LACTIC ACID BACTERIA AS AN INTERVENTION AGAINST SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN BEEF

A Thesis

by

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) are Gram-negative bacteria that are able to cause disease in humans and animals. Beef is a significant transmission vehicle of foodborne outbreaks of STEC, which are commensal inhabitants of the gastrointestinal tracts of cattle and may be shed in the feces of animals and may be transferred onto the surfaces of beef during harvest via cross-contamination. Concern has been expressed surrounding the utility of studies describing the efficacy of antimicrobial interventions targeting the STEC that inoculate chilled versus non-chilled beef carcasses. The objectives of this study were to evaluate the effects of chilling on STEC attachment to brisket surfaces, and the effects of post-inoculation storage on STEC recovery. Paired briskets from split carcasses were separated; one brisket from each pair was kept non-chilled, while the other was chilled to a surface temperature of ≤5 °C prior to inoculation. Briskets were inoculated with a cocktail of eight STEC and then stored at 5 or 25 °C. At 0, 30, 60, 90 and 120 min post-inoculation, 30 cm^2 of tissue was aseptically excised, followed by selective enumeration of strongly and loosely attached STEC. A significant, though small (0.4 $\log_{10}$ CFU/cm^2), difference in the numbers of strongly attached cells was observed between non-chilled and chilled briskets ($p<0.05$). Significant effects on cell attachment by the interaction of chilling and post-inoculation storage period, or chilling and post-inoculation storage temperature, were identified ($p<0.05$). Results indicate beef chilling and post-inoculation storage conditions influenced STEC attachment to beef.

Lactic acid bacteria (LAB) produce antimicrobial substances such as organic acids, bacteriocins, and/or hydrogen peroxide that may have antagonistic activities against STEC. However, additional research is needed to assess the inhibitory potential of LAB interventions against non-O157:H7 STEC in beef. The objective of the subsequent study was to assess the antimicrobial efficacy of a commercial LAB mixture, applied via electrostatic or conventional spray, for the disinfection of STEC on beef strip loins during refrigerated aging in vacuum packaging for 14 or 28 d. Pre-rigor, non-chilled beef was inoculated with a cocktail of eight STEC, chilled for 24 h to ≤ 4 °C, and subsequently treated with a solution containing 8.7 ± 0.1 $\log_{10}$ CFU/ml LAB using either a hand-held pressurized tank air sprayer or an air-assisted electrostatic sprayer. Electrostatic and conventional sprays did not differ ($p>0.05$) in terms of STEC reductions, strip loin surface pH values, nor levels of applied LAB. Application of LAB (6.5 ± 0.1 $\log_{10}$ CFU/cm^2) significantly reduced STEC populations ($p<0.05$) from pre-treatment levels (7.2 ± 0.01 $\log_{10}$ CFU/cm^2), though pathogen numbers enumerated after 14 days of aging were not different ($p\geq0.05$) from pre-treatment counts. Mean STEC numbers on beef sampled after 28 days of aging were significantly lower ($p<0.05$) than pre-treatment means, though the difference was numerically small (0.5 $\log_{10}$ CFU/cm²). Biopreservatives such as LAB can be useful for reducing pathogens on food surfaces, though optimization of the numbers and activity of such interventions must be completed to assure maximal food safety preservation.

DEDICATION

This thesis is dedicated to my dear Aunt Leslie Cloutier, whose support and encouragement instilled in me the confidence to pursue this dream.

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NOMENCLATURE

µg	Microgram(s)
A/E	Attaching and Effacing
ATP	Adenosine Triphosphate
bp	Base Pairs
CDC	United States Centers for Disease Control and Prevention
CFU	Colony Forming Unit
DAEC	Diffusely Adherent *Escherichia coli*
DNA	Deoxyribonucleic Acid
EAEC	Enteroaggregative *Escherichia coli*
EAF	*Escherichia coli* Adherence Factor
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
FDA	United States Food and Drug Administration
FSIS	United States Department of Agriculture – Food Safety and Inspection Service
GRAS	Generally Recognized As Safe
h	Hour(s)
HACCP	Hazard Analysis and Critical Control Points

HC	Hemorrhagic Colitis
HUS	Hemolytic Uremic Syndrome
kg	Kilogram(s)
kPa	Kilopascal(s)
LAB	Lactic Acid Bacteria
LEE	Locus of Enterocyte Effacement
LT	Heat-Labile Enterotoxin
meq	Milliequivalent
min	Minute(s)
ml	Milliliter(s)
ppm	Parts Per Million
Rif^r	Rifampicin Resistant
sp.	Species (Singular)
spp.	Species (Plural)
ST	Heat-Stable Enterotoxin
STEC	Shiga toxin-producing *Escherichia coli*
Stx	Shiga toxin
subsp.	Subspecies
TBARS	Thiobarbituric Acid Reactive Substances
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TTP	Thrombotic Thrombocytopenic Purpura

USDA	United States Department of Agriculture
v/v	Per Volume Basis
w/w	Per Weight Basis

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CHAPTER I

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are Gram-negative bacteria that can cause disease in humans and animals. They are natural inhabitants of the gastrointestinal tracts of some ruminants and may be shed in the feces of cattle. While the internal muscles of cattle are considered to be essentially sterile prior to slaughter, STEC may be transmitted onto beef tissue via cross-contamination during harvest. Biological preservation has been used historically to extend the shelf-life of foods and may be useful for food safety applications. Lactic acid bacteria (LAB) are Gram-positive rods or cocci that synthesize a variety of metabolic end-products inhibitory to microorganisms, including *E. coli*. These organisms are classified as generally recognized as safe for use in beef carcasses, non-intact products, and whole cuts. Reported reductions in *E. coli* O157:H7 numbers in these foods have varied; however, little is known regarding the efficacy of LAB for the disinfection of non-O157:H7 STEC.

The primary objective of the present study was to evaluate the effectiveness of a LAB food safety intervention, applied via electrostatic or conventional spray, for the decontamination of eight Shiga toxin-producing *E. coli* serogroups on beef strip loins during refrigerated aging in vacuum packaging. To provide a thorough understanding of the species *Escherichia coli*, the microbiological characteristics of this group and the virulence factors of pathogenic strains are elaborated in Chapter II of this thesis. Chapter

III includes key information regarding the incidence of STEC in the beef supply chain and an overview of significant occurrences associated with STEC in beef products. Biological interventions are introduced in Chapter IV, followed by a review of previous studies regarding the use of these treatments for the disinfection of STEC in beef products in Chapter V. The research presented in Chapter VI expands upon current knowledge concerning the individual replication rates of eight STEC serogroups and the attachment of these organisms to lean beef tissue. Data relating to the efficacy of a lactic acid bacteria treatment for the disinfection of these pathogens on lean beef is offered in Chapter VII. In the final chapter (Chapter VIII), the potential implications of these studies are explained in reference to existing literature.

CHAPTER II

ESCHERICHIA COLI

Introduction

Escherichia coli (originally termed *Bacterium coli commune*) was first isolated from infant stool samples in 1885 by the pediatrician and bacteriologist, Theodor Escherich. At the time, it was noted that this organism was present in the feces of healthy neonates within 3 to 24 h following birth *(137, 138)*. Subsequent research demonstrating serotype specificity between maternal and neonatal strains provided evidence in support of transmission from mother to infant during delivery *(32, 355)*. Today, *E. coli* are recognized as commensal organisms of the gastrointestinal tracts of warm-blooded species, and the most abundant facultative anaerobes in the human intestinal microflora *(100, 184)*. Within the genus *Escherichia*, the *E. coli* are members of the family *Enterobacteriaceae*, of the order *Enterobacteriales*, a subset of the class *Gammaproteobacteria* in the phylum *Proteobacteria (28, 232)*. While most strains are not harmful to humans, some variants have acquired virulence factors through evolutionary processes and are thus able to cause disease in animals and humans *(186, 235)*. To better understand this diverse species, serological typing (developed in 1943 by Kauffmann for salmonellae) has been applied to *E. coli*. Using this method, strains are first classified according to the O (somatic) antigen, determined by the structure and composition of polysaccharide side chains extending from the outer membrane. The serotype (O:H) is defined by the flagellar H antigen. Strains may be further

differentiated by the capsular antigen (K antigen) *(1)*. Today, more than 200 O groups and 60 H groups have been identified for *E. coli (233, 389)*.

Microbiology

Escherichia coli is a Gram-negative, rod-shaped bacterium ranging from 1.1 to 1.5 µm in width and 2.0 to 6.0 µm in length *(28)*. The cell envelope of these organisms includes the plasma membrane, peptidoglycan layer, and outer membrane. Singer and Nicholson *(441)* described the structure of bacterial membranes with a fluid mosaic model, composed of a bilayer of amphiphilic polar phospholipids with heterogeneous integral proteins dispersed intermittently. The degree of saturation within the lipid constituents of membranes is dynamic and may differ according to environmental conditions like temperature *(97)*. The cytoplasmic cell interior is encased by a selectively permeable phospholipid bilayer called the plasma membrane, which contains embedded transport systems and chemoreceptors *(36)*. Numerous metabolic activities occur in the plasma membrane *(36)*, which is pushed against the cell wall by turgor pressure resulting from intracellular water and compatible solutes relative the solute potentials of the surrounding environment *(99)*. Between the inner and outer membranes is an aqueous periplasmic space that contains essential enzymes, proteins, and chemical receptors *(6)*. This compartment, which constitutes approximately 20 to 40% of the cell volume *(451)*, contains a mono- or bi-layer of covalently cross-linked glycan strands known as peptidoglycan (murein) *(165)*. The peptidoglycan layer, approximately 2 nm in width, maintains the cell structure and shape *(163, 513)*. It is bound to the internal phospholipid layer of the outer membrane with Braun's lipoproteins, which are the most prevalent

lipoproteins in *E. coli (57, 58).* Unlike the symmetrical and homogenous plasma membrane, the exterior portion of the outer membrane bilayer is made of lipopolysaccharides *(413),* which serve as a permeability barrier and protect cells from toxic compounds. These glycolipids are made of a membrane-embedded lipid A subunit covalently bound to an extracellular core polysaccharide, and an immunologically-active O-antigen polysaccharide side-chain that protrudes from the cell. The core polysaccharide confers a slightly negative charge to the outer membrane *(503).* Transport of extracellular components across the outer membrane is facilitated by number channel outer membrane proteins (OmpA, OmpB, OmpC, OmpF) *(135).* The OmpC porin is smaller than the OmpF porin, and has a lower rate of diffusion *(346, 347).* The channels uptake positively charged hydrophilic solutes more rapidly than negatively charged lipophilic solutes *(347).* Expression of OmpC is increased in conditions of low temperature, low osmolarity, and low pH *(194, 206).* When glucose is limited or absent from the environment, *E. coli* synthesize LamB, a membrane bound glycoporin *(110).* This lambda-receptor protein transports glucose, lactose, maltose, and maltodextrins *(496).* Some isolates produce a ScrY porin, which shares 23% amino acid homology with LamB. This outer membrane protein facilitates sucrose uptake *(192).*

E. coli is a chemoorganotroph, possessing both fermentative and respiratory metabolisms *(28).* These organisms ferment D-glucose with the production of acid and gas *(28).* Other fermentable carbohydrates include L-arabinose, maltose, D-mannitol, D-mannose, L-rhamnose, trehalose, and D-xylose *(211).* Most *E. coli* strains are capable of fermenting lactose, though *i*-inositol and D-adonitol are not metabolized *(28).* As a fecal

coliform, *E. coli* can ferment lactose with subsequent acid and gas production within 24 h at 44.5 °C *(468)* and is oxidase-negative, facultatively anaerobic, and non-sporulating *(128, 268)*. In addition, they possess the enzymes catalase and β-galactosidase *(128, 268)*, lack cytochrome *c* oxidase, and can grow in the presence of bile salts *(268)*. Most strains can liberate indole from tryptophan (indole-positive) and produce mixed acids sufficient to lower pH below 4.4 (methyl red test positive), but do not produce acetoin (Voges-Proskauer-negative) or use citrate as a sole carbon source (citrate-negative). However, *E. coli* type II strains are generally indole negative *(268)*. The chromosome of *E. coli* is circular, containing approximately 4.5 to 5.9 million bp *(45, 358)*, with a genetic composition of 52 mol% guanine (G) + cytosine (C) *(293)*. One way in which gene expression is regulated is activation of specific sigma factor subunits (σ) that bind to RNA polymerase holo-enzymes and recognize specific gene promoters *(398)*. Sigma factor activation is dependent upon growth phase, environmental conditions, and exposure to certain stressors. Transcriptional inhibition is caused by the synthesis of anti-sigma factors *(221)*, or RNA polymerase repressor proteins *(120)*. During the exponential phase of growth, transcription is directed by a 70,000 Da sigma factor (σ^{70}), which facilitates the expression of housekeeping genes *(398)*. Upon entrance into stationary phase or and/or conditions of potential stress, the alternate sigma factor σ^{S} (encoded by the *rpoS* gene) controls transcription *(294, 458)*. Compared to exponentially growing cells, *E. coli* in the stationary phase of growth are more tolerant to stressors. This inherent resistance to various adverse conditions is due to activation of σ^{S}, which controls genes involved in the general stress response. Induction of this response results

in physiological changes to the cell structure which provide resilience, mechanisms for damage repair, and accumulation of essential components within the cell *(21)*. In the presence of ferric (Fe^{3+}) citrate, iron-deficient cells will produce a sigma factor (FecI σ) that facilitates the transcription of iron citrate uptake systems *(356)*.

E. coli may exist as motile or non-motile cells. Motility is mediated by the presence of 5 to 10 flagellar filaments that measure approximately 20 nm in width and up to 20 µm in length. Each filament is composed of repeating protein subunits called a flagellin. When present, flagella protrude from cell surface in a peritrichous arrangement *(28)*. Taxis is mediated by an alternative 28 kDa sigma factor ($\sigma^{F \text{ or } 28}$), which regulates the transcription of genes encoding for flagellar production and assembly. Motility of flagellated *E. coli* is dependent upon the rotation of helical filament by a motor complex in the cytoplasmic membrane *(31)*. Counterclockwise rotation steadily propels cells forward in a "run" while clockwise rotation causes a rapid reorientation of the cell, referred to as a "tumble" *(104)*. Flagellar rotation and subsequent motility are powered by an electrochemical transmembrane ion gradient, rather than ATP *(31, 263)*. In addition to taxis, flagella may mediate biofilm initiation *E. coli (378)*. Sub-optimal temperatures limit motility, but do not inhibit flagellar production *(330)*.

Water is an essential component of cell metabolism, nutrient uptake, as well as membrane stability, and thus influences both the growth and survival of bacteria. One of the most significant parameters is substrate water activity ($a_w = p/p_o$), defined as the ratio of water vapor pressure of the medium (p) to that of of pure water (p_o) *(89)*. The minimum water activity for growth of *E. coli* is approximately 0.95 *(233)*. Significant

($p<0.05$) declines in STEC populations have been reported during storage when water activities are reduced to 0.95 or below *(14, 290, 390)*. Changes in environmental water activity may be detected directly by osmosensors or indirectly from subsequent changes in intracellular structures *(508)*. The a_w of a food can be modulated by drying or inclusion of water binding humectants like sodium chloride and/or sucrose *(405)*. The resistance of bacteria to various processing conditions like heat may be influenced by the environmental water activity, as well as the solutes present *(13)*.

The osmotic pressure of a system is determined by the intracellular and extracellular concentrations of compatible solutes and water. The optimal external osmolality for *E. coli* replication in minimal media is approximately 0.3 osmol *(253)*. In hypertonic solutions, the osmotic pressure of cell is lower than that of the substrate. The reverse is true for hypotonic solutions *(393)*. The difference in hydrostatic pressure between the intracellular and extracellular osmotic pressures is defined as the turgor pressure. When the internal and external osmotic pressures are balanced, the elastic plasma membrane is pushed against the cell wall by turgor pressure *(99)*. In general, smaller cells are less susceptible to strain imposed by changes in turgor pressure than larger cells. Rigid structures like the cell wall are more resistant to strain than flexible structures like the plasma membrane *(508)*.

If the extracellular environment is rendered hyperosmotic from loss of water or accumulation of compatible, polar, highly soluble compounds, the change in turgor pressure may cause the plasma membrane to separate from the cell wall. Plasmolysis is caused by the passive diffusion of water from the cell interior, which subsequently

decreases the cell volume and cytoplasmic water activity *(252, 508)*. Within 1 to 2 min of osmotic imbalance, cellular respiration and transportation activities cease *(320, 508)*. Surviving cells will initiate several passive and active processes to restore osmotic balance, including uptake of compatible solutes and cellular remodeling *(508)*. High osmotic pressure activates the sigma factors σ^S, σ^{32}, and σ^E, which facilitate transcription of specific genes encoding for protein repair (cytoplasmic and membrane-bound), proteases, and restoration of membrane integrity *(40)*. Phosphorylation of the OmpR regulator by the intracellular EnvZ sensor alters the ratio of outer membrane porin proteins to include more OmpC and less OmpF variants *(508)*. However, the role of this change in regulating osmotic pressure is unclear *(99, 325)*. The ProP systems are also activated by high osmolality *(323, 508)*, though the compatible solute glycine-betaine is preferentially transported into the cell in these conditions instead of proline *(73)*. In response to accumulation of extracellular solute concentrations, the sensor kinase KdpD phosphorylates the KdPE regulator, which facilitates uptake of potassium salts (K+) via transcription and induction of the Kdp-ATPase system Increased K+ subsequently induces transcription of glycine-betaine influx systems (ProU) *(454)*. Following 20 to 60 min of exposure, potassium and, to a lesser extent, betaine is transported from the medium into the cytoplasmic interior *(117)*. At this time, cellular respiratory functions may slowly recommence at a lower rate *(508)*. After 60 min, protein synthesis and cellular replication activities resume, with simultaneous expression of genes for osmotic regulation *(508)*.

