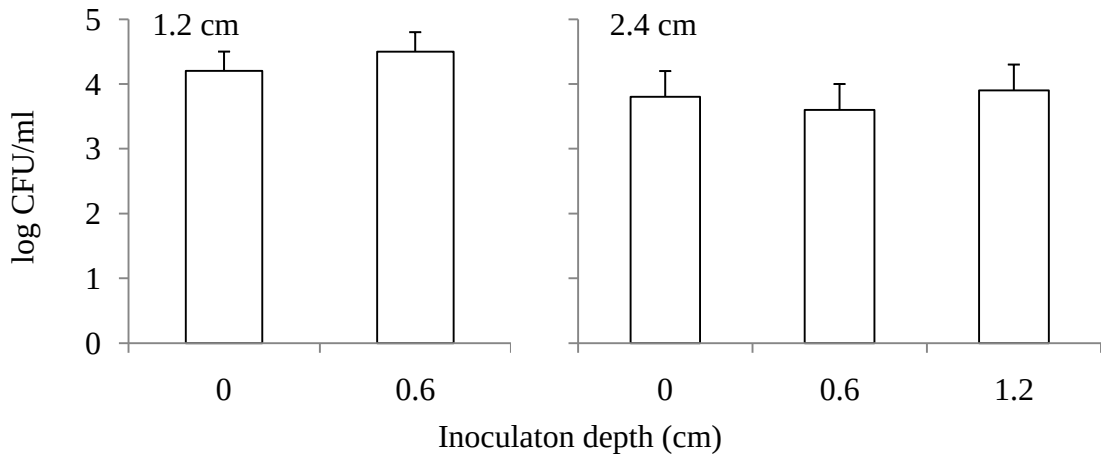

Figure 5.3 (Data in Appendix Table 12). Mean *E. coli* O157:H7 populations (log CFU/ml±SD) in purge from 1.2 and 2.4 cm thick non-intact steaks after thawing at 25°C and before cooking. Individual steaks were inoculated with 3.7 log CFU/cm^2 at single depth of inoculation.

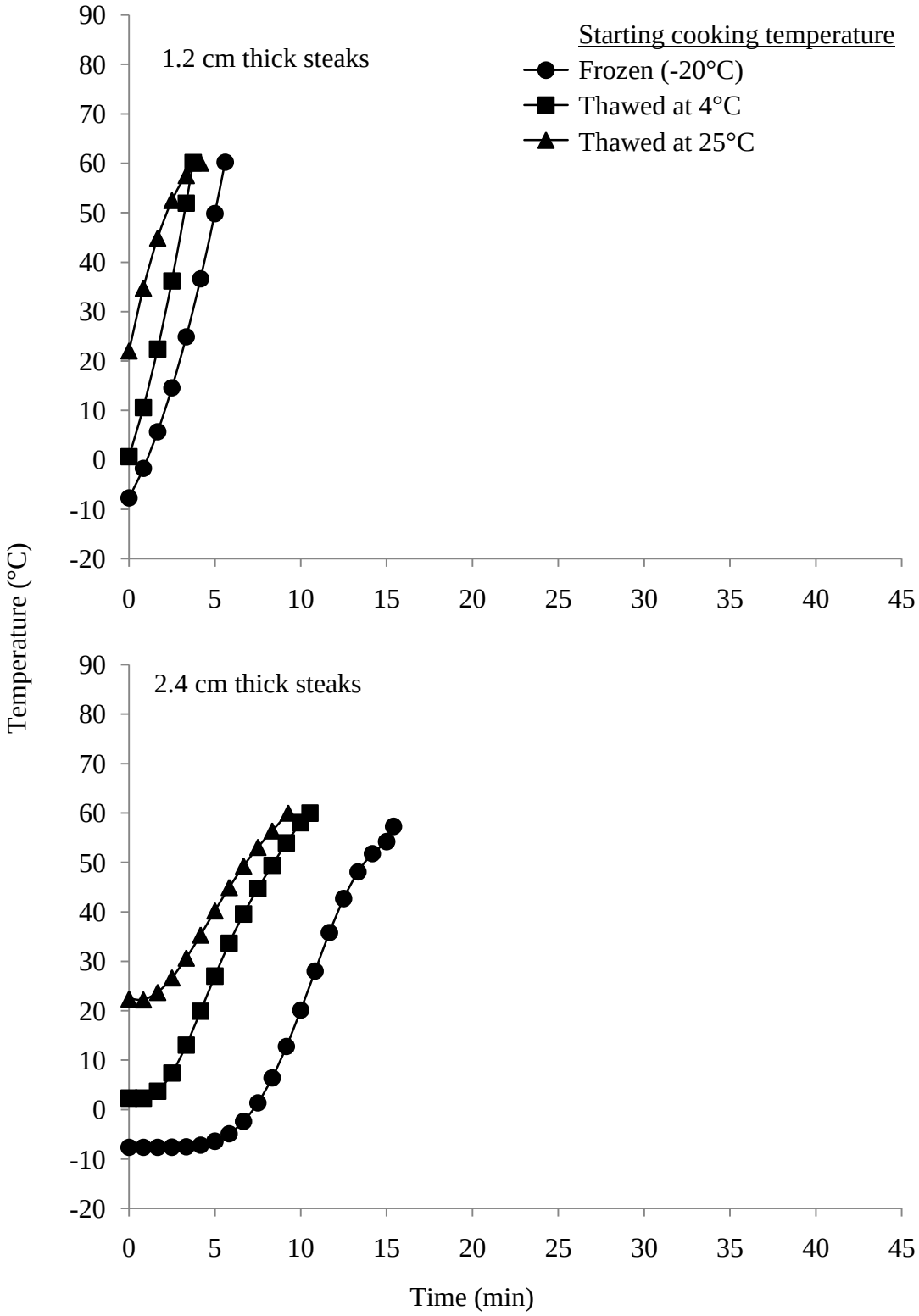

Figure 5.4 (Data in Appendix Table 13). Cooking time (min) and temperature curves for 1.2 and 2.4 cm thick non-intact beef steaks cooked by pan-broiling from frozen or thawed at 4 or 25C.