If the intracellular solute potential is greater than that of the external environment, water will rapidly (within 1 min) diffuse to the interior of the cell, increasing the cytoplasmic water activity and causing it to swell *(313, 508)*. In response, membrane-bound aquaporins transport water from the cell interior *(234)*. In addition, compatible solutes are exported from the cell *(508)*. The downshift in osmotic pressure is sensed by an intracellular sensor (EnvZ), which then signals the porin regulator (OmpR) to promote the synthesis of large, OmpF porins rather than small, OmpC porins *(508)*. Transcription of genes encoding for uptake systems of glycine-betaine (ProU) and proline (ProP) is inhibited in low osmotic pressure *(511)*. After 10 to 20 min, the water channels are closed *(508)*.

Environmental temperature is a significant determinant of *E. coli* growth and survival. As mesophiles, the optimal temperature for growth is approximately 37 °C. However, these organisms have the capacity to replicate in temperatures as low as 7 °C and up to 50 °C *(233)*. An interaction ($p<0.05$) between water activity and temperature on the growth and survival of STEC has been reported in multiple studies *(14, 30, 290, 390)*. Lindqvist and Linblad *(290)* evaluated the inactivation rates of STEC serogroups O157, O103, and O111 in fermented sausage. The rate of cell death in sausages with reduced water activity (0.95) was significantly ($p<0.05$) enhanced in storage temperatures of 20 °C versus 8 °C. Balamurugan et al. *(14)* also observed a significant ($p<0.05$) increase in STEC death when low water activity (0.90 to 0.95 a_w) was combined with high storage temperature (20, or 30 °C) versus storage at 5 °C *in vitro (14)*.

Several physiological changes occur when *E. coli* are exposed to elevated temperatures. When growth temperatures are increased, the structures of cell membrane phospholipids are altered. Specifically, the expression of *cis* acyl-chain double bonds is reduced in favor of *trans* isomer synthesis *(106)*. The increased number of *trans* acyl double bonds increases the lipid phase transition temperature of the bacterial membrane, thus decreasing permeability and restoring fluidity *(97)*. Sublethal exposure to heat can activate mechanisms for thermal resistance, which can make the cells more tolerant to later thermal challenge *(238)*. Protein misfolding may activate the general stress response controlled by the sigma factor σ^S *(203)*. Upon the detection of misfolded or unfolded proteins in the cell cytoplasm, the alternative sigma factor σ^{32} facilitates the rapid, transient expression of heat shock proteins to repair or degrade improper protein structures *(315, 515)*. If periplasmic protein folding is disrupted by thermal shock, another sigma factor (σ^E) is activated. The associated genes encode for molecular chaperones or other proteins that aid in restoring extra-cytoplasmic protein structures *(315, 324)*.

Exposure to sub-optimal temperatures induces several physiological changes in *E. coli* and can lead to decreased efficiency of cellular functions. In addition, protein folding efficiency and ribosomal functions are also impaired *(365)*. RNA translation and transcription are also delayed as a result of cold-induced stabilization of nucleic acid secondary nucleic acid structures *(365, 366)*. The primary cold shock protein expressed by *E. coli* is CspA. This protein serves as an RNA chaperone to facilitate gene expression in low temperatures *(365, 366)*. Rapid cold shock (15-16 °C) of cells grown

at optimal temperature (37 °C) induces accelerated synthesis of cold shock proteins and retards growth for 3 to 6 h *(12, 366, 372)*. As temperatures decrease, cell membrane lipids transition from a liquid-crystalline state to a gel-crystalline state and membrane fluidity is subsequently decreased *(404)*. To restore the fluidity of the membrane, lipid composition is altered to include more unsaturated fatty acids *(404)*.

Another important factor related to bacterial growth and survival is pH. In sub-optimal environments, cell nutrient uptake, enzymatic function, and cytoplasmic membranes are altered. The minimum pH required for *E. coli* replication is approximately 4.5, though this organism grows best in neutral conditions (~pH 7.0) *(233)*. However, an acidic environment is not necessarily bactericidal to *E. coli*, as evidenced by the persistence of this organism in acidic non-pasteurized juice products *(362)*. Acid tolerance of STEC is highly variable and can be influenced by a number of factors, including growth phase, substrate water activity, and storage temperature *(30, 262, 287, 288)*. Numerous studies have demonstrated that declines in STEC populations are affected by the interaction of low water activity and low pH *(14, 380)*. Presser et al. *(380)* observed that the minimum pH required for STEC replication increased in conditions of suboptimal a_w (0.90 to 0.95). Simultaneous exposure of STEC to conditions of low pH and high temperature has been shown to enhance the lethal action of lactic acid *(14, 380)*.

Induction of acid stress response in *E. coli* populations is thought to be mediated by the secretion of signaling proteins by adapted cells and subsequent uptake by acid-sensitive cells *(399)*. In addition, attachment of these organisms has been shown to

influence acid susceptibility. Using a model attachment system for *E. coli*, Poynter et al *(377)* demonstrated enhanced acid tolerance of cells attached to glass beads relative to planktonic cultures. Several mechanisms of acid resistance have been reported for STEC, including two amino acid decarboxylase systems, and an oxidative system *(262, 287, 288)*. *E. coli* acid tolerance may be increased by growth conditions, with fermentative replication making the cells less sensitive *(116)*. This may be due to the acid tolerance response, wherein habituation of bacteria in acidic conditions (pH 4.5 to 6.0) induces expression of genetic elements that enhance acid resistance. The presence of glutamate in *E. coli* prevents the accumulation of alternative anions in the cytoplasm *(391)*. One way in which *E. coli* responds to acid stress is through the expression of various enzymes that alkalinize the pH through various reactions. Production of urease, which generates ammonia and carbon dioxide via hydrolysis of urea, in enterohemorrhagic *E. coli* is controlled by the *Fur* gene cassette *(198)*. The liberated ammonium ions modulate the intracellular and/or extracellular pH. At pH 5.0 or lower, expression of cyclopropane fatty acid synthase is increased. This enzyme facilitates the transformation of unsaturated fatty acids in the membrane into cyclopropane fatty acids, which decrease membrane proton permeability *(85, 97)*. In addition, cells in stationary phase of growth are more resistant to acid, which may be due in part to the higher cyclopropane expression in mature cells versus young or growing cells *(64, 97)*.

Diarrheal *Escherichia coli*

While the majority of strains are nonpathogenic, some possess virulence factors and are able to exert pathogenesis in humans *(416)* and animals *(186)*. *E. coli* was first

recognized as a foodborne pathogen in 1971, when over 200 individuals became ill after consuming imported soft-ripened cheese contaminated with enteropathogenic *E. coli* O124:B17 *(307).* Although *E. coli* was known to cause diarrheal illness during this period, the spectrum of clinical syndromes attributed to the organism had yet to be elucidated. Hemolytic uremic syndrome (HUS) was first associated with STEC human disease in 1985 *(243).* Each year, more than 2.8 million individuals are estimated to be infected with STEC worldwide, resulting in 230 deaths *(305).* In the United States, 54.8% of foodborne STEC infections are attributed to non-O157 STEC serovars in the each year *(416).* Among the non-O157 STEC, the serogroups most frequently isolated in foodborne infections are *E. coli* O26, O111, O103, O121, O45 and O145 *(62).* The reported annual costs associated with foodborne *E. coli* O157:H7 infections in the United States range from $255 million *(209)* to $635 million *(418),* while estimated expenditures for non-O157:H7 STEC foodborne disease are generally lower, ranging from $24 million *(209)* to $154 million *(418).* Variations in cost estimates are due to differences in valuation methods, most notably the inclusion or exclusion of monetized quality-adjusted life years (e.g. value of pain, suffering, and losses due to functional disability) *(209, 418).* While the majority of *Escherichia coli* strains are benign commensals, genes encoding for virulence factors that bestow pathogenicity have been acquired by some strains. The general clinical syndromes associated with pathogenic *E. coli* include urinary tract infections, diarrheal disease, meningitis and/or sepsis *(214, 317, 338, 446).* Diarrheagenic *E. coli* strains can be classified according to patho-histology, production of virulence factors, and toxin synthesis. Six virulence groups are

recognized currently: enteropathogenic, enterotoxigenic, enteroinvasive, enterohemorrhagic, enteroaggregative, and diffusively adhering *(233, 317, 337).*

Enteroaggregative *Escherichia coli*. The defining characteristic of Enteroaggregative *E. coli* (EAEC) is the distinct "stacked brick" adherence pattern to HEp-2 epithelial cells *(338).* Included in this pathotype are *E. coli* serogroups O104, O3, O15, O44, O111, and O127 *(153, 317)*. Members of this pathotype display heterogeneous capacities for toxin synthesis, thought to initiate following colonization of the host intestine *(46).* Some EAEC have plasmids that facilitate expression of an enterotoxin termed enteroaggregative heat-stable toxin 1 (EAST1), which is similar in structure to the ETEC ST toxin *(414).* Several toxins classified as serine protease auto-transporters of *Enterobacteriaceae* (SPATE) have been recovered from EAEC cultures and clinical isolates *(285, 402).* Class I SPATE proteins exert cytotoxic activity to human intestinal epithelial cells and cleave serine *(125),* while class II proteins are non-cytotoxic, display mucin-cleaving activity, and are thought to be involved in colonization *(46, 201)*. Examples of chromosomally encoded class I SPATE toxins produced by EAEC strains include a 107 kDa secreted autotransporter toxin (Sat) *(46),* which is also expressed by some diffusely adherent and uropathogenic *E. coli (185),* a 116 kDa homologue of *Shigella* enterotoxin 1 (ShET1) *(343) (143, 144).*, and a 103 kDa *Shigella* IgA homologue (SigA) *(46).* Another class I SPATE found in EAEC isolates is a 108 kDa plasmid-encoded toxin (Pet) *(139, 343).* Many serogroups within this pathotype synthesize a 109.8 kDa mucin-cleaving class II SPATE protease involved in intestinal colonization (Pic) *(201).* In addition, Shiga toxin *(50, 329)* and cytolethal

distending toxin (CDT) *(4)* have also been recovered from EAEC. The characteristic mucosal adherence pattern of these bacteria is attributed to expression of aggregative adherence fimbriae (AAF) *(47)*, encoded on a 55 to 65 MDa plasmid (pAA) *(341)*. Some strains also express fimbrillar bundle adhesins, which may be accompanied by synthesis of hollow rod, rod, and/or fibrillary adhesins *(493)*. The clinical syndrome of EAEC infection often includes intestinal inflammation caused by induced secretion of IL-8 from host cells mediated by the interaction of bacterial flagellin and intestinal epithelial cells *(193, 448).*

Members of this group have been associated with diarrhea in infants and children in developing countries *(18, 37, 38, 142)*. Diarrheal illness caused by EAEC infection has occurred in individuals following travel from native industrialized countries to developing nations *(423)*. Persistent diarrhea has been reported in patients co-infected with EAEC and human immunodeficiency virus (HIV) *(219)*. The diarrheal syndrome elicited by these pathogens is more frequently persistent (lasting greater than 2 weeks) than acute (lasting less than or equal to 2 weeks) *(38, 95)*. Symptoms associated with EAEC infection usually include watery non-bloody diarrhea, abdominal pain, nausea, vomiting, and fever *(38, 96, 219, 340).*

Enteropathogenic *Escherichia coli.* Members of the group enteropathogenic *E. coli* (EPEC) display a distinctive pattern of localized adherence to HeLa and HEp-2 cells *(492)* and form attaching and effacing (A/E) lesions in the human intestinal epithelium *(317)*. Members of this pathotype, including serogroups O55, O111ab, O119, O126, and O127, do not produce Shiga toxins, nor the enterotoxins ST or LT. However, heat stable

enterotoxin (EAST1) *(439)* and cytolethal distending toxin (CDT) *(424)* have been recovered from EPEC strains. Some, though not all, synthesize 110 kDa EPEC-secreted protein C (EspC) enterotoxin that is classified as a SPATE toxin and exhibits protease activity on host cytoskeletal serine *(316)*. The class II SPATE protein Pic has also been recovered from some strains *(46)*. The non-sequential pathogenesis of EPEC can be generalized as localized adherence to host endothelial tissue, signal transduction, and intimate adhesion *(121)*. Typical EPEC strains possess an *E. coli* adherence factor plasmid (EAF) that contains essential genes for elements that mediate localized adherence to host cells in the small intestine. This plasmid is absent in atypical EPEC variants which therefore do not display this adherence factor *(15, 336)*. Local adherence to host cells and auto-aggregation of EPEC cells are mediated by type 4B fimbriae called bundle forming pili (BFP) *(338)*. While BFP are thought to initiate local adhesion of EPEC to eukaryotic cells, the precise role of these fimbriae in host cell attachment is not fully understood *(122, 155)*. The EPEC chromosome contains a pathogenicity island termed the locus of enterocyte effacement (LEE). This pathogenicity island contains the genetic elements necessary for a type III secretion system, which transports seven different LEE-encoded proteins across the bacterial cytoplasmic and outer membranes, and into the host cytosol *(463)*. The secreted proteins include structural components for translocation systems (EspA, B, and D) and effector proteins that are translocated into host cells (Tir, Map, Esp E and F) *(338)*. The type III secretion system also exports the prophage-encoded protein EspI, is present in some EPEC *(332)*. The EspA filaments interact directly with host cells. Genetic elements required for intimate adherence and

the formation of attachment and effacing (A/E) lesions on host epithelial cells are also present on the LEE pathogenicity island. Tir (translocated intimin receptor) is transported to the host cell via the type III secretion system and inserted into the host cell membrane *(111)*. The LEE-encoded adhesion protein intimin is expressed and embedded into the EPEC membrane. Binding of intimin to Tir initiates formation of actin-rich pseudopods beneath the bacterial cell and subsequent cytoskeletal rearrangement. This rearrangement may also facilitate invasion of host cells *(395)*.

These pathogens have been implicated as the primary agents of infantile diarrhea in developing countries *(338)*. The incidence of EPEC in industrialized countries is low, though outbreaks of this pathotype have occurred in childcare establishments. Infants younger than 6 months of age are especially prone to EPEC infections *(273, 338)*. Associated symptoms include profuse non-bloody diarrhea lasting 5 to 15 days, which may be accompanied by fever and vomiting *(338)*. Chronic syndromes of diarrhea leading to secondary sequelae such as malnutrition, weight loss, and delayed growth have been reported in rare cases *(273)*.

Diffusely adherent *Escherichia coli.* In 1987, Nataro et al. *(339)* observed some members of the virulence group EAEC adhered diffusely to HEp-2 cells. These bacteria, which adhere over the entire surface of the intestinal cells, were later defined as diffusely adherent *E. coli* (DAEC) *(502)*. Binding to host cells and subsequent internalization may be facilitated by adhesion structures (F1845 or fimbrial adhesins) *(178, 351)*. Synthesis of the class I SPATE proteins Sat and SigA has been found in some DAEC strains *(46)*.

These organisms have been isolated from clinical samples of children with diarrhea *(17, 176)*, though the mechanisms of pathogenesis have yet to be defined.

Enteroinvasive *Escherichia coli*. Enteroinvasive *E. coli* (EIEC) were first identified as causative agents of bacillary dysentery in the 1970's *(124, 467)*. The serogroup most frequently associated with human EIEC infection is O124 *(317)*. The organisms in this group are unique in that they are non-motile *(440)*, do not synthesize lysine decarboxylase *(412)*, and do not produce ETEC enterotoxins (ST/LT) or Shiga toxin *(149, 317, 318)*. However, some strains express the class I SPATE SigA *(46)*. These bacteria invade colonic epithelial cells, multiply and spread intracellularly *(180)*. Invasion is mediated by the presence of a large plasmid which contains genetic elements (*ipaBCDA* genes) that encode invasion plasmid antigens (Ipa) *(188)*. The infectious dose of EIEC ranges is high (≥ 8.0 $\log_{10}$ CFU) and the incubation period lasts 12 to 72 h. Symptoms of EIEC are indistinguishable from the clinical syndrome caused by *Shigella dysentariae* 1, characterized by severe abdominal cramps and watery stool that may contain pus, mucus, and/or blood lasting 1 to 2 weeks *(124)*.

Enterotoxigenic *Escherichia coli*. Enterotoxigenic *E. coli* (ETEC) produce heat-labile enterotoxin (LT) and/or heat-stable enterotoxin (ST). Serogroups of this pathotype include O6, O8, O27, O85, and O115 *(317)*. The primary virulence factors are adhesion fimbriae (colonization factor antigens) and enterotoxins (ST and LT), which usually are encoded on the same plasmid and facilitate attachment to the epithelial cells of the host small intestine *(459)*. Colonization factors may express fimbrial (rigid and filamentous), fibrillar (flexible and smaller), or nonfimbrial structure *(164)*. Enterotoxins

may be heat stable (ST1 and ST2) or heat labile (LT-1 and LT-2). Approximately 46% of ETEC express solely ST, while 25% exclusively synthesize LT. The essential elements for both toxin types are encoded in 29% of strains *(506)*. The ST1 is secreted by the cell and binds to the brush-border of the host epithelium. The LT toxins, which are structurally similar to the cholera toxin, are inactivated at within 30 min exposure to 60 °C *(443)*. The B subunit of the LT holotoxin binds preferentially to GM_1 gangliosides on the surface of host intestinal mucosa *(129)*. The enzymatically active A subunit induces cyclic adenosine monophosphate (cAMP) production by facilitating the ADP ribosylation of the adenylate cyclase activator (G protein) and subsequent induction of adenylate cyclase activity *(425)*. Heat labile type-1 enterotoxins are associated human disease, while type-2 variants are usually specific to animals *(429)*. Heat-stable enterotoxins are secreted extracellularly and bind to extracellular guanylate cyclase C receptors on the host cell surface *(385)*. Once bound, host guanylate cyclase is activated and subsequently induces cyclic guanosine monophosphate (GMP) production *(251)*. In addition, the SPATE enterotoxins EatA are synthesized by some strains *(364)*.

This pathotype has been associated with diarrheal illness in both humans and domestic animals *(337)*. They are a leading cause of diarrhea in developing countries, with 300,000 to 500,000 deaths being attributed to these pathogens globally each year *(510)*. Contaminated food and water are the most common vehicles for these pathogens. Weaning infants are especially prone to ETEC infection *(43, 509)*. However, susceptibility generally decreases with exposure through the development of colonization factor antibodies and acquired immunity *(274, 275)*. If not previously

exposed to ETEC, travelers from industrialized countries may be susceptible to ETEC infection. In fact, these bacteria have been cited as the most frequent causative agents of traveler's diarrhea *(42)*. The infectious dose of these pathogens is high, ranging from 8.0 to 10.0 $\log_{10}$ CFU in healthy adults *(455)*. Symptom onset occurs between 8 and 44 h following ingestion, with illness severity ranging from mild diarrhea to profuse rice-water-like stools lasting up to 19 days, usually accompanied by abdominal cramps. Infected individuals may experience nausea and headaches, though fever and vomiting are generally absent *(102, 124)*. In severe cases, the clinical syndrome of ETEC infection may be indistinguishable from cholera *(407)*.

Enterohemorrhagic *Escherichia coli.* The defining characteristics of Enterohemorrhagic *E. coli* include Shiga toxin synthesis and the production of A/E lesions (encoded on the LEE island). Some strains produce enterohemolysin (ehxA) and/or heat-stable enterotoxin (EAST1) *(51)*. However, the role of these virulence factors in human illness is unclear. Production of class I SPATE proteins by EHEC strains has been reported, including a cytotoxin referred to as either EspP or PssA, derived from extracellular serine protease plasmid-encoded (EspP) and protease secreted by STEC (PssA), facilitating attachment to intestinal cells of both humans and cattle *(51, 65, 402, 489)*. The autotransporter proteases Sat *(46)*, and SigA *(46)* have also been recovered from strains in this pathotype. The class II SPATE Pic may also be synthesized by these pathogens *(46)*. Pathogenesis of strains lacking the LEE island may be due in part to the secretion of a plasmid-encoded 111.7 kDa non-cytotoxic protease or

a prophage-encoded 110.4 kDa protein (EspI) *(279)*, though the latter has been recovered from clinical isolates containing LEE as well *(332)*.

The infectious dose of *E. coli* O157:H7 is low (10 to 100 cells), though infectious doses of non-O157 STEC may be greater. The symptoms of EHEC infections begin with abdominal cramps accompanied by watery diarrhea; 80% of O157 STEC and 45% of non-O157 STEC infections will progress to hemorrhagic colitis (HC), characterized by severe abdominal pain and bloody diarrhea. Approximately 3 to 7% of individuals with HC will develop life-threatening chronic sequelae, including hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TP) *(149, 233)*. In the U.S., HUS is the primary cause of renal failure in children *(438)*. The serovars most commonly implicated in human non-O157 STEC infections are *E. coli* O26, O111, O103, O121, O45 and O145 *(62)*. Although all EHEC produce Shiga toxin, Shiga toxin-producing *E. coli* are not limited to EHEC, as some strains lack the genes necessary for the production of A/E lesions *(242)*. The production of Shiga toxin does not necessarily render *E. coli* pathogenic to humans; toxin expression must be paired with other virulence factors *(500)*. For example, the Shiga toxin variant Stx2e is rarely associated with human disease *(34, 160)* but is a significant causative agent of diarrhea in weaned pigs and porcine edema *(35, 304)*.

Shiga toxin. In 1977, strain-specific cytotoxic activity of *E. coli* culture filtrates on Vero cells (kidney epithelial cells extracted from African green monkey) was reported; the causative factor of this phenomenon was termed *verotoxin (254)*. Alternative nomenclatures of *verotoxin* include Shiga-like toxin *(353)* and Shiga toxin,

with the latter being used most frequently currently. It is an AB_5-type holotoxin composed of one enzymatically active A subunit and surrounded by a pentamer of B subunits that facilitate binding to host cell receptors *(156)*. The bacteriophage-encoded genes of toxin subunits A (*stxA*) and B (*stxB*) share a promotor and are transcribed within a single operon. The A subunit is composed of an enzymatically active A_1 component bound to an A_2 component which interacts with the B subunit. The ribosome binding sites encoded by *stxA* and *stxB* differ; enhanced ribosomal binding leads to greater translation of B subunits *(187)*. Holotoxins secreted by *E. coli* are classified as either Stx1, with 3 subtypes (Stx1a, Stx1c, and Stx1d), and Stx2, with at least 7 subtypes (Stx2a-Stx2g) *(162, 230, 420)*. The nucleotide sequences encoding for the A and B subunits of Stx1 and Stx2 share 57% and 60% homology, respectively *(230)*. Toxin potency among Shiga toxin types and subtypes is variable *(162)*, though Stx2-type toxins are more frequently associated with the development of HUS than Stx1 *(437)*. Cytotoxicity is thought to be a function of B subunit binding kinetics to host cell receptors *(197, 353)*. The primary host cell receptor targeted by the Stx B subunit is globotriaosylceramide (Gb_3), though Stx2e (associated with porcine disease) binds preferentially to globotetraosylceramide (Gb_4) *(162, 228, 470)*. The sensitivity of eukaryotic cells to Shiga toxin is dictated by the quantity and fatty acid profiles of surface Gb_3 receptors. The Shiga toxin is then internalized via endocytosis, transferred to the *trans*-Golgi network, then to the endoplasmic reticulum, and is translocated into the cytosol *(411, 457)*. The A_1 subunit of Shiga toxin exhibits *N*-glycosidase activity that inactivates the eukaryotic 60S ribosomal subunit. Specifically, Shiga toxin prevents

chain elongation by preventing the host elongation factor-dependent tRNA from binding to the ribosome by excising a single adenine residue from the 28S ribosomal RNA (rRNA) of the 60S ribosomal subunit *(134, 224)*. Subsequently, host protein synthesis is inhibited.

CHAPTER III

SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* INFECTIONS ASSOCIATED WITH BEEF

Introduction

In the U.S., current estimates suggest that 48 million people acquire a foodborne illness annually, resulting in 128,000 hospitalizations and 3,000 deaths *(80)*. However, only 20% (9.4 million) of domestically acquired foodborne illnesses are linked to a definitive pathogen and food vehicle *(416)(415)*. Although the majority of defined foodborne illnesses are caused by a viral pathogen (5.5 million), approximately 64% of hospitalizations (35,796 of 55,961) and deaths (861 or 1,351) are due to bacteria *(416)*. Animal-derived foods are significant sources of foodborne disease. The greatest number of foodborne illnesses in the U.S. are attributed to dairy (18.0%), poultry (17.9%), and beef (13.2%) *(361)*. Consumption of contaminated beef causes 7.4% and 5.9% of all foodborne illness-related hospitalizations and deaths each year, respectively *(361)*.

Beef consumption has decreased, with approximated 2014 values (10.9 billion kg) being 1.7 billion kg lower than those reported in 2004 (12.6 billion kg). Beef production in the same period declined, with nearly 113.4 million kg of additional beef being produced in 2004 (11.1 billion kg) than 2014 (11.0 billion kg). Beef exports were markedly reduced following the first case of bovine spongiform encephalopathy (BSE) in 2003; beef exports decreased from 9.6% of production in 2003 to 1.9% in 2004. Exports have recovered steadily, with 11.0% of beef production being exported in 2014,

valued at over $6.5 billion. Lower production outputs and increased exports have resulted in higher consumer prices; the mean per kg retail value of choice beef changed from $1.85 in 2004 to $2.71 in 2014 *(472)*. Similar trends have been reported for the retail equivalency value of the U.S. beef industry, which increased from $70 billion in 2004 to $95 billion a decade later *(472)*. While cattle inventories are expected to rise from 29 million head in early 2015 to 94 million in 2024, beef production and consumption are projected to decrease through 2017 due to retention of cattle for breeding. Increases in beef production are expected to occur between 2017 (10.3 billion kg) and 2024 (11.8 billion kg); greater production will likely decrease beef prices through 2022. Although lower costs may stimulate per capita beef consumption after 2017 (22.0 kg), 2013 levels (25.5 kg) will not be achieved by 2024 (23.8 kg) *(473)*.

Outbreaks and Regulations Pertaining to Shiga Toxin-Producing *E. coli*

From 1982 to 2002, a total of 183 foodborne outbreaks of *E. coli* O157:H7 were reported in the United States. The most common vehicle of disease was ground beef, which was linked to 41% (75 of 183) of these incidents *(383)*. One of the most significant foodborne disease outbreaks during this period occurred between 1992 and 1993. Over 500 cases of foodborne *E. coli* O157:H7 were attributed to undercooked hamburger served by a single fast food chain (Jack in the Box). Hospitalization was required in 151 cases; development of HUS occurred in 45 individuals, and four did not survive the disease. The median age of infected individuals was 7.5 years old *(24, 25)*. This outbreak was a pivotal event for food safety regulation in the United States, and highlighted the need for *E. coli* O157:H7 control measures in the beef chain.

In 1994, the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) declared raw ground beef to be adulterated if contaminated with *E. coli* O157:H7, unless further processed to eliminate the pathogen *(479)*. The USDA-FSIS issued a rule on July 25, 1996 which required: 1) the development, institution, and record maintenance of sanitation standard operating procedures by all meat and poultry establishments; 2) implementation of scheduled generic *E. coli* testing to verify process controls by slaughter establishments prior to January 27, 1997; 3) construction and implementation Hazard Analysis and Critical Control Point (HACCP) systems prior to January 26, 1998 (employers of ≥ 500 individuals), January 25, 1999 (employers of 10 to < 500 individuals), or January 25, 2000 (employers of < 10 individuals or establishments with < $2.5 million in annual sales); and 4) establishment of pathogenic *Salmonella* reduction performance standards by all producers of raw ground product in accordance with the HACCP system deadlines *(479)*.

On January 19, 1999, the USDA-FSIS expanded the definition of adulteration to include confirmed positive isolation of *E. coli* O157:H7 from all raw beef products which have been or are intended to be processed in such a way that the meat interior would be exposed to potential migration of pathogens, including mechanical tenderization (e.g. needling, Frenching, or reconstruction), comminution (e.g. chopping, mincing, or grinding), and manufacturing trimmings *(474)*. In spite of the enhanced food safety regulations, another significant multi-state outbreak of *E. coli* O157:H7 occurred in July of 2002. After ground beef from a Colorado producer (ConAgra Beef Company) was implicated as the transmission vehicle in 43 cases of foodborne illness, a nationwide

recall was issued involving 8.4 million kg of beef (trim and ground) *(487)*. Soon after, a Wisconsin firm recalled approximately 1.3 million kg of ground beef after the same pathogen was detected *(433)*. These incidents, combined with new data regarding the prevalence of *E. coli* O157:H7, prompted the USDA-FSIS to mandate reassessment of HACCP systems for all raw beef producers *(476)*.

Several outbreaks involving mechanically tenderized beef products occurred between 2003 and 2005, highlighting the need for pathogen control measures by non-intact beef producers. In 2003, foodborne diseases were attributed to the consumption of bacon wrapped beef fillet; each filet was blade tenderized and injected with marinade prior to the addition of bacon. Testing indicated matching two-enzyme pulsed field gel electrophoresis (PFGE) patterns in 11 cases of *E. coli* O157:H7 spanning five states; two-enzyme PFGE patterns obtained from five samples of the implicated product were identical to case patterns. Three of the infected individuals required hospitalization and one illness progressed to HUS, though no deaths were reported. After reviewing the standard operating procedures of the producer, cross contamination via injection and tenderization needles was cited as the likely source of *E. coli* O157:H7 in the beef fillets. Approximately 335,200 kg of beef were voluntarily recalled by the producing establishment; needle cleaning and sanitizing schedules were increased from once per week to daily *(260)*. Another outbreak of *E. coli* O157:H7 associated with mechanically tenderized beef occurred in 2004. In total, four cases of foodborne disease were linked to the consumption of tenderized, marinated beef steak from a national restaurant. Once the isolates from all four cases were confirmed to have matching PFGE patterns, a voluntary

recall was issued by the beef steak producer; subsequently, nearly 184,160 kg of product was destroyed. In light of these occurrences, the USDA-FSIS issued a notice requiring all manufacturers of cooked and raw non-intact beef products to reevaluate their HAACP plans to account for *E. coli* O157:H7 *(477)*. However, a subsequent outbreak of *E. coli* O157:H7 involving marinated, injected steaks occurred in 2007, resulting in 8 confirmed cases and a recall involving over 117,900 kg of beef *(481, 484)*. In the same year, needle tenderized tri-tip beef linked to a restaurant facility was implicated in an outbreak of *E. coli* O157:H7 involving 124 cases and 8 hospitalizations. While no recall was issued and the source of adulteration could not be identified, improper sanitation by non-intact producer was cited as a contributing factor *(77, 484)*. Another outbreak involving blade tenderized beef steaks occurred between 2009 and 2010. In total, 21 individuals across 16 states were infected with *E. coli* O157:H7, resulting in 9 hospitalizations, 1 case of HUS and a recall of nearly 112,500 kg of beef products, although no conclusive source of cross-contamination was identified *(82)*.

The first confirmed outbreak of non-O157:H7 attributed to beef occurred in August of 2010. *E. coli* O26 cultures from 3 individuals were found to have identical PFGE patterns to isolates recovered from suspected ground beef samples. The Pennsylvanian producer issued the first beef recall (~3,856 kg) involving non-O157 Shiga toxin-producing *E. coli (369)*. More recently, 18 individuals acquired *E. coli* O157:H7 infections from the consumption of contaminated beef and beef products from a processor Alberta, Canada, 4 of which were attributed to mechanically tenderized meat. This outbreak resulted in a recall of over 4,000 tons of product and a request by

Health Canada for a risk assessment of mechanically tenderized beef. Insufficient sanitation procedures and poor plant management were cited as possible contributing factors to this outbreak *(277)*. Outbreaks of non-O157 STEC attributed to beef products have also occurred in the United States, France, Denmark, Germany, Australia and Italy *(54, 68, 75, 133, 140, 154, 247, 369, 381, 498)*.

On September 20, 2011, the USDA-FSIS issued a publication in the Federal Register which stated that raw, non-intact beef and intact beef intended to be processed into non-intact beef would be considered adulterated if found to be positive for *E. coli* serogroups O26, O45, O103, O111, O121, and/or O145 *(478)*. A risk assessment was published by the USDA-FSIS in May of 2012, which indicated that the risk of infection with STEC from mechanically tenderized beef was low and comparable to intact beef products *(480)*. A subsequent notice was issued, directing that routine verification testing of raw beef trimmings would be expanded on June 4, 2012 to include the previously mentioned *E. coli* serogroups *(483)*. On June 5, 2014, the USDA-FSIS announced that, starting on the 29th of the June, it would assay all ground beef submitted for STEC testing for the presence of *Salmonella (475)*.

Incidence of Shiga Toxin-Producing *E. coli* in Cattle and Beef

Presently, the estimated economic burden from foodborne illnesses attributed to beef in the United States is $1.36 billion *(22)*. Beef products have been implicated as the primary food vehicle in outbreaks of *Escherichia coli* O157:H7 (199 of 186) *(361)*. Although cattle are a significant reservoir of STEC, the primary host cell receptor targeted by Shiga toxin (Gb_3) is not expressed in bovine endothelial tissue *(76, 382)*. As

a result, infected animals are generally asymptomatic and difficult to detect in the absence of microbiological analysis *(33, 349, 434)*. The primary source of pathogens in beef is thought to be fecal contamination *(205)*. Fecal assays from U.S.-harvested cattle at slaughter have indicated rates of *E. coli* O157:H7 carriage ranging from 0.0% (n=38) *(130)*, 0.3% (n= 307) *(19)*, 2.3% (n=301) *(461)*, 12.9% (n= 287) *(19)*, 52.0% (n=48) *(130, 501)*. Prevalence of *E. coli* O157:H7 in cattle feces is seasonal and influenced by age. Carriage is thought to be higher in the summer and early fall and decreased in the winter and early spring *(86, 189, 432)*. Further, young claves are more likely to shed this pathogen than mature, adult cattle *(168, 501, 517, 518)*.