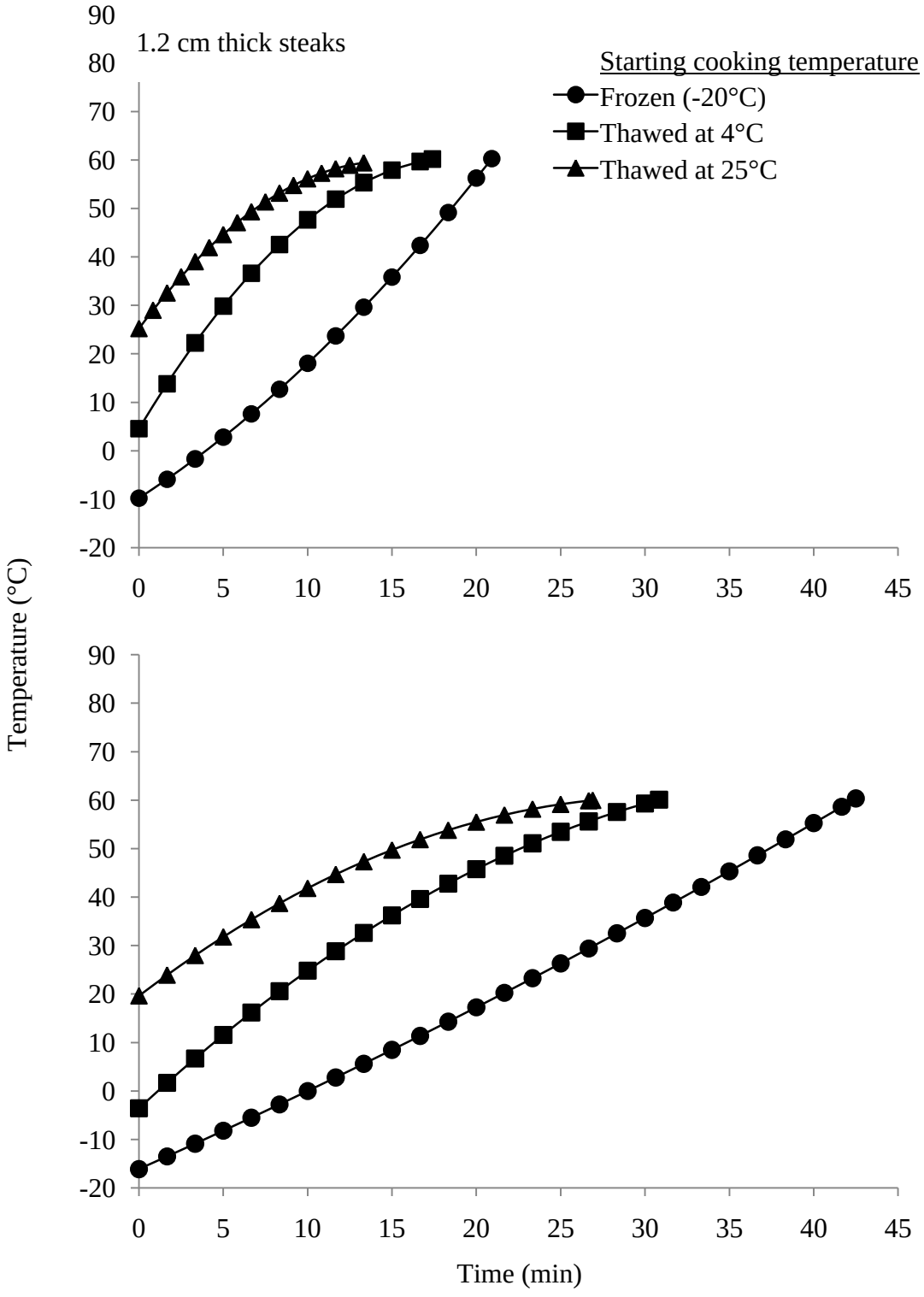

Figure 5.5 (Data in Appendix Table 14). Cooking time (min) and temperature curves for 1.2 and 2.4 cm thick non-intact beef steaks cooked by roasting from frozen or thawed at 4 or 25C.

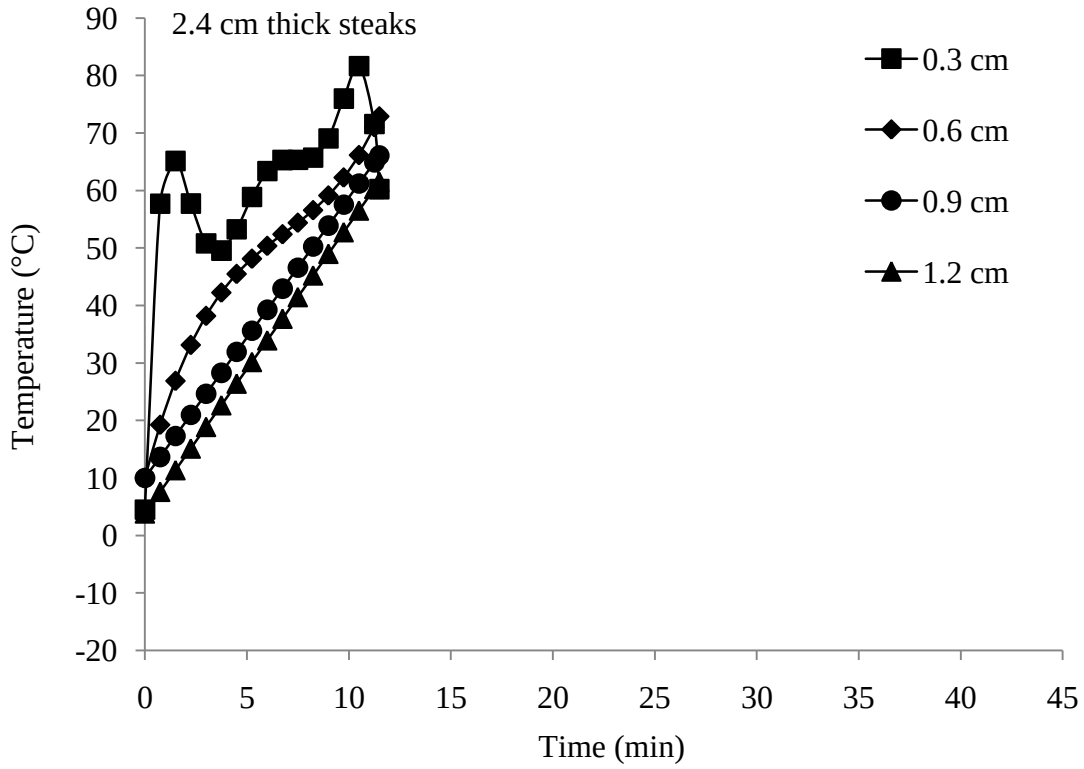

Figure 5.6 (Data in Appendix Table 15). Cooking time and temperature curves of non-intact beef steaks cooked by pan-broiling to 60°C at the geometric center.

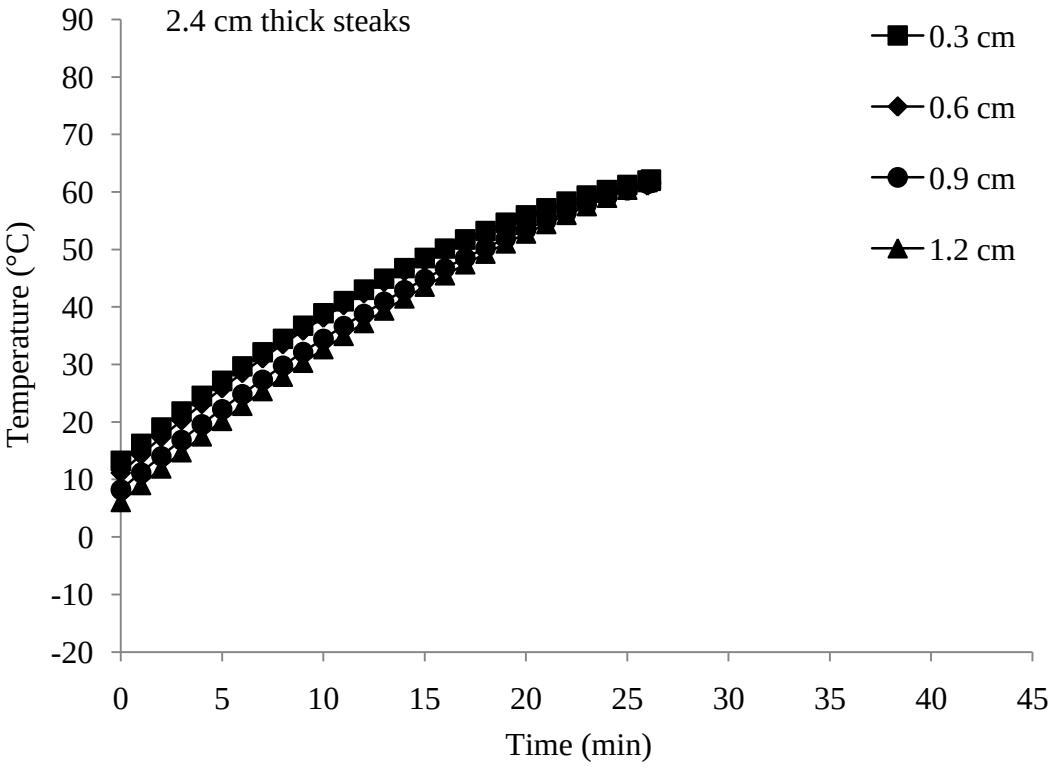

Figure 5.7 (Data in Appendix Table 16). Cooking time and temperature curves of non-intact beef steaks cooked by roasting to 60°C at the geometric center.