The variability between reported infection rates may be due in part to the existence of super shedding cattle, wherein the fecal matter of some animals contains more than 4.0 $\log_{10}$ CFU/g *E. coli* O157:H7 *(9)*. Elevated secretion of O157 STEC is associated with colonization of the bovine terminal rectum *(344)*. It is estimated that 80.0% of O157 STEC infections are transmitted by 20.0% of infected cattle, namely the super shedders *(310)*. The phenomena of enhanced shedding has been described for non-O157 STEC in dairy cows *(319)*. Available data suggest that between 8.0% (n=48; United States) *(130)*, 22.3% (n=811; Argentina) *(309)*, 39.0% (n=38; United States) *(130)* of cattle feces at slaughter contains at least one non-O157 STEC serotype. Once shed by infected cattle, STEC may persist in bovine feces for up to 18 weeks *(159, 161)*; animal hides may become contaminated with fecal matter in the environment. While these pathogens survive for a relatively short period (<9 days) on hides, continuous shedding may result in recurrent contamination with viable cells *(10)*.

Diet may also influence the carriage of STEC in beef cattle. Diez-Gonzalez et al. *(115)* reported that the feces of grain-fed cattle contained more *E. coli* than hay-fed cattle. This is in agreement with the findings of Garber et al. *(168)*, who observed greater incidence of *E. coli* O157:H7-positive feces in dairy calves that consumed grain than those that were fed hay or clover. Dargatz et al. *(103)* similarly found that barley-fed feedlot cattle were more likely to harbor this pathogen than cattle with corn or wheat-based diets. Further, no correlation between consumption of corn silage and pathogen detection in bovine feces was observed by Garber et al. *(167)*. In contrast, Herriott et al. *(204)* indicated that diets consisting of corn silage may contribute to the incidence of *E. coli* O157:H7 in cattle feces, while others have suggested that diet is not related to pathogen carriage in bovine animals *(69, 302, 462)*. Clearly, additional research is needed to assess relationship between diet and fecal prevalence of STEC in cattle so that feed formulations can be optimized to minimize the incidence of these pathogens.

The incidence of O157 STEC on animal hides at slaughter is generally higher than that of feces, ranging from 5.4% (n=1277; Canada) *(447)*, 7.3% (n=1500; Ireland) *(354)*, 17.6% (n=301; Ireland) *(461)*, 29.4% (n =100; Australia) *(146)*, 60.6% (n=1288; United States) *(19)*, 73.8% (n=305; United States) *(19)*. Not surprisingly, seasonal hide prevalence is similar to fecal prevalence, with a higher incidence of STEC in the summer to early fall *(19, 261)*. In the United States, non-O157 STEC were isolated from 77.7% (n=332) of hides in the fall and 43.0% (n=305) of hides in the spring *(19)*. While less is known regarding the incidence of non-O157 STEC on beef cattle hides, available reports

indicate that rates of contamination are similar to, if not greater than, O157 STEC *(19, 33, 326, 491)*.

Carcass contamination is significantly correlated with the prevalence of STEC in bovine feces and hides *(131)*. Elder et al. isolated STEC O157 from 28% (n=327), 11% (n=355), and 2% (n=330) of bovine feces, hides, and pre-chill carcasses, respectively at a U.S. meat processor *(131)*. In contrast, Fegan et al. reported that STEC O157 were found more frequently in hide samples (44%; n=100) than fecal samples (10%; n=68) at an Argentinian abattoir, though pre-chill carcass prevalence was similar (6%; n=100) *(146)*. Likewise, a study involving five U.S. beef slaughtering plants discovered that 36.7% (n=30) of hides and 13.3% (n=30) of fecal samples were positive of O157 STEC, though no carcasses (n=30) were found to be contaminated *(384)*. Barkocy-Gallagher et al. *(19)* assessed the frequency of both O157 and non-O157 STEC in a U.S. beef processing plant. The results from this study indicated that *E. coli* O157:H7 was recovered less frequently from feces (5.9%; n=1189) than hides (60.6%, n=1,288), though non-O157 STEC serotypes were more prevalent in fecal (34.3%) and hide (92.0%) samples than O157:H7 overall. Over a quarter of carcasses sampled prior to evisceration (n=1,288) were O157-positive (26.7%) and almost all contained a different STEC serotype (96.6%). The application of an unspecified intervention reduced, but did not eliminate STEC from beef carcasses; O157:H7 and non-O157:H7 STEC persisted on 1.2% and 16.2% of pre-chill carcasses, respectively.

While the internal muscle tissue of cattle is considered to be essentially sterile, beef carcasses may be contaminated by the hide or GI tract contents during harvest

(233). Alternatively, carcass surfaces may be cross-contaminated by a number of vectors in the slaughter and processing environments, including plant workers and equipment *(173)*. The reported prevalence of the non-O157 STEC is highly variable, ranging from 2.4% to 30.0% in ground beef and from 1.7 to 58.0% on whole carcasses *(222)*. Reported prevalence of STEC in subprimals is generally lower than that of ground product. In 2009, Gill et al. estimated that 0.2% of intact beef products procured in the United States were contaminated with *E. coli* O157:H7 *(171)*. Other researchers have reported similar incidences of <0.083% (n=1,199) *(244)*, 0.2% (n=1,014) *(200)* 0.7% (n=150) *(237)*. Liao et al. analyzed 1,129 ground beef samples procured in 24 states for and found that all were negative for six non-O157 serotypes (O26, O45, O103, O111, O121, and O145) *(283)*. These differences could be due several factors such as geographical distribution, season of sampling, beef production and processing practices, experimental design and/or method of STEC detection.

Risk Profile of Shiga Toxin-Producing *E. coli* in Non-Intact Beef

Non-intact beef products have garnered increased attention due to the potential introduction of pathogenic microorganisms below the tissue surface. Included in this category are meats that have been injected, mechanically tenderized, ground, minced, cubed, and reconstructed *(488)*. Mechanical tenderization is a process in which blades or needles are inserted into muscle tissue for the purpose of breaking muscle fibers and connective tissue to increase product tenderness and palatability *(169, 301)*. According to the National Cattlemen's Beef Association, approximately 18% of retail beef products are mechanically tenderized or injected *(342)*. In 2008, the FSIS published the *E. coli*

Checklist, which indicated that 37% (850 of 2,323 respondents) of beef operations surveyed use mechanical tenderization, with cumulative production volume of over 276 million kg of product annually. Further, 88% of these establishments indicated that they did not use pre-enhancement interventions and 94% cleaned and sanitized equipment at a frequency of once per day following production. In regards to microbiological analysis, 83% and 82% of processors reported no pre- or post-tenderization testing for *E. coli* O157:H7 *(5)*.

The potential for vertical translocation of microorganisms, including pathogens, from beef surfaces into deep tissues following mechanical tenderization has been demonstrated *(127, 174, 220, 297, 298)*. Following blade tenderization, the greatest numbers of cells are translocated to a depth of approximately 1 cm of the meat surface *(127, 297, 298, 445)*. Luchansky et al. *(297)* evaluated the mean transfer rates of *E. coli* O157:H7 at varying levels of inoculum (0.5, 1.5, 2.5, or 3.5 $\log_{10}$ CFU/cm^2) into top beef butts following single-pass blade tenderization of the lean side. The authors reported that the mean transfer rates of *E. coli* O157:H7 at depths of 1, 2, 3 and 4 cm were 32 to 41%, 1.25 to 6.18%, 0.04 to 7.5%, and 0 to 0.1%, respectively. This is in agreement with Sporing *(445)*, who reported 3-4% of *E. coli* O157:H7 inoculated on the surface of beef top sirloin can be transferred into the geometric center (2 to 4 cm in depth) following single-pass blade tenderization. Luchansky et al. *(298)* reported comparable rates of translocation and depth of penetration following single-pass blade tenderization for *E. coli* O157:H7 and non-O157 STEC, with 61.3 and 53.8% recovered within 1 cm of depth from the surface and 1.0 and 1.9% recovered from the remaining 5 segments of the

core samples, respectively. The influence of surface inoculum level on the numbers of microorganisms translocated into interior meat tissue following mechanical tenderization has been investigated. While the percentage of bacteria transferred into beef subprimal tissues following blade tenderization did not differ appreciably between high and low levels of inoculum, the overall numbers of internalized microorganisms and penetration depth increased with greater initial surface microbial load *(172, 297)*. Luchanksy et al. *(297)* found no significant difference between the total levels of *E. coli* O157:H7 transferred into top beef butts when the lean or fat side was inoculated and blade tenderized, or when the subprimal received a single- or double-pass. Similarly, Gill and McGinnis *(172)* saw no significant difference in the number of aerobic bacteria translocated following one or eight blade incisions. Additional research is needed to validate the efficacy of existing interventions in reducing *E. coli* O157:H7 and non-O157:H7 Shiga toxin-producing *Escherichia coli* in mechanically tenderized products.

CHAPTER IV

LACTIC ACID BACTERIA

Introduction

Lactic acid bacteria (LAB) were first described and named by Orla-Jensen in 1919 *(241)*. The organisms in this group are Gram-positive, non-sporulating, microaerophilic bacteria that produce lactic acid as the primary end-product of fermentation *(11, 494)*. They are generally catalase-negative, non-motile, and exhibit either bacillus (*Lactobacillus, Bifidobacterium, Carnobacterium*) or coccoid (*Lactococcus, Leuconostoc, Enterococcus, Pediococcus, Streptococcus, Aerococcus, Tetragenococcus*, *Vagococcus*) shape *(109)*. The genetic composition of these organisms is less than 50 mol% G + C *(375)*. Most strains are non-pathogenic to humans and may even be used as probiotics *(292)*, though rarely strains may act as opportunistic pathogens in immunocompromised individuals *(3, 20)*. Members of the LAB are often applied in foods to enhance the flavor, texture, and prevent microbial spoilage. However, some members of this group produce metabolic derivatives that contribute to the development of undesired flavors, odors, or slime associated with food spoilage *(148)*. In addition, some members of the LAB have been shown to inhibit foodborne pathogens *(55, 56, 59)*.

Lactic acid bacteria use substrate-level phosphorylation to generate ATP from ADP. These organisms are considered fermentative because they use an endogenous electron acceptor, derive energy from the oxidation of an organic molecule, and do not

employ the electron transport chain.These organisms are classified as either homofermentative, which produce lactic acid as the primary end product of glucose catabolism, or heterofermentative, which generate lower quantities of lactic acid from glucose metabolism. Homofermentative LAB primarily degrade glucose to pyruvate via glycolysis, usually via the Embden-Meyerhof-Parnas (EMP) pathway. The net output of this metabolism is two ATP molecules per 1 mole glucose. In contrast, heterofermentative LAB favor the pentose phosphate pathway which is less efficient and (net output=1 ATP per 1 mole glucose). The streptococci, pediococci, and homolactic lactobacilli are considered homofermentative, while the leuconostocs and heterolactic lactobacilli are heterofermentative. In addition to glucose, LAB are able to ferment a number of carbohydrates *(241, 327)*. However, organic acid production is dependent upon the extrinsic factor of temperature and will not occur in refrigeration *(328)*.

The antagonistic mechanisms include competition for nutrients and/or attachment sites, production of antimicrobial metabolites (e.g. reuterin, diacetyl, and fatty acids), inhibitory enzymes (e.g. lysozyme), bacteriocins (e.g. nisin, lactococcin B, and pediocin PA) and organic acids (e.g. lactic acid and acetic acid) *(233, 357)*. In fact, the U.S. Food and Drug Administration (FDA) has categorized some strains as generally recognized as safe for use in non-intact, whole muscle, and ready-to-eat meat and poultry products or whole carcasses at levels of 6.0 to 8.0 $\log_{10}$ CFU/g *(471, 486)*. Holzapfel et al. *(213)* developed a series of guidelines for the selection of appropriate protective cultures for use in foods. Most importantly, strains should exert no pathogenesis in humans or synthesize detrimental metabolic end products. In addition, the organisms should not

negatively alter the sensorial properties of the inoculated food product or contribute to spoilage. To ensure that treatment efficacy is consistent, the isolates should be resistant to mutation and synthesize antimicrobial compounds in a predictable manner. Ideal strains would also behave as indicators of temperature abuse.

Antagonistic Metabolites

Organic acids. The inhibitory action of the LAB is due in part to the production of organic acids and the subsequent microbial interactions of the acidic products *(108, 357)*. The inhibitory capacities of organic acids in foods is influenced by intrinsic attributes of the system matrix, extrinsic environmental factors, and innate structural or metabolic properties of the specific organism *(105, 227)*. Intrinsic factors include the availability of essential nutrients, water activity, composition, buffering capacity, pH, and the oxidation and reduction potential of the food commodity. The physiochemical attributes and microflora of foods may be altered during processing and production, which may subsequently change the bacteriostatic or bactericidal actions of acids. The extrinsic environmental conditions of that affect the antimicrobial efficacies of organic acids include holding temperature, atmospheric conditions, properties of packaging and time of storage *(105)*

An acid can be classified as "strong" or "weak" in accordance with the specific propensity for dissociation, which is quantified by the specific acid dissociation constant (pK_a). The pK_a is the pH value at which the concentration of undissociated acid molecules is equivalent to that of the dissociated forms. Below the respective pK_a, organic acid molecules exist as non-ionic, lipid-soluble, undissociated molecules, while

anions and protons dominate in pH values above the dissociation constant *(158, 289)*. Weak acids, composed of small fatty acids with less than 3 carbons acidify the cytoplasmic interior of bacterial cells in acidic environmental conditions *(452)*. Weak acids such as acetic ($pK_a = 4.75$), lactic ($pK_a = 3.79$), and propionic ($pK_a = 4.87$) acids dissociate in relatively high pH conditions and exist in non-ionic, undissociated forms in pH conditions relevant to many food commodities *(105, 158)*. Lipophillic acids, including acetic and lactic acids, can be transported across the bacterial membrane in the undissociated form. Upon transport into the near-neutral cytoplasm of the bacterial cell, the acid molecule will dissociate *(394)*. Accumulation of anions in the cytoplasm may contribute to the toxic effects of weak acids *(116, 403)*. The cytoplasmic interior is subsequently acidified by the newly liberated H^+ ions *(233, 380)*. Acidification of the cell interior may inhibit the functions of pH-sensitive enzymes by alteration of substrate charge and/or denaturation of protein structures. The innate buffering capacity of bacterial cells is thought to be due to the phosphate constituents in the genetic material (DNA and/or RNA), as well as the presence of aspartate and glutamate in protein side-chains. However, high levels of dissociated anions may overwhelm the buffering capacities and thus exert bacteriostatic or bactericidal effects *(394)*. Some weak acid compounds, including nitrite and sulfite, display a high degree of reactivity and may exert deleterious effects on essential metabolic constituents (enzymes, co-factors, and/or metabolites) *(403)*. It is important to note however, that hydrophilic weak acids like succinic, tartaric, and citric, generally cannot overcome the negative charge of the bacterial membrane to enter the cytoplasm of the cell and thus exhibit bacteriostatic

activity *(453)*. In neutral pH conditions, small acids and medium sized acids with less than 7 carbons destabilize the bacterial membrane *(452)*. However, the behavior of small and medium acids may mimic that of large acids in high concentrations and induce cell lysis by rupturing the cell membrane. Further, the activity is also influenced by the hydrophobicity of the acid molecules. For example, lactic, citric, and other hydrophilic acids tend to dissociate extracellularly in low pH conditions but exhibit a propensity for chelation of metal ions in alkaline pH ranges. Conversely, the inhibitory activity of esters (destabilization of the cell membrane) is delineated to a greater extent by lipid solubility rather than environmental pH. In contrast, strong acids, including hydrochloric and sulfuric acids, dissociate readily across a wide range of pH conditions. Therefore, strong acids tend to exist in food products as dissociated, ionic molecules that inhibit bacterial growth through reduction of environmental pH *(452)*. Membrane bound proteins sense acid anions in the periplasmic and environmental pH and may facilitate the induction of various acid resistance mechanisms *(150)*. Bacteria are generally more tolerant to decreases in environmental pH and more sensitive to cytoplasmic acidification *(394)*. Lactic acid bacteria resist the antagonistic effects of organic acids by employing several systems for adaptation and tolerance. Some species, including *L. lactis* subsp. *lactis* and *L. acidophilus*, synthesize adenosine triphosphatase, which restores intracellular pH by translocating H+ into the environment *(259, 335)*. *L. lactis* acid tolerance may also be achieved through induction of the arginine deaminase pathway, which facilitates the production of ammonia *(373)*. Cellular pH may also be restored with amino acid decarboxylation reactions *(410)* and/or utilization of the citrate

transport system, characterized by the uptake of citrate, production of acetate and oxaloacetate via citrate lyase, and subsequent decarboxylation of oxaloacetate *(295, 308)*.