CHAPTER 6

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APPENDIX

TABLES

Appendix Table 1 (Figure 3.1 and 3.2). Effect of hydration during inoculation and fluid flow during storage and on extent of loosely and firmly attached *Escherichia coli* O157:H7 populations (log CFU/cm^2 ± standard deviation) and biofilm strength of attachment (Sr ± standard deviation) on stainless steel coupons stored for 4 days at 15°C under static and fluid flow (60 rpm) conditions in tryptic soy broth diluted 10-fold with sterile distilled water.

		Static			Fluid flow			
Inoculum hydration	Day	Total attached[a]	Loosely attached[b]	Firmly attached[c]	Total attached	Loosely attached	Firmly attached	Sr[d]
Dry[e]	0	3.1 ± 0.4 L	3.0 ± 0.5	2.6 ± 0.3	3.1 ± 0.4 L	3.0 ± 0.5	2.6 ± 0.4	0.18 ± 0.07 a
	0.25	3.5 ± 0.4 JKL	3.4 ± 0.5	2.4 ± 0.4	3.4 ± 0.7 KL	3.3 ± 0.7	2.4 ± 0.7	0.08 ± 0.07 cde
	0.5	3.5 ± 0.5 JKL	3.5 ± 0.5	2.2 ± 0.7	3.9 ± 0.6 HIJ	3.9 ± 0.6	2.2 ± 0.5	0.04 ± 0.07 e
	1	4.3 ± 0.2 H	4.3 ± 0.2	2.8 ± 0.6	5.1 ± 0.4 FG	5.1 ± 0.4	2.8 ± 0.3	0.03 ± 0.07 e
	2	5.8 ± 0.6 E	5.7 ± 0.6	4.8 ± 0.7	6.4 ± 0.5 CD	6.4 ± 0.6	4.8 ± 0.6	0.06 ± 0.07 de
	4	7.3 ± 0.2 A	7.2 ± 0.2	6.7 ± 0.3	7.0 ± 0.2 AB	6.9 ± 0.3	6.7 ± 0.2	0.15 ± 0.07 ab
Hydrated[f]	0	3.4 ± 0.2 KL	3.4 ± 0.2	2.2 ± 0.4	3.4 ± 0.2 KL	3.4 ± 0.2	2.2 ± 0.4	0.04 ± 0.07 e
	0.25	4.0 ± 0.2 HI	4.0 ± 0.2	3.1 ± 0.2	3.6 ± 0.1 IJK	3.5 ± 0.1	2.9 ± 0.3	0.08 ± 0.07 cde
	0.5	4.3 ± 0.2 H	4.3 ± 0.2	3.0 ± 0.4	3.9 ± 0.2 HIJ	3.8 ± 0.2	2.9 ± 0.4	0.04 ± 0.08 e
	1	5.3 ± 0.2 F	5.3 ± 0.2	4.0 ± 0.3	4.8 ± 0.3 G	4.8 ± 0.3	3.8 ± 0.4	0.04 ± 0.07 e
	2	6.7 ± 0.3 BC	6.6 ± 0.3	5.8 ± 0.3	6.2 ± 0.3 DE	6.1 ± 0.3	5.6 ± 0.5	0.11 ± 0.07 bcd
	4	7.0 ± 0.2 AB	6.9 ± 0.3	6.3 ± 0.4	6.7 ± 0.5 BC	6.6 ± 0.6	5.9 ± 0.2	0.14 ± 0.08 abc

[a]Total = log(loosely attached (CFU/cm^2) + Firmly attached (CFU/cm^2)).
[b]*E. coli* O157:H7 populations removed from stainless steel coupons with 30 s of vortexing at 1,000 rpm.
[c]*E.coli* O157:H7 populations remaining on stainless steel coupon after vortexing for 30s at 1,000 rpm.
[d]Sr = Total – Loosely attached. Fluid flow did not affect Sr; therefore, LSmeans presented which average the values observed for the static and fluid flow conditions.
[e]Coupons were inoculated by placing the inoculum (0.1 ml) directly on to the coupon and then spreading it over the entire surface with a sterile bent glass and allowed to dry for 10 minutes.
[f]Coupons were inoculated by submersion in a liquid suspension (40 ml) of the inoculum for 10 minutes.
Means with different uppercase letters are different (P<0.05).
Means with different lowercase letters are different (P<0.05).

Appendix Table 2 (Figure 3.3 and 3.4). Effects of 10-fold diluted tryptic soy broth (dTSB), filter-sterilized beef-grinder washings (FSBGW), and non-sterile beef-grinder washing (NSBGW) and fluid flow (60 rpm) during storage on attached *Escherichia coli* O157:H7 populations (log CFU/cm^2 ± standard deviation) and strength of attachment (Sr ± standard deviation) on stainless steel coupons stored for 4-days at 15°C.

		Static			Fluid Flow			
Substrate	Day	Total attached[a]	Loosely attached[b]	Firmly attached[c]	Total attached	Loosely attached	Firmly attached	Sr[d]
dTSB	0	3.2 ± 0.3 GHI	3.1 ± 0.3	2.0 ± 0.4	3.2 ± 0.3 GHI	3.1 ± 0.3	2.0 ± 0.4	0.05 ± 0.09 d
	1	3.9 ± 0.3 F	3.9 ± 0.3	2.5 ± 0.4	3.1 ± 0.2 KI	2.9 ± 0.2	2.4 ± 0.3	0.08 ± 0.09 cd
	2	5.1 ± 0.3 D	5.1 ± 0.3	3.4 ± 0.5	4.7 ± 0.3 E	4.7 ± 0.3	3.5 ± 0.1	0.02 ± 0.09 d
	4	6.4 ± 0.5 AB	6.4 ± 0.5	5.0 ± 0.4	6.3 ± 0.2 B	6.2 ± 0.2	5.2 ± 0.3	0.03 ± 0.09 d
FSBGW	0	3.2 ± 0.3 GHI	3.0 ± 0.3	2.0 ± 0.2	3.2 ± 0.3 GHI	3.0 ± 0.3	2.0 ± 0.2	0.05 ± 0.09 d
	1	3.4 ± 0.3 G	3.4 ± 0.3	2.1 ± 0.3	2.0 ± 0.2 JK	2.0 ± 0.2	1.0 ± 0.5	0.04 ± 0.09 d
	2	4.4 ± 0.5 E	4.3 ± 0.5	3.3 ± 0.3	3.3 ± 0.3 GHI	3.3 ± 0.3	2.1 ± 0.2	0.04 ± 0.09 d
	4	6.7 ± 0.6 A	6.6 ± 0.6	5.9 ± 0.6	5.6 ± 0.3 C	5.5 ± 0.3	4.6 ±0.3	0.07 ± 0.09 cd
NSBGW	0	3.2 ± 0.1 GHI	3.2 ± 0.1	2.3 ± 0.2	3.2 ± 0.1 GHI	3.2 ± 0.1	2.3 ± 0.2	0.05 ± 0.09 d
	1	3.0 ± 0.3 I	2.9 ± 0.4	1.9 ± 0.2	2.0 ± 0.6 JK	1.8 ± 0.6	1.4 ± 0.9	0.13 ± 0.09 c
	2	2.3 ± 0.4 J	2.1 ± 0.6	1.8 ± 0.2	1.1 ± 0.5 L	0.7 ± 0.4	0.8 ± 0.6	0.32 ± 0.09 a
	4	1.8 ± 0.4 K	1.7 ± 0.4	1.2 ± 0.6	0.6 ± 0.0 M	0.3 ± 0.0	0.3 ± 0.0	0.22 ± 0.09 b

[a]Total = log(loosely attached (CFU/cm^2) + Firmly attached (CFU/cm^2)).
[b]*E. coli* O157:H7 populations removed from stainless steel coupons with 30 s of vortexing at 1,000 rpm.
[c]*E.coli* O157:H7 populations remaining on stainless steel coupon after vortexing for 30s at 1,000 rpm.
[d]Sr = Total – Loosely attached. Fluid flow did not affect Sr; therefore, LSmeans presented which average the values observed for the static and fluid flow conditions.
Coupons were inoculated by submersion in a liquid suspension (40 ml) of the inoculum for 10 minutes.
Means with different uppercase letters are different (P<0.05).
Means with different lowercase letters are different (P<0.05).

Appendix Table 3 (Figure 3.5). Effect of substrate during incubation on the extent of attached total bacterial counts (log CFU/cm^2 ± standard deviation) on stainless steel coupons inoculated (3 log CFU/cm^2) with *E. coli* O157:H7 and stored for 4 days at 15°C.

	Static			Fluid Flow		
Day	dTSB	FSBGW	NSBGW[a]	dTSB	FSBGW	NSBGW
0	3.1 ±0.3 eA	3.0 ± 0.3 bA	3.3 ± 0.2 cA	3.1 ± 0.3 dA	3.0 ± 0.3cA	3.3 ± 0.2 cA
1	3.1 ± 0.2 cC	2.0 ± 0.2 cD	4.0 ± 0.5 bB	3.9 ± 0.3 cB	3.4 ± 0.4 cBC	4.8 ± 1.4 bA
2	4.7 ± 0.3 bCD	3.3 ± 0.3 bE	5.7 ± 0.6 aAB	5.1 ± 0.3 bBC	4.4 ± 0.4 bD	6.0 ± 0.1 aA
4	6.3 ± 0.2 aAB	5.6 ± 0.3 aB	6.0 ± 0.3 aAB	6.4 ± 0.5 aA	6.7 ± 0.5 aA	6.2 ± 0.4 aAB

Mean values with different lowercase letters in the same column are different (P<0.05).
Mean values with different uppercase letters in the same row are different (P<0.05).
dTSB: 10-fold diluted tryptic soy broth.
FSBGW: filter-sterilized beef-grinding residues.
NSBGW: beef-grinding residues (non-sterile).

Appendix Table 4 (Figure 3.6). Effects of substrates and fluid flow during storage on the extent of planktonic *Escherichia coli* O157:H7 (log CFU/ml ± standard deviation) recovered in substrates stored for 4 days at 15°C under static or fluid flow conditions.

	Static			Fluid Flow		
Day	dTSB	FSBGW	NSBGW	dTSB	FSBGW	NSBGW
0	3.4 ± 0.2 dA	3.3 ± 0.2 dA	3.2 ± 0.3 bA	3.4 ± 0.2 dA	3.3 ± 0.2 dA	3.2 ± 0.3 aA
1	4.8 ± 0.1 cB	4.1 ± 0.2 cC	4.0 ± 0.2 aC	5.4 ± 0.2 cA	4.8 ± 0.2 cB	3.3 ± 0.2 aC
2	6.6 ± 0.7 bB	4.9 ± 0.6 bC	2.9 ± 0.4 bD	7.6 ± 0.6 bA	6.4 ± 0.4 bB	2.8 ± 0.8 bD
4	9.0 ± 0.3 aA	7.3 ± 0.3 aC	3.1 ± 1.3 bD	9.4 ± 0.1 aA	8.5 ± 0.3 aB	2.7 ± 0.7 bD

Mean values with different lowercase letters in the same column are different ($P<0.05$).
Mean values with different uppercase letters in the same row are different ($P<0.05$).
dTSB: 10-fold diluted tryptic soy broth.
FSBGW: filter-sterilized beef-grinder washings.
NSBGW: beef-grinder washings (non-sterile).

Appendix Table 5 (Figure 3.7). Effects of substrate and fluid flow during storage on extent of planktonic total bacterial (log CFU/ml ± standard deviation) stored for 4 days at 15°C under static or fluid flow conditions.

	Static			Fluid Flow		
Day	dTSB	FSBGW	NSBGW	dTSB	FSBGW	NSBGW[a]
0	3.3 ± 0.1 dB	3.3 ± 0.2 dB	4.4 ± 0.9 dA	3.3 ± 0.1 dB	3.3 ± 0.2 dB	4.4 ± 0.9 dA
1	4.8 ± 0.1 cC	4.0 ± 0.2 cD	6.1 ± 0.6 cA	5.3 ± 0.2 cB	4.8 ± 0.1 cC	6.4 ± 0.1 cA
2	6.4 ± 0.1 bB	4.9 ± 0.5 bC	7.9 ± 0.6 bA	7.6 ± 0.6 bA	6.4 ± 0.4 bB	7.9 ± 0.6 bA
4	8.9 ± 0.4 aB	7.3 ± 0.3 aD	8.5 ± 0.6 aB	9.4 ± 0.2 aA	8.5 ± 0.3 aBC	9.0 ± 0.2 aAB

Mean values with different lowercase letters in the same column are different ($P<0.05$).
Mean values with different uppercase letters in the same row are different ($P<0.05$).
dTSB: 10-fold diluted tryptic soy broth.
FSBGW: filter-sterilized beef-grinding residues.
NSBGW: beef-grinding residues (non-sterile).

Appendix Table 6 (Figure 3.8). Effects of incubations on pH values[a] (pH ± standard deviation) of substrates stored for 4 days at15°C.

	Substrate		
Day	dTSB	FSBGW	NSBGW
0	5.99 ± 0.04 bA	6.15 ± 0.03 bA	6.01 ± 0.08 cA
1	5.94 ± 0.04 bA	6.07 ± 0.05 bA	5.62 ± 0.44 dB
2	5.80 ± 0.13 bB	6.00 ± 0.05 bB	6.85 ± 0.75 bA
4	6.62 ± 0.46 aB	6.59 ± 0.41 aB	7.69 ± 0.19 aA

[a]Fluid flow did not have a significant (P<0.05) effect on pH values therefore it is not presented in the table.
Mean values with different lowercase letters in the same column are different (P<0.05).
Mean values with different uppercase letters in the same row are different (P<0.05).
dTSB: 10-fold diluted tryptic soy broth.
FSBGW: filter-sterilized beef-grinder washings.
NSBGW: beef-grinder washings (non-sterile).

Appendix Table 7 (Figure 3.9). Effect of sanitizer challenge and growth substrate on the attachment of *Escherichia coli* O157:H7 (log CFU/cm^2 ± standard deviation) on stainless steel coupons after exposure to the manufacturer's maximum recommended concentration of sanitizer.