Bacteriocins. Bacteriocins are antimicrobial peptides that are produced by most know bacteria. The genetic elements for bacteriocin production often exist on a plasmid or transposable element and may be transferred between strains and species *(229, 499)*. These compounds generally exhibit bactericidal activity on organisms that are closely related to the producer. As the LAB are Gram-positive, bacteriocins produced by these organisms are active against Gram-positive bacteria. However, Gram-negative bacteria may be susceptible if the outer membrane integrity is disrupted *(181, 291, 450)*. Klaenhammer *(250)* reviewed the genetic properties and physiochemical attributes of bacteriocins produced by LAB, including a thorough explanation of the different classifications of these antimicrobial peptides. Bacteriocins synthesized by these organisms are grouped according to genetic, structural, and biochemical similarities. In terms of size, the class I lantibiotics are the smallest bacteriocins (<5 kDa) produced by LAB which contributes to the thermal stability of these peptides. They also include amino acid subunits that are not usually present in bacteriocins, including lanthionine and enzymatically dehydrated molecules. The primary antimicrobial activity is disruption of microbial membranes and examples include nisin and lacticin 481 *(250, 469)*. Class II bacteriocins such as pediocin PA-1, lactococcin A, and lactacin F may be slightly larger than lantibiotics but measure less than 10 kDa. These peptides display similar antagonistic functions to class I types in that they disrupt cell membranes. They

also exhibit moderate to high tolerance and do not contain lanthionine amino acid subunits *(250)*. The largest bacteriocins (>30 kDa) are the heat-liable class III types such as lactacin A, lactacin B, and acidophilucin A. Class IV includes the complex bacteriocins lactocin 27 and pediocin SJ-1 *(39, 67, 94, 109, 202, 250, 321, 359, 367, 456, 516)*.

Bacteriocins are not strain-specific and may be produced by multiple genera of LAB or multiple species within the same genera. For example, both *Pediococcus* spp. and *L. plantarum* are able to produce pediocin PA-1 *(321)*, while salivaricin A1 production has been observed in *Streptococcus pyogenes, S.* agalactiae, and *S. dysgalactiae* subsp. *equisimilis (499)*. In addition, multiple bacteriocins may be produced by specific LAB, as demonstrated by the synthesis of Enterocin A and B by *Enterococcus faecium* CTC492 *(348)*. Production is governed by a number of intrinsic and extrinsic factors. Growth-phase may induce, inhibit, or have no influence on production. Production of sakacin A by *L. sakei* can occur at all phases of growth, while *L. curvatus* synthesis of curvacin A is limited to logarithmic replication *(210, 495)*.

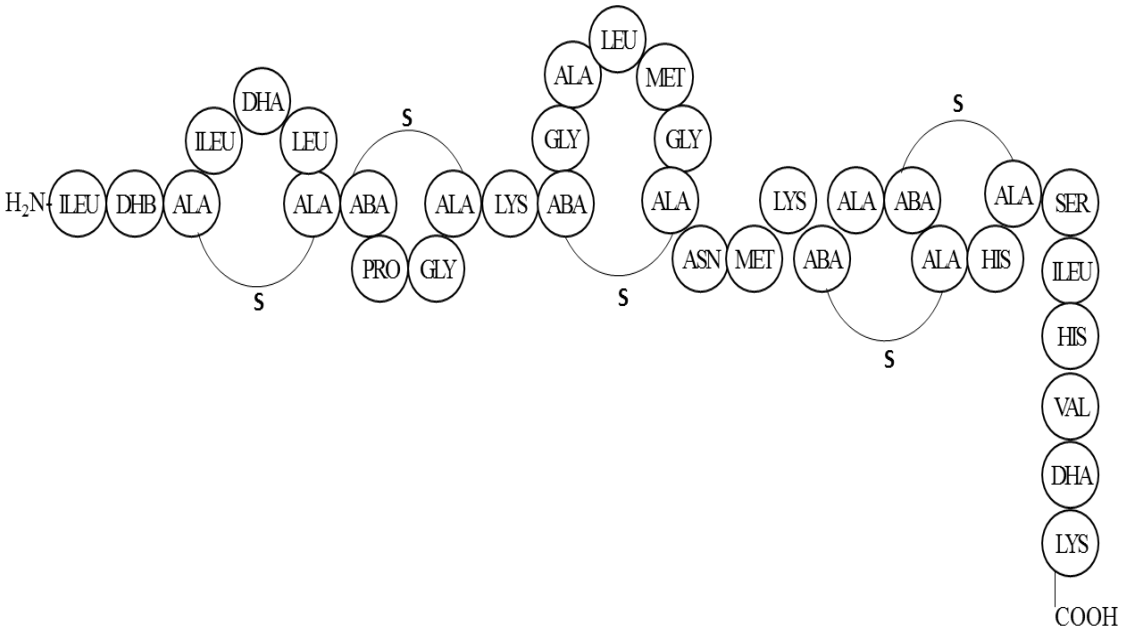

Figure 1. The structure of nisin. Adapted with permissions from Gross, E., and J.L. Morell, 1971 *(183)*.

In 1928, the first bacteriocin produced by a member of the LAB was discovered. Today, this polypeptide is known as nisin (Group N *Streptococcus*-recovered inhibitory substance) *(392)*. However, it was not used for food preservation until the 1950's *(208)*. Nisin is approved for use in foods as a biopreservative *(145)*, however this substance has limited utility in raw meats because it complexes with glutathione, fats, and proteins *(269, 421, 449)*. The biosynthesis and antimicrobial activity of nisin were reviewed by Lubelski et al. *(296)*. The genetic elements encoding for sucrose hydrolysis, nisin production, and nisin resistance are encoded on a chromosomally linked transposon *(70, 119)*. Translation of these genes produces a precursor called prenisin which does not display antagonistic activity *(61, 465)*. Prenisin is composed of 57 amino acid residues

(436), 23 of which are subunits of an N-terminal peptide. This leader peptide mediates recognition of unmodified prenisin by NisB dehydratase *(257, 280, 436)*, which enzymatically dehydrates serine and threonine amino acids in the prenisin structure *(257)*. Once dehydrated, these modified residues are enzymatically coupled to cysteine by NisC, to form the characteristic lanthionine rings of class I antibiotics *(280, 422)*. A specific ABC-type transporter (NisT) then exports the modified, inactive prenisin across the cytoplasmic membrane *(256)*, where the leader peptide is cleaved by the extracellular serine peptidase NisP *(258)*. Excision of this amino acid sequence produces the active form nisin *(371)*, which functions as a cationic detergent and exerts bactericidal activity via destabilization and of the cytoplasmic membrane and inhibition of cell wall production *(60, 63)*. Mature extracellular nisin molecules bind to lipid II on the surface of target cells *(490)* and complex to form a pore, causing leakage of intracellular constituents and increasing permeability *(195)*. The cell proton motive force is lost as a result of this membrane disruption, causing a rapid decrease in intracellular ATP *(66, 67)*. Cell lysis has been reported following exposure to nisin, though this phenomenon is likely due to activation of host cell autolytic enzymes *(401)*. It is antagonistic to Gram-positive bacteria, including *Leuconostoc* spp., *Lactobacillus* spp., *Clostridium perfringens, C. botulinum, Bacillus cereus, Listeria* spp. and *Staphylococcus aureus.* However, Gram-negative bacteria, yeasts, and molds are resistant to its inhibitory activity *(386)*. *Lactococcus lactis* subsp. *lactis*, is a nisin-producing organism with GRAS status *(352, 471).* The inherent immunity of this organism to the lethal effects of nisin is derived from the expression and synergistic functions of ABC transporters

(NisFEG), which exports nisin from the cellular interior, and nisin intercepting lipoprotein (NisI) *(136, 435)*. In addition to nisin, some *L. lactis* spp. synthesize the bacteriocins lacticin 481, lactococcin and dricin. Lacticin 481 has inhibitory activity against Gram-positive spoilage bacteria, including *Lactobacillus, Lactococcus, Leuconostoc,* and *Clostridium tyrobutyricum (367)*. Lactocin is a plasmid-encoded bacteriocin with bacteriocidal activity to *C. tyrobutyricum, Lactobacillus helveticus,* and *Streptococcus thermophilus (123)*. However, the host range of dricin is narrow, and has little application for foods *(376)*.

Pediococcus acidilactici is also classified GRAS for use in certain foods *(352, 471)* and is used as a starter culture in the production of fermented meats and vegetables. In meat matrices, the optimal temperatures for fermentation and acid production of this organism range from 35 to 46.1 °C *(387)*. This species may act as an opportunistic pathogen in immunocompromised individuals and has been associated with bacteremia *(20)*.Pediocin AcH is produced by several *P. acidilactici* strains, including E, F, H, M, and AcH. Translation of the plasmid-encoded *pap* gene produces an inactive form of pediocin AcH called prepediocin AcH; prepediocin AcH is composed of 62 amino acids. In low pH conditions, the inactive form undergoes post-translation modification, wherein 18 amino acids are cleaved from the N_2-terminal end and the activated pediocin AcH is then excreted from the cell *(109)*. Excretion of this bacteriocin is greatest during stationary phase in pH conditions of 4.0 or less. In pH conditions greater than 5.0, a significant decrease in excretion occurs *(41, 331)*. This bacteriocin is heat-stable, resistant to extreme pH, and retains activity in temperatures below 0 °C. This bacteriocin

exhibits inhibitory action towards *Pediococcus, Leuconostoc*, *Lactococcus, Lactobacillus, Bacillus, Staphylococcus, Listeria*, and *C. botulinum* Type E *(39, 278, 514)*. In sensitive cells, Pediocin AcH binds host cell receptors and is then transported through the cell wall and subsequently destabilizes the cytoplasmic membrane of Gram-positive organisms, causing cell death. Resistant cells uptake this bacteriocin, though it is not transported through the cell wall to interact with the cytoplasmic membrane *(39, 41)*. Bactericidal action has been reported in Gram-negative bacteria (including *Pseudomonas, E. coli, Salmonella, Yersinia,* and *Aeromonas* spp.) that were previously exposed to sub-lethal freezing, weak acid, and heating *(16)*. The outer membrane of Gram-negative bacteria does not have appropriate Pediocin AcH receptors; however, sub-lethal injury may damage the outer membrane, exposing the cytoplasmic membrane to the destabilizing activity of the bacteriocin and possibly cell death. Like nisin, host cell lysis has been reported following exposure to Pediocin AcH, though this is thought to be attributable to exposure to autolytic enzymes excreted by adjacent cells after death *(39, 41)*.

L. acidophilus is considered generally recognized as safe (GRAS) for use in specific food products *(352, 471)*. These organisms are acid-tolerant, microaerophilic, chain-forming rod-shaped bacteria that exhibit homofermentative metabolism *(241)*. Lactic acid may be produced from fructose, galactose, glucose and maltose *(7)*. Health benefits associated with probiotic supplementation of *L. acidophilus* in humans, include colon cancer prevention *(507)* and improved digestion of lactose *(107, 175)*. *L. acidophilus* strains have been shown to synthesize a number of bacteriocins, including

lactacin, lactocidin, acidolin, and acidophilin *(7)*. The majority of these antimicrobials are heat stable, low molecular weight peptides *(516)*. While most lack antagonistic activity towards Gram-negative bacteria, others may be antimicrobial to both Gram positives and Gram-negatives *(97)*. The antagonism towards Gram-negative cells may require membrane destabilization prior to exposure, however *(249)*.

Reactive oxygen species. Reactive oxygen species such as hydrogen peroxide, superoxide anions, and hydroxyl radicals may be produced by LAB from aerobic respiration *(464)*. Hydrogen peroxide may be formed as an end product of reduced nicotinamide adenine dinucleotide (NADH), lactate, and/or pyruvate oxidation by flavoprotein oxidases *(241)*, as well as through enzymatic reactions of superoxide dismutase. The antagonistic activity of hydrogen peroxide is due to peroxidation of membrane lipids and subsequent changes in membrane permeability *(196)*. In addition, it may react and produce free hydroxyl and superoxide radicals that may damage cellular proteins or nucleic acids. Furthermore, hydrogen peroxide is highly reactive and may form other inhibitory compounds through interactions with other components *(91, 241)*. Gram-negative bacteria are highly susceptible to the bactericidal effects of hydrogen peroxide, though Gram-positive bacteria, yeasts, and molds may also be inactivated in its presence. However, the concentrations of hydrogen peroxides produced in typical food systems would likely be inhibitory, rather than lethal (204). Hydrogen peroxide-producing LAB are also susceptible to its antimicrobial effects *(84)*. These organisms express enzymes which degrade reactive oxygen species in response to oxidative stress. While most LAB are catalase-negative, production of this hydrogen peroxide degrading

enzyme has been reported in some strains, including *Pediococcus acidilactici (505).* Catalase-negative LAB including *L. acidophilus* may synthesize NADH peroxidase, which degrades toxic hydrogen peroxide into water and NADH oxidase, which forms water or hydrogen peroxide from the dismutation of superoxide anions*(91, 408).* GUptake of glutathione by *L. lactis* has been shown to increase resistance to hydrogen peroxide through activation of glutathione peroxidase and reductase *(282).* Another system employed by LAB strains is the thioredoxin-thioredoxin reductase system *(428).*

Ethanol. Ethanol is one end-product of heterofermentative metabolism. It is amphiphilic in structure, water soluble, and may be bactericidal. The antimicrobial activity of ethanol may be due to membrane destabilization, denaturation of proteins, and/or binding of water with subsequent reductions in water activity. Protein denaturation is thought to be due to destabilization of non-covalent bonds of tertiary structures, which is enhanced in the presence of water. Due to vapor pressure and evaporative procedures, aqueous ethanol solutions containing 70% ethanol (v/v) are more effective in eliminating bacteria than 95% ethanol (v/v) *(239, 264).* This alcohol is transported into cells via passive diffusion *(497).* Exposure to ethanol may increase membrane fluidity, resulting in leakage of ions and solutes from the cell *(360, 409).* Additional inhibition may result from hydrogen bonding of water molecules with ethanol, which reduces the availability of free water for metabolic reactions *(239).* Tolerance may be induced in these organisms when grown in the presence of sublethal concentrations of ethanol. Specifically, membrane phospholipid composition is changed to include more unsaturated fatty acids *(29, 225, 226).*

CHAPTER V

CONTROL OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* USING LACTIC ACID BACTERIA

Introduction

Biological preservation has been used historically for elongation of shelf life and prevention of food spoilage. Consumer demand for minimally processed foods has increased in recent years. This, combined with the growing concern regarding stress adaptation of pathogens following exposure to chemical and thermal treatments, has highlighted the need for alternative solutions for beef decontamination. Lactic acid bacteria (LAB) are known to synthesize a number of antimicrobial metabolites, including bacteriocins, organic acids, and low molecular weight peptides. However, the inhibitory activity of many of these compounds is often limited to Gram-positive bacteria. The exterior layer of the Gram-negative outer membrane provides a permeability barrier against hydrophobic compounds such as nisin *(199)*. In spite of this, *in vitro* studies have demonstrated that these organisms may suppress the growth pathogenic *E. coli.* Extrapolation of these results to complex food matrices may be unreliable, as planktonic cells display markedly increased susceptibility to bacteriocins and acids compared to attached cells *(207)*. Numerous studies have also sought to exploit the inhibitory mechanisms of specific protective cultures for the inhibition of pathogenic or spoilage bacteria in meats, with varying results *(56, 59, 212)*. While some have reported the production of organic acids by LAB during refrigerated storage *(48,*

118), many do not synthesize these in such conditions *(328)*. In addition, exposure of STEC to sub-lethal acidic conditions may induce adaptations that provide enhanced tolerance to reduced pH conditions and other sublethal stressors such as heat *(151, 431)*. Organic acid production is also dependent upon intrinsic properties of the surrounding medium, including the availability of fermentable carbohydrates. The level of available glucose in meats after the completion of rigor is low, ranging from 4.5 to 7 µmol/g. Without the supplementation of fermentable carbohydrates, chilled meats are not a suitable substrate for organic acid production and reduction in pH would likely be negligible *(328, 387)*.

The outer membrane of Gram-negative bacteria may act as a barrier against the bacteriocins produced by LAB. However, destabilization of divalent cations of the non-covalent cross-bridges that bind the lipopolysaccharides together can induce sensitivity to hydrophobic bacteriocins. Metal chelators can sequester divalent cations from the structure, therein allowing entry of bacteriocins, which subsequently attack the cytoplasmic membrane *(190, 270)*. Castellano et al. *(78)* investigated the antimicrobial efficacy of *L. curvatus* CRL705 and *L. lactis* CRL1109, combined with a the chelator disodium ethylenediamine tetraacetate (Na_2EDTA; 18 g per kg/48 mM), for the disinfection of an Stx-negative *E. coli* O157:H7 in ground beef patties stored at 5 °C for 9 days. Pathogen numbers in samples treated with 7.0 $\log_{10}$ CFU/g LAB were not different ($p \geq 0.05$) than untreated controls throughout the storage period. However, significant reductions ($p < 0.05$) in *E. coli* were observed in samples treated with both LAB and the chelating agent. Compared to untreated samples, the combined treatment

reduced numbers of *E. coli* by 1.6, 2.0, 3.4, and 3.9 $\log_{10}$ CFU/g following 2, 3, 6, and 9 days' storage (5 °C), respectively *(78)*. Millette et al. *(322)* similarly observed that *E. coli* O157:H7 was inhibited in the presence of LAB and the chelator EDTA (10 mM), but pathogen growth was not affected in the absence of the chelator. Purified bacteriocins, including lactocin 705 (produced by *L. curvatus* CRL705), lactocin AL705 (produced by *L. curvatus* CRL705), and nisin (produced by *L. lactis* CRL11109) have been reported to inhibit *E. coli in vitro* when added in conjunction with chelator (EDTA or sodium lactate) *(23)*. While *E. coli* may become sensitive to bacteriocins following membrane disruption, these antimicrobial peptides are not effective against intact, viable cells.