		Growth Substrate	
Sanitizer	Exposure time	Beef Grinder Washings	Ten-fold diluted TSB
Water	1	4.9 ± 0.1 aB	5.4 ± 0.1 aA
	10	4.9 ± 0.1 aB	5.5 ± 0.1 dA
Peroxyacetic acid / Octanoic acid mixture (0.26%)	1	0.6 ± 0.1 dA	0.7 ± 0.1 dA
	10	0.6 ± 0.1 dA	0.6 ± 0.1 dA
Quaternary ammonium compound (300 ppm)	1	1.4 ± 0.1 bB	2.8 ± 0.1 bA
	10	0.6 ± 0.1 dA	0.8 ± 0.1 dA
Sodium Hydroxide (200 ppm)	1	0.9 ± 0.1 cB	1.5 ± 0.1 cA
	10	0.6 ± 0.1 dA	0.6 ± 0.1 dA

Mean values with different lowercase letters in the same column are different (P<0.05).
Mean values with different uppercase letters in the same row are different (P<0.05).

Appendix Table 8 (Figure 3.10). Effect of hydration before sanitizer challenge on extent of attached *Escherichia coli* O157:H7 populations (log CFU/cm^2 ± standard deviation) on stainless steel coupons after exposure to the manufacturer's maximum recommended concentration of sanitizer.

	Initial biofilm hydration level[a]	
Sanitizer	Dried	Hydrated
Water	4.6 ± 0.1 aB	5.8 ± 0.1 aA
Peroxyacetic acid / Octanoic acid mixture (0.26%)	0.6 ± 0.1 dA	0.6 ± 0.1 dA
Quaternary ammonium compound (300 ppm)	1.2 ± 0.1 bB	1.8 ± 0.1 bA
Sodium Hydroxide (200 ppm)	0.9 ± 0.1 cA	0.9 ± 0.1 cA

[a]Stainless steel coupons with attached bacteria were allowed to dry a room temperature for 30 minutes or kept hydrated before sanitizer challenge.
Mean values with different lowercase letters in the same column are different (P<0.05).
Mean values with different uppercase letters in the same row are different (P<0.05).

Appendix Table 9 (Figure 3.11). Effect of sanitizer exposure time and growth substrate on extent of attached total bacterial population (log CFU/cm^2 ± standard deviation) on stainless steel coupons after exposure to the manufacturer's maximum recommended concentration of sanitizer.

		Growth Substrate	
Sanitizer	Exposure time	Beef Grinder Washings	Ten-fold diluted TSB
Water	1	5.9±0.6 aA	5.4±0.6 aA
	10	5.9±0.6 aA	5.5±0.6 aA
Peroxyacetic acid / Octanoic acid mixture (0.26%)	1	1.5±0.6 cA	0.7±0.6 dB
	10	0.9±0.6 cA	0.6±0.6 dA
Quaternary ammonium compound (300 ppm)	1	2.9±0.6 bA	2.9±0.6 bA
	10	1.7±0.6 cA	0.8±0.6 dB
Sodium Hydroxide (200 ppm)	1	2.6±0.6 bA	1.6±0.6 cB
	10	0.7±0.6 cA	0.6±0.6 dA

Mean values with different lowercase letters in the same column are different (P<0.05).
Mean values with different uppercase letters in the same row are different (P<0.05).

Appendix Table 10 (Figure 5.1). Mean *E. coli* O157:H7 populations[a] (log CFU/cm^2±SD) from 1.2 and 2.4 cm thick non-intact steaks cooked to a geometric center temperature of 60°C by pan-broiling or roasting[b].

			Starting cooking temperature (°C)		
Cooking method	Thickness (cm)	Depth of Inoculation	Frozen(-20°C)	Thawed at 4°C	Thawed at 25°C
Pan-broiling	1.2	0	2.2 ± 0.7 c	2.3 ± 0.3 bc	2.1 ± 0.6 b
		6	3.3 ± 0.2 a	3.1 ± 0.3 a	3.0 ± 0.2 a
	2.4	0	1.3 ± 0.6 d	1.2 ± 0.7 d	0.9 ± 0.5 c
		6	2.6 ± 0.5 bc	2.1 ± 0.3 c	2.0 ± 0.4 b
		12	3.2 ± 0.2 a	2.7 ± 1.0 ab	2.7 ± 0.2 a
Roasting	1.2	0	3.4 ± 0.2 a	3.1 ± 0.3 a	3.3 ± 0.1 a
		6	3.1 ± 0.5 ab	3.0 ± 0.2 a	3.2 ± 0.2 a
	2.4	0	3.3 ± 0.2 a	2.9 ± 0.5 a	3.2 ± 0.3 a
		6	3.4 ± 0.3 a	3.2 ± 0.2 a	3.2 ± 0.3 a
		12	2.3 ± 1.3 c	2.8 ± 0.4 a	2.9 ± 0.2 a

[a]Steaks were inoculated with contained 3.8±0.4 log CFU/ cm^2 *E. coli* O157:H7 before cooking.

[b]The the starting cook temperature after thawing did not have an effect ($P \geq 0.05$) on *E. coli* O157:H7 populations.

Means with different lowercase letters within the same column are different ($P < 0.05$).

Appendix Table 11 (Figure 5.2). Mean *E. coli* O157:H7 populations[a] (log CFU/cm^2±SD) at different depths of a 2.4 cm inoculated (3.7 CFU/cm^2) non-intact steaks after cooking at 149°C to a geometric center temperature of 60°C.

	Cooking method	
Depth of inoculation (cm)	Pan-broiling	Roasting
0.0	0.3±0.6 bA	1.5±1.0 bA
0.3	0.8±0.8 bA	2.1±1.1 abA
0.6	1.5±0.9 abA	2.6±0.8 aA
0.9	2.5±0.5 aA	3.1±0.3 aA
1.2	2.5±0.6 aA	2.9±0.5 aA

[a]Steaks contained 3.7±0.2 log CFU/cm^2 E. coli O157:H7 before cooking.

Means with different lowercase letters within the same column are different (P<0.05).

Means with different uppercase letters within the same row are different (P<0.05).

Appendix Table 12 (Figure 5.3). Mean *E. coli* O157:H7 populations[a] (log CFU/ml±SD) in purge from non-intact steaks before cooking and after thawing.

		Starting cooking temperature (°C)		
Thickness (cm)	Depth of Inoculation	Frozen(-20°C)[b]	Thawed at 4°C [c]	Thawed at 25°C[c]
1.2	0	-	-	4.2±0.5
	6	-	4.7±0.3	4.5±0.4
2.4	0	-	-	3.8±0.6
	6	-	-	3.6±0.5
	12	-	4.1±0.3	3.9±0.5

[a]There were no differences ($P \geq 0.05$) in pathogen populations recovered from purge. All values reported are similar.

[b]Non-intact steaks were cooked from the frozen state, therefore, purge was not allowed to form.

[c]Non-intact steaks were thawed at a temperature (4°C) that doesn't support *E. coli* O157:H7 growth, therefore, pathogen populations from 1.2 and 2.4 cm thick non-intact steaks inoculated at the geometric center (0.6 and 1.2 cm, respectively) were only analyzed.

Appendix Table 13 (Figure 5.4). Cooking time (min) and temperatures at the geometric center for 1.2 and 2.4 cm thick non-intact beef steaks stored at -20°C for 5 days then cooked by pan-broiling from the frozen state or after thawing at 4 and 25°C.

		Cooking time (min)																
Thickness (cm)	Start cooking temperature (°C)	0.0	1.7	3.3	3.6	3.7	4.2	5.0	5.6	6.7	8.3	9.3	10.0	10.4	11.7	13.3	15.0	15.5	Average
1.2	Frozen (-20°C)	-7.7	5.7	24.9			36.6	49.8	60.2										5.6±0.8
	Thawed at 4°C	0.6	22.4	51.9		60.1													3.7±0.5
	Thawed at 25°C	22.0	44.9	57.5	60.0														3.6±0.4
2.4	Frozen (-20°C)	-7.6	-7.6	-7.2			-6.4	-4.9		1.3	12.8		28.0		42.7	51.8	57.3	60.0	15.5±4.1
	Thawed at 4°C	2.3	3.7	13.1			19.9	27.0		39.6	49.4		58.1	60.0					10.4±2.5
	Thawed at 25°C	22.4	23.7	30.7			35.3	40.2		49.3	56.4	60.0							9.3±0.6

[a]time points were normalized to the average time to reach a geometric center temperature of 60°C by pan-broiling to account for the variation in cooking time oberseved among 1.2 and 2.4 cm thick steaks cooked from the frozen state or thawed at 4 or 25°C.

Appendix Table 14 (Figure 5.5). Cooking time[a] (min) and temperatures at the geometric center for 1.2 and 2.4 cm thick non-intact beef steaks stored at -20°C for 5 days then cooked by roasting from the frozen state or after thawing at 4 and 25°C.

		Cooking time (min)																	
Thickness (cm)	Start cooking temperature (°C)	0.0	3.3	6.7	10.0	13.6	16.7	17.4	20.0	22.8	23.3	26.7	26.9	30.0	32.5	33.3	36.7	40.0	45.0	Average
1.2	Frozen (-20°C)	-9.8	-1.7	7.6	18.0	29.6	42.4		56.3	60.3										22.8±3.5
	Thawed at 4°C	4.5	22.3	36.6	47.6	55.3	59.7	60.2												17.4±3.2
	Thawed at 25°C	25.2	39.0	49.3	56.1	59.4														13.6±1.6
2.4	Frozen (-20°C)	-16.1	-10.9	-5.5	0.0	5.6	11.4		17.3		23.3	29.4		35.7		42.1	48.6	55.3	60.3	45.0±6.7
	Thawed at 4°C	-3.6	6.7	16.2	24.8	32.6	39.6		45.8		51.1	55.6		59.3	60.1					32.5±6.3
	Thawed at 25°C	19.6	28.0	35.4	41.8	47.3	51.9		55.5		58.2	59.9	60.0							26.9±3.7

[a]time points were normalized to the average time to reach a geometric center temperature of 60°C by roasting to account for the variation in cooking time oberseved among 1.2 and 2.4 cm thick steaks cooked from the frozen state or thawed at 4 or 25°C.

Appendix Table 15 (Figure 5.6). Cooking time (min) and temperatures (°C) at different depths (cm) of 2.4 non-intact beef steaks storaged at 4°C for 5 days then cooked by pan-broiling to a geometric center temperature of 60°C.

	Cooking time (min)																
Depth (cm)	0.0	0.8	1.5	2.3	3.0	3.8	4.5	5.3	6.0	6.8	7.5	8.3	9.0	9.8	10.5	11.3	11.5
0.3	4.5	57.7	65.1	57.7	50.8	49.5	53.3	58.9	63.4	65.3	65.3	65.7	69.1	76.0	81.6	71.6	60.2
0.6	10.0	19.3	26.9	33.1	38.2	42.2	45.5	48.1	50.4	52.4	54.4	56.6	59.1	62.3	66.2	71.0	72.9
0.9	10.0	13.7	17.3	21.0	24.6	28.3	31.9	35.6	39.3	42.9	46.6	50.2	53.9	57.6	61.2	64.9	66.1
1.2	3.8	7.6	11.4	15.1	18.9	22.7	26.4	30.2	33.9	37.7	41.5	45.2	49.0	52.8	56.5	60.3	61.6

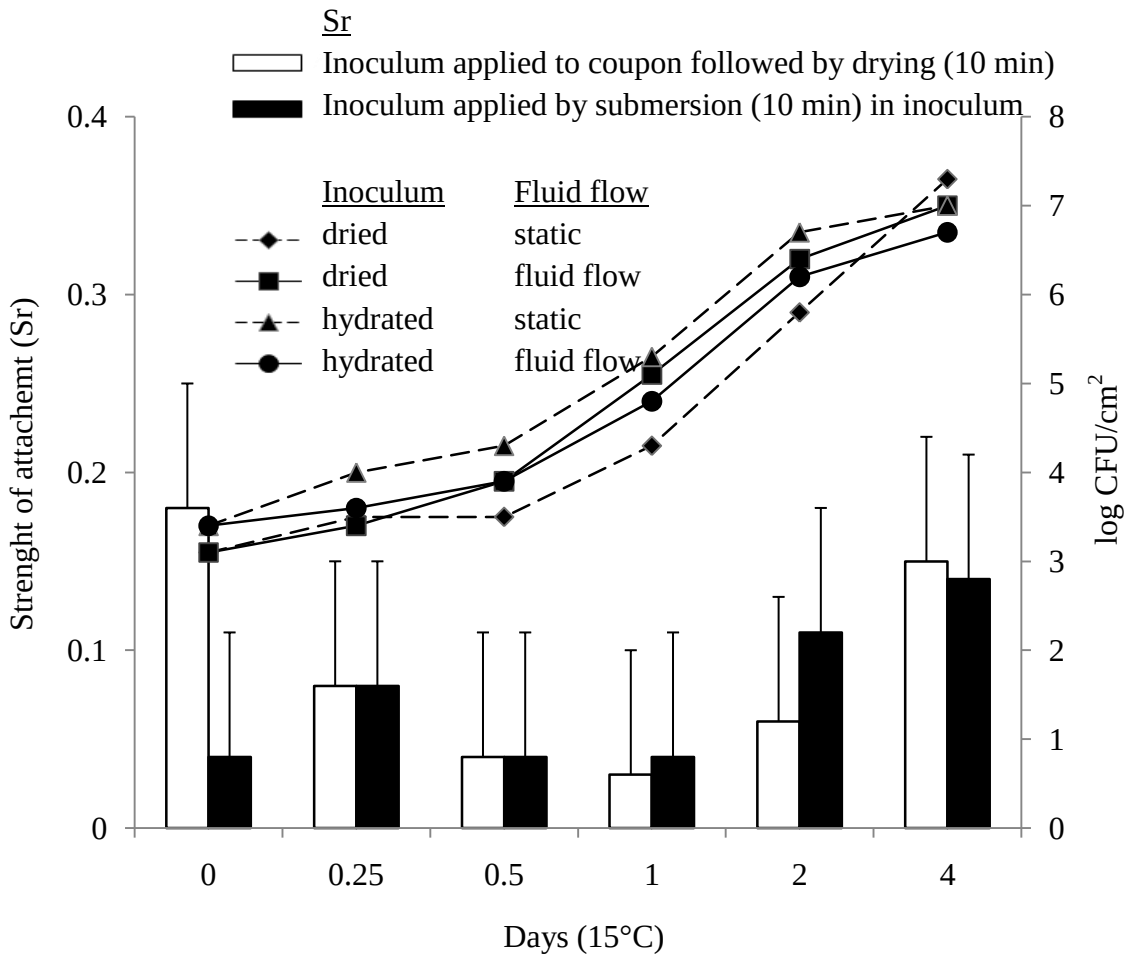
[a]time points were normalized to the average time (11.5±1.2 min) to reach a geometric center temperature of 60°C by pan-broiling to account for the variation in cooking time oberseved among steaks.

Appendix Table 16 (Figure 5.7). Cooking time [a] (min) and temperatures (°C) at different depths (cm) of 2.4 cm non-intact beef steaks stored at 4°C for 5 days then cooked by roasting to a geometric center temperature of 60°C.