Antagonistic Activity of Lactic Acid Bacteria in Beef

Ruby and Ingham *(400)* evaluated the antagonistic activity of *Lactobacillus sakei* 10-EGR-s in ground beef for the inhibition of two strains of *E. coli* O157:H7 (strains 380-94 and 9A). No significant inhibition ($p \geq 0.05$) was observed for either pathogenic strain in ground beef samples co-inoculated with 5.0 $\log_{10}$ CFU/g *L. sakei* and 3.0 $\log_{10}$ CFU/g *E. coli* throughout storage for up to 9 days at 5 or 10 °C. Subsequently, a second study was conducted to determine whether co-inoculation of ground beef with 7.0 $\log_{10}$ CFU/g *L. sakei* and 3.0 $\log_{10}$ CFU/g *E. coli* would produce reductions under the same conditions (5 or 10 °C) for up to 6 days. Numbers of both pathogen strains evaluated (*E. coli* 380-94 and 9A) in untreated and treated samples did not change ($p \geq 0.05$) following 3 days' storage at 5 °C. While significant increases ($p < 0.05$) were observed in control samples for both strains after 6 days at 5 °C, *E. coli* populations in co-inoculated samples

remained constant ($p \geq 0.05$). Pathogen growth occurred in all samples stored in abusive conditions (10 °C), regardless of treatment status. However, only *E. coli* O157:H7 380-94 displayed sensitivity to treatment after 3 days, with control populations being greater ($p < 0.05$) by 0.9 $\log_{10}$ CFU/g. Significant inhibition ($p < 0.05$) was observed for both strains after 6 days at 10 °C, with treated samples being 2.0 $\log_{10}$ CFU/g and 2.3 $\log_{10}$ CFU/g lower than untreated for isolates 9A and 380-94, respectively. In Study 3, the researchers sought to determine whether inhibition was solely attributed to the population of *L. sakei,* or if the observed differences between treated and untreated samples were a function of *L. sakei* and *E. coli* population ratio. In this study, ground beef was inoculated with 6.0 $\log_{10}$ CFU/g of *L. sakei* and 2.0 $\log_{10}$ CFU/g of *E. coli* O157:H7 (either 380-94 or 9A). Although the methodology in this experiment was identical to Study 2, pathogen numbers in treated samples were not different than control samples following storage at 5 or 10 °C for up to 6 days *(400).*

In a similar study, Muthukumarasamy et al. *(333)* evaluated the antagonistic activity of *Lactobacillus reuteri* (3.0 or 6.0 $\log_{10}$ CFU/g) combined with glycerol (250 mM/kg) in ground beef for the disinfection of *E. coli* O157:H7 inoculated at low (3.0 $\log_{10}$ CFU/g) and high (6.0 $\log_{10}$ CFU/g) levels of contamination. Following inoculation, individual beef samples were packaged in nylon/ethylene vinyl alcohol/polyethylene bags (oxygen transmission rate= 2.3 ml/m^2 for 24 h at 23 °C), which were then flushed with nitrogen, vacuum sealed, and stored at 4 °C for 0, 5, 10, 15, 20, or 25 days. Pathogen numbers in ground beef samples co-inoculated with high LAB and low *E. coli* populations were significantly ($p < 0.05$), though not substantially (0.2 $\log_{10}$ CFU/g)

reduced by day 5. For this treatment, no viable pathogens were detected at the 10 day sampling point. In beef samples treated with low LAB and low *E. coli* numbers, pathogen numbers were significantly lower after 10 days (0.2 $\log_{10}$ CFU/g recovered) and were not subsequently detected at later sampling points. Treatment of beef samples contaminated with high loads of *E. coli* with 3.0 $\log_{10}$ CFU/g of LAB reduced pathogen numbers to below the limit of detection after 20 days of storage. Reductions achieved using elevated LAB numbers (6.0 $\log_{10}$ CFU/g) were similar to those reported using low LAB levels (3.0 $\log_{10}$ CFU/g) for samples inoculated with both high and low pathogen numbers in the presence of glycerol.

To assess the inhibitory function of individual LAB strains, Smith et al. *(442)* co-inoculated ground beef with one of four *Lactobacillus acidophilus* isolates (NP3, NP7, NP35, and NP51) to a level of ~ 7.0 $\log_{10}$ CFU/g and one strain of *E. coli* O157:H7 (~5.0 $\log_{10}$ CFU/g). Numbers of *E. coli* in vacuum-packaged beef were significantly reduced ($p<0.05$) by *L. acidophilus* NP35 within 4 days of storage at 5 °C, though samples treated with *L. acidophilus* NP3, NP7, and NP51 were not significantly different than untreated samples on day 4. All strains inhibited ($p<0.05$) *E. coli* populations within 8 days of storage, with a >1.5 $\log_{10}$ CFU/g difference from controls. This appeared to be the maximal level of inhibition, however, as pathogen numbers were not different ($p\geq0.05$) between 8 and 12 days' storage. Next, artificially contaminated ground beef (~5.0 $\log_{10}$ CFU/g *E. coli* O157:H7) was treated with a cocktail of the same *L. acidophilus* strains (NP3, NP7, NP35, and NP51) to a level of ~8.0 $\log_{10}$ CFU total LAB/g. Relative to untreated control samples, the LAB cocktail effectively reduced

($p<0.05$) pathogen numbers by 2.0 $\log_{10}$ CFU/g after 3 days of storage at 5 °C in vacuum packaging. Additional storage yielded greater effects, with *E. coli* numbers in treated beef samples being over 3.0 $\log_{10}$ CFU/g lower than untreated at day 5. Together, these studies suggest that a cocktail of LAB strains within the same species may have a synergistic effect on pathogen numbers. To exploit the heterogeneous production of antimicrobial metabolites by LAB, the use of multiple genera and species has been investigated. Hoyle et al. *(215)* evaluated a cocktail of LAB-inoculated ground beef with *E. coli* O157:H7 and varying concentrations of lactic acid bacteria (10^6, 10^7, and 10^8 CFU/g), then stored samples at 5 °C in traditional overwrap packages. Significant ($p<0.05$) reductions in the numbers of *E. coli* were observed following 3 days of storage (2.7 to 2.8 $\log_{10}$ CFU/g), with additional reductions after 5 days (3.7 to 4.0 $\log_{10}$ CFU/g); reductions achieved in this study were not influenced by LAB concentration.

The composition (fat:lean ratio), structure, nutrient availability, and water activity of ground beef is different than that of intact whole beef cuts and carcasses. Researchers have sought better understanding of the antagonistic potential of protective cultures in these food matrices. In one such study, boneless strip loin (*M. longissimus dorsi*) was inoculated with *E. coli* O157:H7, and then treated by submersion to: a) 2.0 $\log_{10}$ CFU/ml *Pseudomonas fluorescens*; b) 6.0 $\log_{10}$ CFU/ml *P. fluorescens*; c) 2.0 $\log_{10}$ CFU/ml *Lactobacillus plantarum*; or d) 4.0 $\log_{10}$ CFU/ml *L. plantarum (466).* Senne and Gilliland *(427)* reported that treatment of beef steak with 7.0 $\log_{10}$ CFU/g of *L. delbrueckii* subsp. *lactis* RM2-5 was not effective in controlling low level (<3.0 $\log_{10}$ CFU/g) of *E. coli* O157:H7 contamination during storage at 5 °C. Total coliform counts

on treated samples were 0.3, 0.6, 0.7, and 0.8 $\log_{10}$ CFU/g lower than untreated samples after 0, 3, 6, and 9 days of aerobic refrigeration, respectively. While numerically lower, coliforms were not ($p \geq 0.05$) influenced by treatment. In a subsequent study by the same group, 7.0 $\log_{10}$ CFU/cm^2 *L. delbrueckii* subsp. *lactis* RM2-5 was applied to the surface of beef carcass excisions for the disinfection of *E. coli* O157:H7. Total coliform bacteria enumerated after aerobic storage at 5 °C for 8 days were significantly reduced by 1.1 $\log_{10}$ CFU/cm^2 in treated samples, while coliform populations in untreated beef were decreased by only 0.5 $\log_{10}$ CFU/g in the same period. Other researchers have compared the inhibitory utility of LAB to conventional interventions. In a study by Echeverry et al. *(126)*, refrigerated choice-grade strip loins were artificially contaminated with *E. coli* O157:H7 (5.0 $\log_{10}$ CFU/cm^2) and treated with LAB (7.7 $\log_{10}$ CFU/ml), acidified sodium chlorite (1,000 to 1,200 ppm), or 3% lactic acid (pH=2.21). Following refrigerated vacuum aging for 14 or 21 days, surface pathogen populations did not change ($p \geq 0.05$) from day 0 levels for any treatment. A subsequent study by the same group *(127)* used a protocol identical to that already described; populations of *E. coli* O157:H7 on beef strip loins treated with the same concentration of LAB (7.7 $\log_{10}$ CFU/ml) in the same manner and aged for 21 days at 4.4 °C were not different ($p \geq 0.05$) than samples aged for 14 days. However, this study failed to disclose the day 0 pathogen numbers on LAB treated samples, as well as the relevance of the day 0 populations on LAB treated beef in reference to those recovered post-aging.

Sensorial Attributes of Beef Treated with Lactic Acid Bacteria

As previously stated, LAB can contribute to food spoilage. For this reason, researchers have sought to determine the mechanisms by which LAB contribute to the sensorial properties of meat and meat products. Senne and Gilliland *(427)* observed sensorial properties of beef steak were influenced by mode of treatment application. Specifically, samples dipped in solutions of *L. delbrueckii* subsp. *lactis* underwent visible bleaching of color, whereas no lightening was observed when treatments were directly pipetted onto the meat surface.

Hoyle Parks et al. *(218)* compared the spoilage characteristics of ground beef with or without supplementation of 9.0 $\log_{10}$ CFU/g LAB (equal volumes of *L. acidophilus* NP51, *Lactobacillus crispatus* NP35, *Pediococcus acidilactici* and *La. lactis* subsp. *lactis*). Beef was stored at an abusive temperature (10 °C) in traditional packaging, overwrapped in polyvinylchloride film (oxygen transmission rate=21,700 cm^3 per m^2 per 24 h). At 0, 12, 24, and 36 h, the spoilage characteristics of untreated and treated beef samples were evaluated by trained panel (lean color, percent discoloration and immediate off odor), an untrained panel (lean color, purchase intent, freshness of odor, and likelihood of consumption), instrumental color analysis (Hunters *L**, hue angle, and saturation) and quantification of thiobarbituric acid reactive substances (TBARS). No significant treatment effects ($p \geq 0.05$) were reported for any of the spoilage characteristics evaluated by trained panels, untrained panels, or instrumental color analysis at all time points. However, TBARS values were observed to be significantly higher in untreated meat than treated ($p < 0.05$). This indicates that the

inclusion of LAB reduced the accumulation of lipid oxidation end products, which could be attributed to inhibition of lipid oxidation or utilization of end products by LAB.

In a separate study by Hoyle Parks et al. *(217)*, ground beef samples were treated with a mixture of the aforementioned strains of LAB (9.0 $\log_{10}$ CFU/g), packaged in traditional packaging, overwrapped in polyvinylchloride film (oxygen transmission rate=21,700 cm^3 per m^2 per 24 h), and stored at 0 °C for up to 84 h. Trained panel scoring indicated that treated and untreated beef patties exhibited non-differing ($p \geq 0.05$) lean color, percent discoloration, and immediate off-odor. Consumer panels identified no differences ($p \geq 0.05$) between treated and untreated beef lean color, intent to purchase, freshness/odor, and likelihood of consumption. Instrumental analysis revealed that Hunter *L** and hue angle values were not influenced by the inclusion of LAB ($p \geq 0.05$). However, saturation values were significantly higher ($p < 0.05$) in traditionally stored treated beef. While differences were not detected in immediate off-odor (trained panel) or freshness of odor (consumer panel), analysis of TBARS indicated suppressed lipid oxidation in treated samples ($p < 0.05$) versus controls. To determine whether results were influenced by packaging, ground beef was inoculated identically with LAB and then individually sealed in modified atmosphere (80% O_2 and 20% CO_2; package oxygen transmission rate: <20 cm^3 m^{-2} 24 h^{-1} at 4.4 °C and 100% relative humidity). Samples were stored at 0 °C for up to 84 h prior to analysis. No effects were observed by treatment for trained panel lean color, percent discoloration, nor immediate off-odor scores. These findings were in agreement with consumer panel rankings, which indicated no differences by treatment for lean color, purchase intent, freshness of odor, and

likelihood of consumption. Additionally, supplementation of LAB did not significantly affect ($p \geq 0.05$) instrumental color analysis values (Hunter *L**, hue angle, and saturation) or TBARS.

The sensorial attributes of LAB-treated ground beef were also evaluated by Smith et al. *(442).* Beef samples were treated with 7.0 $\log_{10}$ CFU/g of *L. acidophilus* (strains NP3, NP7, NP25, and NP51) and stored at 4 °C for 6 h in oxygen-permeable film overwrapping or 7 days in vacuum packaging. An untrained panel (24 individuals) was unable to differentiate treated samples from untreated for either storage condition. Djenane et al. *(118)* inoculated beef steaks with 4.0 to 5.0 $\log_{10}$ CFU/cm² of *Lactobacillus* CTC 711 or *Lactobacillus sakei* CT 372. Samples were stored in modified atmospheric packaging (70% O_2, 20% CO_2, 10% N_2, or 60% O_2, 40% CO_2) at approximately 1 °C for up to 28 days. A trained panel (6 individuals) detected no significant differences in off-odor between treated and untreated samples for up to 22 days. After 28 days, untreated beef stored in 20% CO_2 was found to have significantly ($p<0.05$) greater off-odor than treated beef. Hydrogen sulfide production by *Lactobacillus* spp. has been reported in vacuum packaged beef samples following refrigerated storage *(48).*

Electrostatic Spray

The method in which an intervention is applied to a food product can impact its antimicrobial efficacy. One application system that has been recently explored is electrostatic spray (ESS), which produces finely atomized droplets that are electrically charged. These charged droplets become attracted to target surfaces possessing opposite

polarity and adhere with enhanced uniformity to surfaces with different orientations *(132, 265)*. Solutions applied with this technology have been shown to achieve significantly greater spray deposition when compared to conventional hydraulic-atomizing sprayers, reducing the amount of solution required to achieve equivalent coverage *(267, 300, 406)*. Due to potential environmental and economic benefits from more efficient solution utilization, ESS has been investigated for a wide array of uses including the application of pesticides (biological and chemical) to crops *(266, 267, 419)*, antimicrobials to food contact surfaces *(300)*, antioxidants to ground beef *(334)*, lactic acid to beef carcasses *(460)*, and even sunless tanner to the human body *(93)*. The viability of bacterial biocontrol agents applied using ESS has been demonstrated *(267, 419)*. Peracetic acid (0.02%) applied with a conventional sprayer, followed by malic (3%), octanoic (3%) or fumaric acid (saturated solution) to beef trim prior to grinding reduced STEC after 0, 1, 2, and 7 days of storage at 4 °C, comparable to that achieved using a conventional sprayer with less usage of antimicrobial *(112)*. However, little research regarding the application of viable protective cultures to foods has been conducted.

CHAPTER VI

GROWTH OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* AND IMPACTS OF CHILLING AND POST-INOCULATION STORAGE ON BACTERIAL ATTACHMENT TO BEEF SURFACES*

Introduction

According to the U.S. Centers for Disease Control and Prevention (CDC), approximately 48 million people acquire a foodborne illness annually in the United States *(81)*. Scallan et al. *(416)* estimated a mean annual disease case incidence of domestically acquired Shiga toxin-producing *Escherichia coli* (STEC) of 205,781 cases, 54.8% of which were attributed to members of the non-O157 STEC. The serotypes most commonly implicated in non-O157 STEC human foodborne disease have been reported to belong to *E. coli* O26, O111, O103, O121, O45 and O145 *(62)*. Outbreaks involving STEC have been linked to a variety of food commodities, in particular not ready-to-eat (NRTE) and non-intact beef products *(182, 361)*. The growing food safety concerns regarding the non-O157 STEC are evidenced by the U.S. Department of Agriculture Food Safety Inspection Service (USDA-FSIS) declaring the afore-mentioned six non-O157 STEC adulterants in beef products or the components of non-intact NRTE beef products *(482)*. According to the USDA Economic Research Service (USDA-ERS), approximately 11.7 billion kg of beef were consumed domestically in 2012 *(472)*.

*Reprinted from Food Microbiology, 44, Kirsch, K.R., Taylor, T.M., Griffin, G., Castillo, A., Marx, D.B., and Smith, L., Growth of Shiga toxin-producing *Escherichia coli* (STEC) and impacts of chilling and post-inoculation storage on STEC attachment to beef surfaces, Pages 236-242, Copyright (2014), with permission from Elsevier.