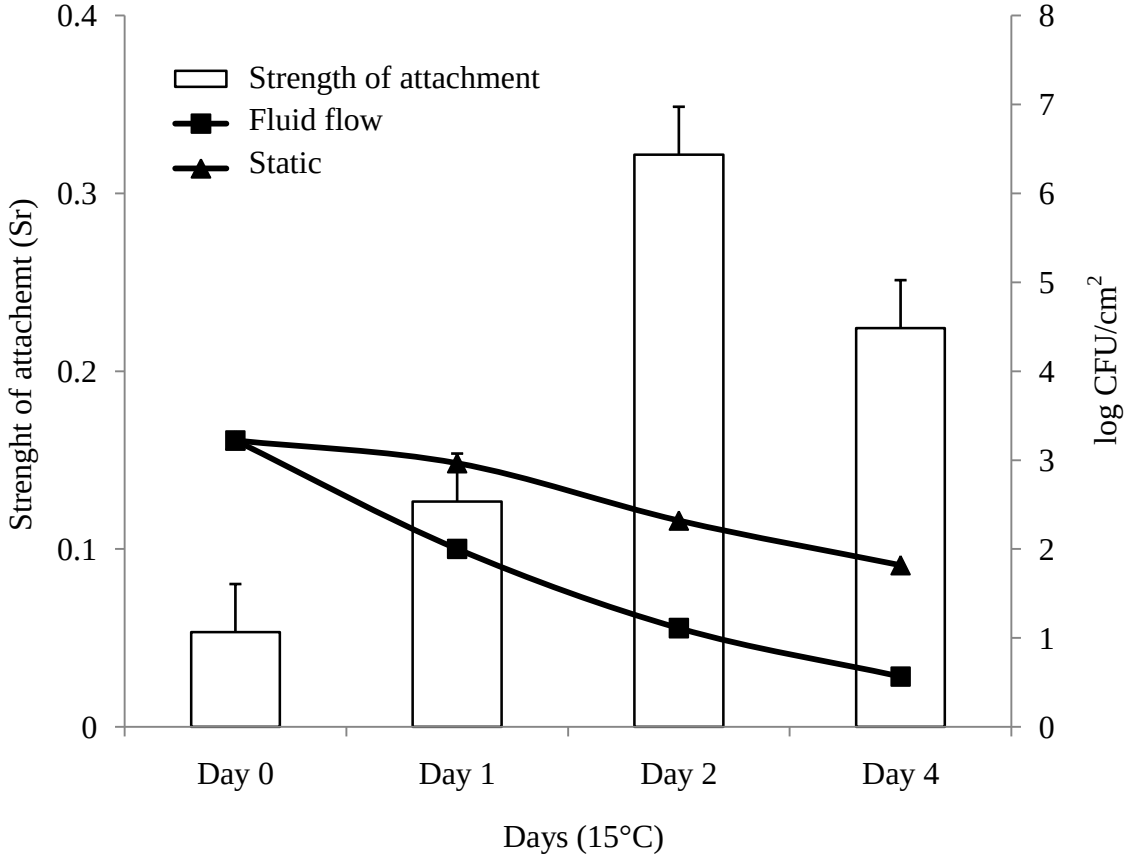
	Cooking time (min)													
Depth (cm)	0.0	2.0	4.0	6.0	8.0	10.0	12.0	14.0	16.0	18.0	20.0	22.0	24.0	25.0
0.3	13.2	19.1	24.5	29.7	34.5	38.9	43.0	46.8	50.2	53.2	55.9	58.3	60.3	61.2
0.6	11.1	17.3	23.1	28.5	33.5	38.1	42.4	46.2	49.7	52.7	55.4	57.7	59.6	60.4
0.9	8.2	14.0	19.6	24.8	29.8	34.4	38.8	42.9	46.7	50.2	53.5	56.4	59.1	60.3
1.2	6.1	11.9	17.5	22.8	27.9	32.7	37.2	41.5	45.5	49.3	52.8	56.0	59.0	60.4

[a]time points were normalized to the average time (26.2 ± 4.3 min) to reach a geometric center temperature of 60°C by roasting to account for the variation in cooking time oberseved among steaks.

FIGURES



Appendix Figure 1 (Figure 3.1 and 3.2). Effects of initial (day-0) hydration level during incubation on strength of attachment (Sr ± standard deviation) and growth (log CFU/cm^2 ± standard deviation) of *Escherichia coli* O157:H7 on stainless steel coupons stored for 4 days at 15°C in 10-fold diluted tryptic soy broth. Coupons were inoculated by placing the inoculums (0.1 ml) directly on the coupon and then spreading it over the entire surface with a sterile bent glass rod and dried for 10 min (dried) or submerged in a liquid suspension (40 ml) of inoculum for 10 min (hydrated) and stored under static and agitated conditions (60 rpm) to simulate a fluid flow.



Appendix Figure 2 (Figure 3.3 and 3.4). Effect of non-sterile beef-grinder washings (NSBGW) containing meat residues and natural flora on strength of attachment (Sr ± standard deviation) and growth (log CFU/cm^2 ± standard deviation) of Escherichia coli O157:H7 on stainless steel coupons stored for 4 days at 15°C under static or fluid flow (60 rpm) conditions.