During this period, 0.15% of raw ground beef samples (n=11,405) collected from federally inspected plants were found to be positive for *E. coli* O157:H7 *(485)*. Similarly, samples of raw ground beef components taken for trim verification programming revealed contamination rates of 0.53% for *E. coli* O157:H7 (n=2263) and 0.91% for non-O157 STEC (n=1533) *(485)*. A high level of variation exists between reports of the prevalence of non-O157 STEC in beef, ranging from <1.0% to 30.0% in ground beef and from 1.7% to 58.0% on whole carcasses *(8, 49, 170, 222, 272, 363, 426, 504)*. It is estimated that 39.4% of *E. coli* O157 and 29.7% of non-O157 STEC foodborne disease cases are acquired through the consumption of contaminated, improperly prepared beef products, though in many instances the transmission vehicle is not identified *(361, 415)*. In 2008 the largest domestic outbreak of non-O157 STEC was recorded; 341 individuals acquired O111:NM-derived foodborne disease after eating at a restaurant in Oklahoma, though the food vehicle was not identified *(53)*. The first recall request by the USDA-FSIS involving a member of the non-O157 STEC occurred in August 2010 after three cases of disease caused by *E. coli* O26 occurred following exposure to contaminated ground beef. After samples of the product were confirmed to be contaminated with this serotype, approximately 3,856 kg of ground beef were recalled *(369)*. Outbreaks of non-O157 STEC attributed to beef products have occurred in the U.S., France, Denmark, Germany, Australia and Italy *(75, 133, 141, 247) (79, 369, 498)*.

While less is known regarding the efficacy of antimicrobial interventions against non-O157 STEC versus *E. coli* O157, available studies have reported comparable

reductions of non-O157 STEC serotypes to those observed for *E. coli* O157:H7 *(231, 240, 369, 370)*. However, observed efficacy of an antimicrobial intervention may be influenced by procedures used in the inoculation of STEC onto beef and the strength of STEC attachment to meat surfaces *(152, 303, 345, 430)*. Bacterial attachment to meat is influenced by several factors, including pH, ambient temperature, meat and/or bacterial surface charge, cell concentration, attachment period, and bacterial cell components *(90, 113, 157, 271, 281, 388)*. Prendergast et al. *(379)* conducted studies evaluating Listeria innocua attachment to meat surfaces and the impact of inoculation of meat pre- or post-chilling. Nevertheless, little research exists comparing the initial attachment of STEC (O157 and non-O157) under conditions relevant to processing. Prior to the initiation of antimicrobial intervention studies or validation trials, the ability to apply microorganisms to beef surfaces in a predictable and reproducible manner should be experimentally verified. Further, those needing to validate the efficacy of antimicrobial interventions should consider the impacts of inoculation procedure on resulting data, particularly when plate counts of inoculated organisms are used as evidence of intervention efficacy, as chilling of meat (temperature or water activity (a_w) decline during rigor mortis) was reported to potentially lead to enhanced survival to post-chilling intervention usage *(379)*. The objectives of this study, therefore, were to evaluate the effects of chilling (non-chilled, chilled to surface temperature of ≤5 °C) on non-O157 and O157:H7 STEC attachment to beef briskets and post-inoculation storage on STEC (Table 1) survival and recovery.

Table 1. Shiga toxin-producing *Escherichia coli* isolate identification, sources, and serotype-specific mean generation times.

STEC Serotype	Isolate ID[a]	Source	Sorbitol Utilization[b]	Generation Time (min)[c]	SEM
O104:H4	TY-2482 ATCC BAA-178	Human stool	+	33.1A	0.6802
O157:H7	USDA-FSIS 380-94	Salami isolate	−	31.1A	
O26:H11	H30	Infant diarrhea	+	26.7B	
O103:H2	CDC 90-3128	Human stool	+	26.5B	
O45:H2	CDC 96-3285	Human stool	+	26.3B	
O145:NM	83-75	Human stool	+	24.9BC	
O111:H-	JB1-95	Clinical isolate	+	23.6BC	
O121:H19	CDC 97-3068	Human stool	+	22.8C	

[a]Isolate identifiers are identical to those provided by J. Luchansky (USDA-Agricultural Research Service, Wyndmoor, PA).
[b]Sorbitol utilization was identified following 24 h incubation at 35 °C on surfaces of Sorbitol MacConkey Agar (SMAC) following isolate revival and streaking.
[c]Values depict least square means of quadruplicate identical replications (n = 4). Generation times for STEC isolates were calculated from the linear portion of the exponential phase of growth for each serotype *(397).* Means within the column, across STEC serotypes, with no letters (ABC) in common differ at $p < 0.05$, determined by one-way analysis of variance (ANOVA), with means separated by Tukey's Honestly Significant Differences (HSD) test.

Materials and Methods

Bacterial revival and identification procedures. Rifampicin-resistant (0.1 g/L; Rif^R) isolates of Shiga toxin-producing *E. coli* corresponding to serotypes O26:H11, O45:H2, O103:H2, O104:H4, O111:H-, O121:H19, O145:NM, and O157:H7 (STEC8) were supplied by Dr. John Luchansky (USDA Agricultural Research Service, Wyndmoor, PA) (Table 1). Upon receipt, isolates were resuscitated in 9.9 ml sterile tryptic soy broth (TSB; Becton, Dickinson and Co., Sparks, MD), followed by overnight

aerobic, static incubation at 35 °C. One loopful of each culture was then used to inoculate 9.9 ml TSB, and tubes were incubated in identical fashion. Following revival, Rif^R capacity and sorbitol utilization were determined by streaking a loopful of each isolate onto Sorbitol MacConkey agar (Becton, Dickinson and Co.) containing 0.1 g/liter rifampicin (Sigma–Aldrich Co., St. Louis, MO) (SMAC-R). Biochemical identification of isolates was completed using the API® 20E™ (bioMérieux N.A., Inc., Durham, NC) according to manufacturer instructions. Stock cultures of each strain were then prepared and stored at 5 °C on tryptic soy agar (TSA; Becton, Dickinson and Co.) slants prior to use.

Determination of STEC isolate-specific generation times. Isolates were resuscitated from slants by two consecutive transfers into tubes containing 9.9 ml sterilized, tempered TSB. Tubes were incubated statically at 35 °C for 18–24 h; this process was repeated once in identical fashion. Following completion of duplicate sequential passages, cultures were aseptically transferred into separate sterile 15 ml conical tubes and centrifuged at 2205 × g for 10 min at 25 °C to pelletize bacterial cells. Following removal of supernatants, bacterial pellets were suspended in 9.9 ml sterile phosphate buffered saline (PBS; Becton, Dickinson and Co.), and the centrifugation and washing procedure was completed once more identically. Suspended cells were serially diluted in 0.1% (w/v) peptone diluent (Becton, Dickinson and Co.) and used to inoculate individual test tubes containing 9.9 ml sterile TSB to a target of 2.0 ± 0.1 $\log_{10}$ CFU/ml. Following inoculation, test tubes were incubated statically at 35 °C. At 0, 1, 2, 4, 6, 8, 12 and 24 h post-inoculation, numbers of STEC isolates were enumerated by serial dilution

in 0.1% peptone and pour plating using sterilized, tempered (45 °C) molten TSA. Colonies were recorded following incubation of Petri dishes for 24 h at 35 °C. Plate counts were $\log_{10}$-transformed and means calculated from like samples over four replicates (n=4). Mean generation times were determined using the portion of the growth curve representing the exponential phase for each serotype according to previously reported methods *(397)*.

Inoculation of briskets and enumeration of loosely and strongly attached STEC. One loopful of each STEC isolate from prepared slants was streaked onto a Petri dish containing TSA supplemented with 0.1 g/liter rifampicin (TSA-R). Following overnight incubation (18 h) at 35 °C, isolated colonies from Petri dishes were picked into test tubes containing 5.0 ml sterile TSB (one isolated colony/serotype/tube). Inoculated tubes were vortexed and incubated statically at 35 °C for 18–24 h. Findings from growth experiments confirmed the ability of STEC isolates to achieve a predictable number following 24 h incubation at 35 °C, providing for approximately equivalent numbers of STEC to be mixed in cocktail for attachment studies. The contents of each tube were then combined into a 50 ml sterile conical tube and centrifuged at 2880 × g for 17.0 min at 25 °C, a modification of the procedure reported by Huang et al. *(223)*. Resulting supernatants were gently poured off and cell pellets suspended in 40.0 ml sterile 0.1% peptone. The cocktail (hereafter referred to as STEC8) was then serially diluted to 8.4 ± 0.1 $\log_{10}$ CFU/ml and applied to beef surfaces.

Four pairs of beef briskets from split pre-chill carcasses were obtained from a USDA-FSIS-inspected facility located in Texas over three visits; each visit was

completed on a separate date and was treated as a distinct replicate (n=3). Immediately after removal from the carcass, briskets were placed into sterile plastic bags, coded, and closed with zip ties. Briskets were transported in insulated coolers and returned to the Food Microbiology Laboratory (Department of Animal Science, Texas A&M University, College Station, TX) within 2.0 h after being obtained. Half of the briskets were transported in a sanitized cooler that did not contain any chilling pouches to minimize loss of heat, while the other half of obtained briskets were transported in a sanitized cooler containing cooling pouches to initiate the chilling process. Upon arrival at the Food Microbiology Laboratory, briskets destined for inoculation post-chilling were immediately placed in a walk-in cooler and left undisturbed until chilled to a surface temperature of ≤5 °C. Prior to inoculation, each brisket was aseptically removed from packaging and placed lean side up in a sterilized, covered plastic tub. A sterile template marking an area of 350 cm^2 was then attached to the lean side of each brisket. Briskets were inoculated to 6.3 ± 0.1 $\log_{10}$ CFU/cm^2 by pipetting 3.0 ml STEC8 cocktail within the template area and then spreading for 2.0 min (vertically, horizontally and diagonally) using a sterile disposable plastic spreader. Inoculated briskets were then stored at 5 or 25 °C. At 0, 30, 60, 90 and 120 min post-inoculation, samples consisting of three 10 cm^2 pieces of inoculated tissue, selected randomly from within the template area, were aseptically excised using flame-sterilized scalpel and forceps.

Loosely attached cells were quantified by compositing excised tissue pieces in a wide-mouth plastic bottle containing 99 ml 0.1% peptone (previously sterilized and tempered), and agitating via 15 inversions according to previously reported methods

(72). Loosely attached STEC8 were enumerated by serially diluting and spreading onto surfaces of Possé medium modified by the addition of 0.1 g/liter rifampicin and 6.0 g/liter l-sorbose, removal of potassium tellurite and novobiocin, and reducing bile salts to 1.5 g/liter (mPossé) *(276, 374)*. The composited excisions were then aseptically transferred into a virgin stomacher bag containing 99 ml sterile 0.1% peptone diluent and pummeled for 2 min. Strongly attached cells were enumerated by serial dilution and spreading onto surfaces of mPossé medium. All inoculated Petri dishes were incubated for 48 h at 35 °C prior to counting colonies; resulting plate counts were then adjusted to $\log_{10}$ CFU/cm^2 (n=3).

Statistical analysis. One-way analysis of variance (ANOVA) was used to determine whether significant differences occurred: (i) between mean generation times for STEC isolates; (ii) in numbers of loosely versus strongly attached STEC8 as a function of brisket chilling, storage temperature and period, and; (iii) in numbers and strength of attachment of STEC8 as a function of treatment-specific effects. Attachment strength (S_R) was calculated by the following formula: (strongly attached cells)/(strongly attached cells + loosely attached cells) *(72)*. Main effects tested were level of bacterial attachment (strong, loose) brisket chilling status (non-chilled, chilled), post-inoculation storage temperature (5, 25 °C), post-inoculation storage period (0, 30, 60, 90, 120 min), and the interactions of these main effects, with means being separated by Tukey's Honestly Significant Differences (HSD) (p=0.05). Plate counts of STEC8 were $\log_{10}$-transformed to meet the assumptions of the model, and the GLIMMIX procedure was utilized for all data analyses (SAS v.9.3, SAS Institute, Inc., Cary, NC).

Results and Discussion

Growth of STEC in liquid medium and generation times for STEC isolates.

The ability to accurately predict the growth of microorganisms in a microbiological medium is essential for effective procedural and experimental design, in allowing prediction of entry into exponential or stationary phases, or for the dilution of overnight cultures to a desired extent for further study. In the present study, differences ($p<0.05$) were observed in mean generation times of STEC isolates (Table 1). This finding is in agreement with previous studies that have reported variable growth rates for non-O157 STEC in different microbiological media *(306).* Although no difference ($p\geq0.05$) was identified between generation times of *E. coli* serotypes O104 and O157 (33.1 and 31.1 min, respectively), these serotypes had lower generation times than the other serotypes evaluated ($p<0.05$) (Table 1). Mean generation times of serotypes O26, O45, O103, O111, and O145 did not differ from one another ($p\geq0.05$), growing at a more rapid rate in TSB than did *E. coli* O104 and O157. Nonetheless, *E. coli* O121 was determined to possess the shortest generation time of the STEC isolates (22.8 min), significantly less ($p<0.05$) than all other STEC isolates excepting only *E. coli* O145 and O111 (Table 1). Gonthier et al. *(179)* evaluated the growth of O157 and non-O157 STEC (including *E. coli* O103:H2, O26:H11, and O111) in Mueller Hinton broth at isolate-specific optimum growth temperatures (40.2 and 41.2 °C for O157 and non-O157 STEC, respectively), and reported generation times ranging from 19.8 to 34.7 min, similar to those presented in the current study. Such differences in STEC growth rates may be a function of the use of differing STEC isolates, the incubation of STEC at a

sub-optimal growth temperature in the current study, nutritional differences between the growth media used in this study versus others, or a combination of these factors. It should be noted that all isolates were observed to achieve 9.0 ± 0.3 $\log_{10}$ CFU/ml within 24 h at 35 °C, despite differences in generation times, thus allowing for equivalent numbers of each STEC serotype to be pooled together to form STEC8 cocktails for beef inoculation (data not shown). Variability in STEC growth rates may be relevant for analytical purposes with respect to the desire to prepare cocktailed cultures containing equivalent numbers of organisms or for the preparation of inoculum using organisms harvested during lag, exponential (early, mid, late), or even early stationary phases of population development.

Attachment of STEC to non-chilled or chilled beef surfaces. In the present study, briskets were inoculated to 6.3 ± 0.1 $\log_{10}$ CFU/cm² with the STEC8 cocktail. Non-inoculated control samples were negative for Rif^R E.coli in all assays performed. Beef chilling status was found to be an important factor in bacterial attachment. Chilled briskets were found to have significantly greater STEC8 attachment (4.0 $\log_{10}$ CFU/cm^2) compared to STEC8 recovery from non-chilled briskets (3.6 $\log_{10}$ CFU/cm^2) ($p<0.05$) (Table 2). This result was surprising, as findings by other researchers *(379)* and research team discussions led to the hypothesis that non-chilled meat would support greater initial attachment versus chilled meat. In the present study, numbers of strongly attached cells recovered from beef brisket (3.7 $\log_{10}$ CFU/cm²) were lower ($p<0.05$) than the mean numbers of loosely attached cells (3.9 $\log_{10}$ CFU/cm²). Previous studies investigating attachment of *E. coli* to beef muscle have reported higher numbers of loosely versus

strongly attached cells on meat, though differences in inoculated tissue type, experimental design, methods of attachment analysis, and *E. coli* serotypes/strains (including *E. coli* O157:H7 and the non-O157 STEC) make direct comparison of others' findings to the present study difficult *(281, 388)*.

Table 2. Least square (LS) means of STEC8 attachment on beef briskets as a function of brisket chilling status, post-inoculation storage period, and STEC8 attachment status.

Main Effect	Least Square Mean (log_{10} CFU/cm^2)	SEM (P-value)
Brisket Chilling		
Non-Chilled	3.6^{a}	0.041 (p=<0.0001)
Chilled (≤5.0 °C)	4.0^{b}	
Post-Inoculation Storage Period (min)		
0.0	4.2^{a}	0.065 (p=<0.0001)
30.0	3.8^{b}	
60.0	3.7^{cb}	
90.0	3.7^{cb}	
120.0	3.3^{c}	
STEC8 Attachment Status		
Strongly Attached	3.7^{a}	0.041 (p=0.025)
Loosely Attached	3.9^{b}	

^{abc}Values depict LS means from triplicate identical replications (n = 3). Means within a main effect with no superscripts (^{a,b,c}) in common differ at p<0.05, determined by one-way analysis of variance (ANOVA) with differing means being separated by Tukey's Honestly Significant Differences (HSD) test.