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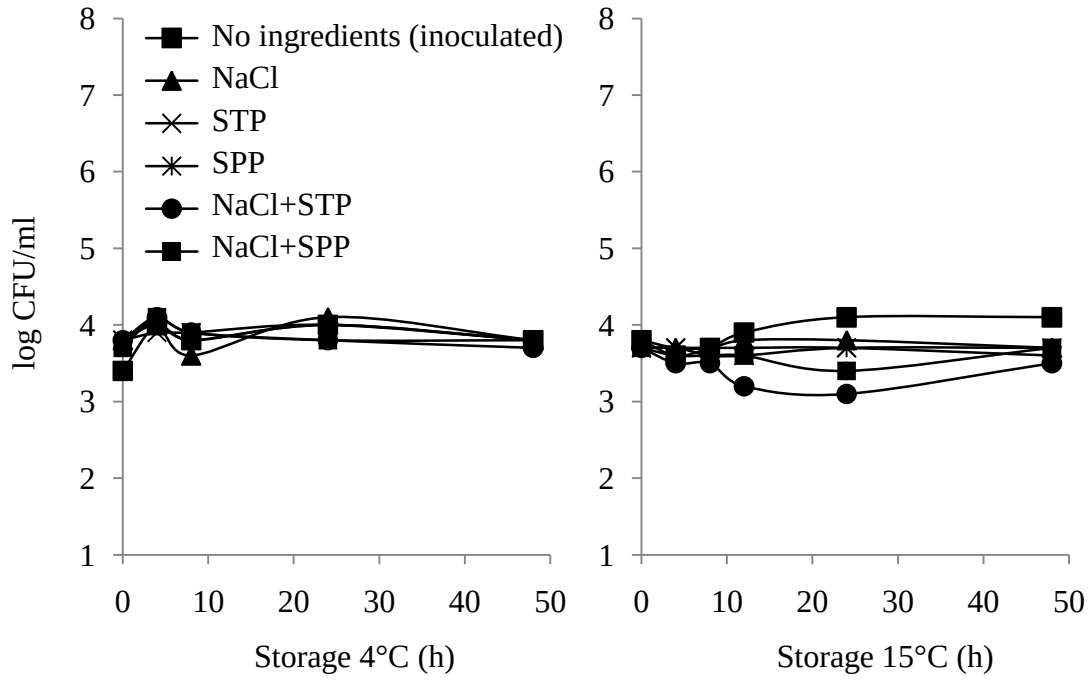
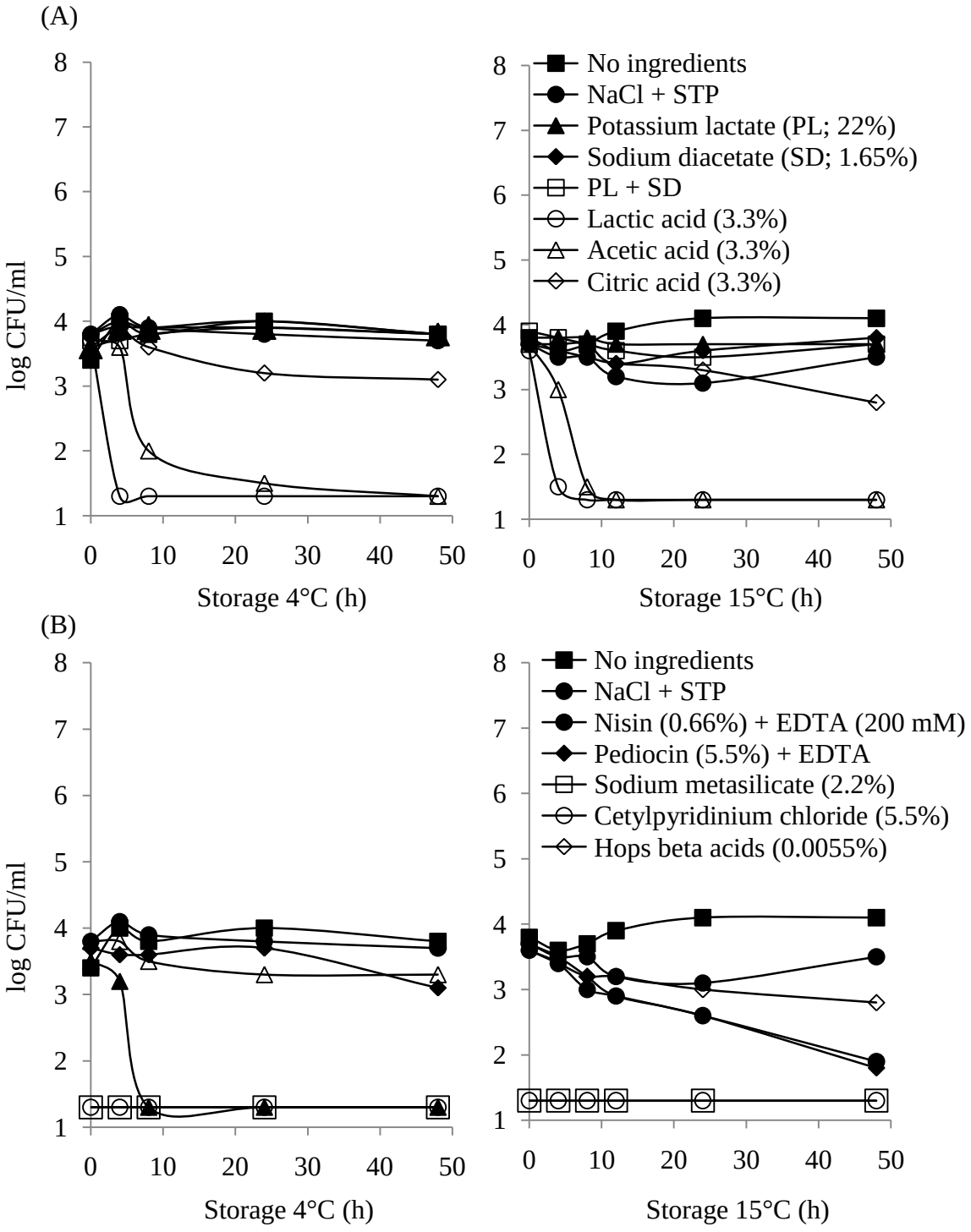
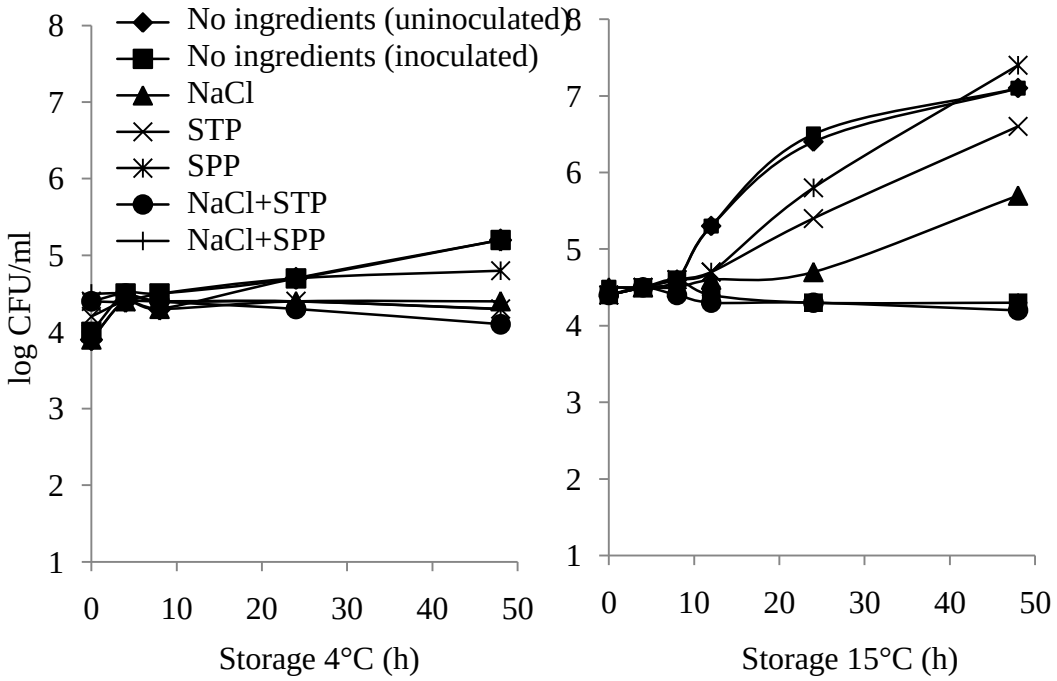


Appendix Figure 3 (Table 4.5 and 4.6). Mean *Escherichia coli* O157:H7 populations (log CFU/ml ± standard deviation) recovered on tryptic soy agar +rifampicin (100 µg/ml) from control brines containing salt (NaCl; 5.5%), sodium tripolyphosphate (STP; 2.75%), sodium pyrophosphate (SPP; 2.75%), or their combination prepared with 3% meat residues and stored at 4 and 15°C



Appendix Figure 4 (Table 4.5 and 4.6). Mean *Escherichia coli* O157:H7 populations (log CFU/ml ± standard deviation) recovered on tryptic soy agar +rifampicin (100 µg/ml) from brines containing salt (NaCl; 5.5%) and sodium tripolyphosphate (STP, 2.75%) and organic acids (A) or and other anitimicrobial compounds (B) prepared with 3% meat residues and stored at 4 and 15°C.



Appendix Figure 5 (Table 4.7 and 4.8). Mean total bacterial populations (log CFU/ml ± standard deviationl) recovered on tryptic soy agar from brines containing salt (NaCl; 5.5%), sodium tripolyphosphate (STP; 2.75%), sodium pyrophosphate (SPP; 2.75%), or their combination prepared with 3% meat residues and stored at 4 and 15°C.