Differences in means of attached STEC8 were observed with respect to elapsed attachment period (Table 2). Recovery of attached cells was highest at 0 min (4.2 log_{10} CFU/cm^2), with STEC8 recovery differing (p<0.05) from all other incubation

period-specific plate counts. Others have reported that bacterial attachment to meat can occur rapidly, within the first few minutes of contact *(52, 157, 368)*. In the present study, differences were not observed between numbers of STEC8 at 30, 60, and 90 min post-inoculation ($p \geq 0.05$). The lowest number of recovered strongly attached STEC8 was identified at 120 min (3.3 $\log_{10}$ CFU/cm^2), though this was not different ($p \geq 0.05$) from STEC8 counts at 60 or 90 min. Reports of the influence of incubation period on bacterial attachment to meat and meat animal carcass surfaces have varied. Multiple researchers have reported a linear increase in the numbers of attached bacterial cells on chicken skin during post-inoculation incubation *(88, 284, 350)*. Dickson *(113)* observed similar trends for the adherence of *Listeria monocytogenes* and *Salmonella enterica* serovar Typhimurium on lean beef over a 20 min attachment period. Conversely, others have reported that increased attachment period failed to result in increased numbers of attached bacterial cells to meat surfaces *(26, 314)*. Reasons for such differences include the variety of tissue surfaces from differing meat-yielding animals, differences in microbes (including differences within various *E. coli* serotypes used in both previous studies as well as the current study), and differences in bacterium preparation and inoculation procedures. Finally, while the role of background microbiota was not evaluated in this study, previous research has indicated the lactic acid bacteria are capable of impeding *S. enterica* attachment to surfaces of produce *(286)*. Although research has been conducted elaborating pathogen antagonism by fermentative microbes on meat surfaces, such studies have largely ignored the role of this bacterial grouping in preventing the initial attachment of a cross-contaminating pathogen to a meat surface

(166, 255, 299). An interaction ($p<0.05$) between beef chilling status and post-inoculation storage temperature on STEC8 was observed (Figure 2).

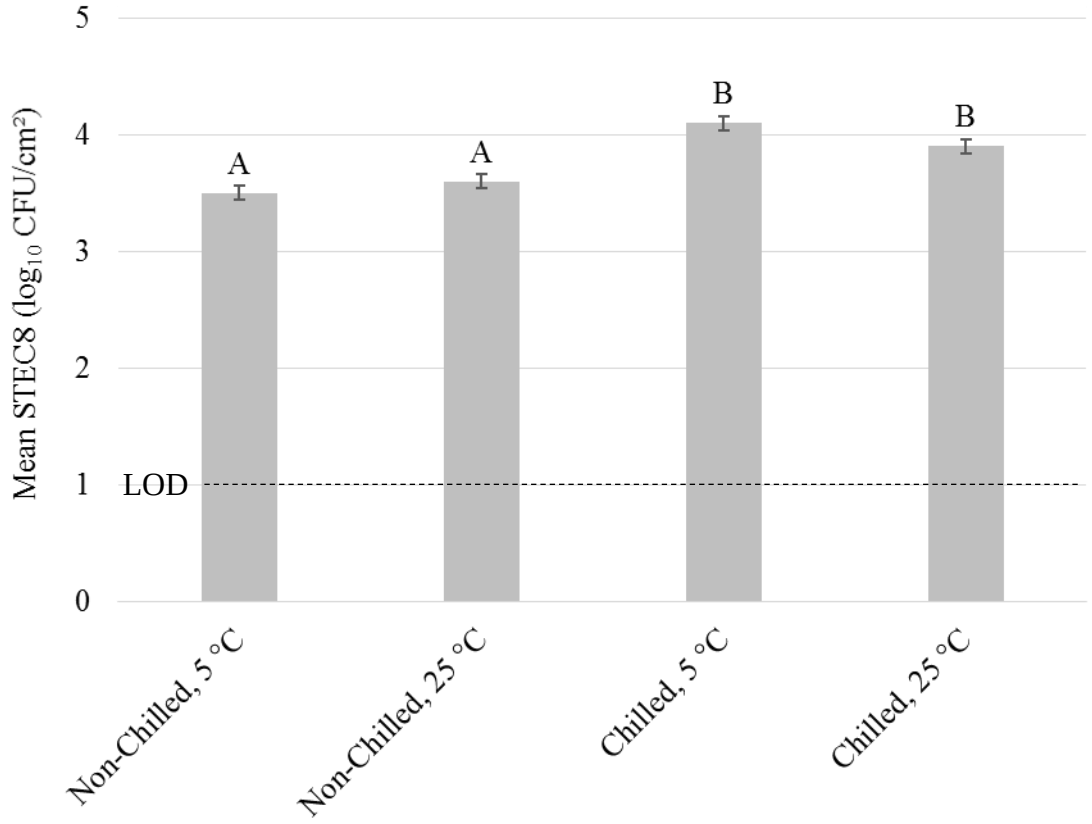

Figure 2. Least square means of the interaction of beef chilling × post-inoculation storage temperature for STEC8 attachment to beef briskets ($p=0.017$). Columns depict interaction means from triplicate identical replicates ($n = 3$), with error bars depicting the SEM=0.06. Dashed line indicates limit of detection. Columns with no letters (A, B) above the column in common differ at $p<0.05$, determined by one-way analysis of variance (ANOVA) and means separation via Tukey's Honestly Significant Differences (HSD) test.

Mean STEC8 on non-chilled briskets were lower (0.4–0.5 $\log_{10}$ CFU/cm^2) than corresponding STEC8 counts from chilled carcasses ($p<0.05$), though no differences in STEC8 attachment were observed between non-chilled briskets stored at 5 or 25 °C, or between chilled briskets stored at 5 or 25 °C (Figure 2). Zulfakar et al. *(520)*, likewise, reported that post-inoculation storage temperature did not affect the numbers of attached *E. coli* O157, members of the non-O157 STEC, and *S. enterica* serovars to muscle cells in cell culture stored at 10 or 37 °C. Chagnot et al. *(83)* observed maximal attachment of *E. coli* O157:H7 to extracellular matrix proteins (collagen I and III) at 25 °C post-inoculation with less adhesion at 7 and 4 °C, potentially a result of increased cellulose and fimbriae production at the higher incubation temperature *(512)*. Once adhered, however, bacterial attachment was not changed with exposure to reduced temperatures (4 or 7 °C for 2 h). Zulfakar et al. *(519)* evaluated attachment of *E. coli* to immobilized extracellular matrix proteins (collagen I, fibronectin, collagen IV, and laminin) *in vitro* and found greater STEC attachment at 37 °C to fibronectin, collagen IV, and laminin versus other incubation temperatures (4, 25 °C), though attachment to collagen I was greatest at 4 °C.

An interaction effect on STEC8 attachment was also identified for brisket chilling status by post-inoculation storage period (Figure 2). The mean attachment of STEC8 to non-chilled brisket at 0 min (4.2 log10 CFU/cm²) was not different ($p\geq0.05$) from that on chilled briskets at 0, 30, and 60 min, which bore mean STEC8 counts of 4.3, 4.0, and 4.1 log10 CFU/cm², respectively. Also, STEC8 counts on non-chilled briskets at 30 min (3.6 log10 CFU/cm²) did not differ from those on chilled briskets at

90 min or 120 min (3.8 log10 CFU/cm²). All other storage period-specific strongly attached STEC8 recoveries (log10 CFU/cm2) differed ($p<0.05$) between non-chilled and chilled briskets

With the exception of 0 min, non-chilled brisket STEC8 means were lower than all post-chilled brisket mean numbers of attached STEC8. The declining STEC8 attachment over the 120 min storage period post-inoculation observed in the current study is similar to previous reports where researchers determined a non-statistically significant decline of 0.9 $\log_{10}$ CFU/cm^2 in *L. innocua* counts on non-chilled and chilled briskets over 24 h storage at 4 °C *(379)*. Crowley et al. *(98)* also reported insignificant declines in *E. coli* O157:H7 numbers on non-chilled beef stored at 4 or 12 °C over 6 h. Whereas in the present study numbers of STEC8 on non-chilled briskets declined significantly between 0 and 30 min of incubation, no significant decline in numbers of attached STEC8 was observed thereafter. Similarly, while statistical analysis identified significant declines in numbers of attached STEC8 on chilled briskets, numerical total decline in STEC8 attached to chilled briskets over 120 min of storage approximated 0.5 $\log_{10}$ CFU/cm^2.

Strength of attachment of STEC on non-chilled or chilled beef. The S_R value is the quotient of the numbers of strongly attached cells on a food surface and the sum of total bacterial population on the surface that are strongly and loosely attached, with higher S_R values indicating higher numbers of strongly attached cells versus loosely attached cells on a surface *(114)*. Attachment strength values for *E. coli* isolates attached to beef surfaces have been reported to vary. Li and McLandsborough *(281)* reported *E.*

coli attachment to beef muscle in low and high ionic strength buffer, with S_R values ranging from 0.05 to 0.39. Following 5 min attachment to lean beef tissue, Dickson and Koohmaraie *(114)* reported an S_R of 0.12 for *E. coli* O157:H7 on lean beef. Benito et al.*(27)* reported S_R = 0.24 for *E. coli* on lean beef tissue following 20 min attachment at 25 °C. In the present study, S_R values were significantly ($p<0.05$) influenced by brisket chilling status, storage temperature, and time, with no interactions between main effects detected (Table 3). With respect to chilling status, briskets inoculated prior to chilling had higher S_R values than those inoculated post-chilling (0.598 and 0.524, respectively). Beef stored at 5 °C bore greater S_R values than beef stored at 25 °C (0.602 and 0.520, respectively) ($p<0.05$). An extended attachment period enhanced the strength of bacterial attachment, with Sr values increasing from 0.439 at 0 min of post-inoculation storage to 0.626 after 120 min of post-inoculation storage, although significant differences in S_R were observed to occur after 60 min of post-inoculation storage ($p<0.05$) (Table 3). These findings are similar to those of Firstenberg-Eden *(147)*, who reported increased attachment strength of *E. coli* K12 to beef with extended attachment. Fratamico et al. *(157)* reported increased *E. coli* O157:H7 attachment to beef over a period of 30 min post-inoculation. However, this is contrary to the findings of researchers that reported no change in attachment strength of *E. coli* O157:H7 over a period of 3 h on briskets stored at 4 °C *(71)*. In the current study, no correlation was observed between S_R and the numbers of enumerated attached bacterial cells, an observation in agreement with previous reports involving bacterial attachment to beef *(26, 27, 72)*. This may have resulted from secretion of curli or other attachment-mediating fimbriae by some

members of the STEC8 during post-inoculation storage without simultaneous observation of significant increases in STEC8 counts, given likely STEC8 generation times on meat surfaces held at temperatures lower than that used to calculate generation times *in vitro* (Table 3) *(87, 88)*.

Table 3. Least square (LS) means of STEC8 strength of attachment (S_R) on beef briskets as a function of brisket chilling status, post-inoculation storage period and temperature[a].

Main Effect	**Least Square Mean[b] (log_{10} CFU/cm^2)**	**SEM (P-value)**
Brisket Chilling		
Non-Chilled	0.598[b]	0.0203 (p = 0.0117)
Chilled (≤5.0 °C)	0.524[c]	
Post-Inoculation Storage Period (min)		
0.0	0.439[b]	0.0322 (p = 0.0008)
30.0	0.557[bc]	
60.0	0.579[c]	
90.0	0.604[c]	
120.0	0.626[c]	
Post-Inoculation Storage Temperature (°C)		
5.0	0.602[b]	0.0203 (p = 0.005)
25.0	0.520[c]	

[bc]Values depict LS means of S_R from triplicate identical replications (n = 3). Means within a main effect with no superscripts ([b,c]) in common differ at $p < 0.05$, determined by one-way analysis of variance (ANOVA) with differing means being separated by Tukey's Honestly Significant Differences (HSD) test. [a]S_R values were determined as strongly attached cells (CFU/cm^2)/strongly attached cells (CFU/cm^2) + loosely attached cells (CFU/cm^2) *(72)*.

An understanding of STEC growth characteristics and the influences of beef chilling, storage temperature and time on STEC adherence to beef surfaces can assist in

the proper development of experimental inoculation procedures for researches determining the antimicrobial/anti-STEC8 efficacy of a particular antimicrobial intervention. These data indicate that attachment of STEC serotypes may be observed as a function of meat chilling status, and post-inoculation storage condition(s). Given the availability of differing process interventions that are approved for use at differing stages of beef products manufacture, data presented in the current study are particularly significant for the optimal development of antimicrobial intervention validation procedures (*92*, *486*). It is recommended that researchers complete the inoculation of beef muscle in a manner that not only simulates the most likely point of cross-contamination in the process chain, but also accounts for potential shifts in numbers of microbial organisms as a function of post-inoculation handling, so as to gain accurate data for the validation of food safety intervention efficacy in the control of STEC on beef.

CHAPTER VII

LACTIC ACID BACTERIA AS AN INTERVENTION AGAINST SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* ON BEEF DURING VACUUM AGING

Introduction

Each year, approximately 176,000 domestically-acquired Shiga toxin-producing *Escherichia coli* (STEC O157 and non-O157) infections attributed to the consumption of contaminated food occur the United States *(417)*. Consumption of foods bearing these pathogens can lead to onset of gastroenteritis, hemorrhagic colitis, and secondary sequelae including hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP), which may result in renal failure or death *(233)*. The food commodity most frequently implicated in foodborne outbreaks of *E. coli* O157:H7 is beef *(361)*. Biological means of preservation have been used historically to inhibit food spoilage. Lactic acid bacteria (LAB) produce antimicrobial metabolites such as organic acids, bacteriocins, and/or hydrogen peroxide *(11)* which may inhibit foodborne pathogenic bacteria. The U.S. Food and Drug Administration has classified certain strains and mixtures of strains as generally accepted as safe for use in various meat systems, including intact beef cuts and carcasses *(55, 56, 59)*.

The primary objective of this study was to investigate the inhibition of eight Shiga toxin-producing *E. coli* (STEC8) by a mixture of commercially available LAB in vacuum packaged beef strip loins during refrigerated aging for 14 or 28 days. In addition, conventional application was compared against electrostatic application to

determine whether the novel spray would confer greater pathogen disinfection and/or loss of protective culture viability.

Materials and Methods

Bacterial culture preparation and maintenance. Rifampicin-resistant (Rif^R) strains of Shiga toxin-producing *E. coli* corresponding to serotypes O26:H11 (H30), O45:H2 (CDC 96-3285), O103:H2 (CDC 90-3128; human stool isolate), O104:H4 (TY-2482), O111:H- (JB1-95), O121:H19 (CDC 97-3068), O145:NM (83-75), and O157:H7 (USDA-FSIS 380-94) (provided by Dr. John Luchansky, U.S. Department of Agriculture-Agricultural Research Service; Wyndmoor, PA) were obtained from the Department of Animal Science Food Microbiology Laboratory culture collection (Texas A&M University, College Station, TX). The source and growth rate of each isolate are listed in Table 1. Biochemical confirmation of each isolate was performed using the API® 20E™ (bioMérieux N.A., Inc., Durham, NC) according to manufacturer instructions. Cultures were individually revived from -80 °C storage by inoculating one cryobead for each isolate into 10 ml Tryptic Soy Broth (TSB; Becton, Dickinson and Co., Sparks, MD) and incubating at 35 °C for 18-24 h. After incubation, one loopful of each isolate was used to inoculate separate sterile 10 ml TSB and incubated identically. Thereafter, stock cultures of each strain were maintained on separate tryptic soy agar (TSA; Becton, Dickinson and Co.) slants at 4 °C. Slants were prepared by streaking one loopful of fresh culture on the surface of each slant and incubating statically at 35 °C in aerobic conditions for 18-24 h.

Inoculum preparation. For each experiment, working cultures of each isolate were prepared by transferring a loopful of culture from TSA slants into 10 ml of sterile TSB and incubating statically at 35 °C in aerobic conditions for 18-24 h. Each isolate was sub-cultured by inoculating sterile 50 ml TSB supplemented with 0.1% rifampicin (Sigma-Aldrich, St Louis, MO) with one loopful of fresh culture, and incubating statically at 35 °C in aerobic conditions for 18-24 h. Each bottle containing culture was then sealed and wrapped in Parafilm M® (Bemis, Oshkosh, WI) to prevent contamination during transport. Sealed bottles were collected in an insulated cooler containing frozen ice packs for transport to the Biosafety Level (BSL) 2 Laboratory at the Texas A&M Agrilife Research and Extension Center in Stephenville, TX. A calibrated inoculum-misting bottle was immersed in 70% ethanol for 5 min and then flushed in triplicate with sterile distilled water. Immediately prior to use, a bacterial cocktail was prepared by transferring 50 ml of each STEC8 culture into the sanitized misting bottle. A 10 ml aliquot of the inoculum was pipetted into a sterile conical tube, which was then stored in an insulated cooler containing frozen ice packs for transport and plating in the Food Microbiology Laboratory (Texas A&M University). Upon receipt, inoculum concentration was verified by serial dilution in 0.1% peptone water (Becton, Dickinson and Co.) and spread plating on TSA supplemented with 0.1% rifampicin (TSAR). Colonies were enumerated following incubation at 35 °C for 18 to 24 h.

Sampling and microbiological analysis. Samples for microbiological analysis consisted of three 10 cm^2 tissue pieces excised from the lean side of the strip loins. At

designated times (pre-inoculation, post-inoculation, pre-treatment, or post-treatment), three 10 cm^2 outlines were marked in the lean tissue surface using a flame-sterilized stainless steel borer and excised to a depth of 1 to 2 mm using flame-sterilized scalpels and forceps. Each sample, consisting of three excised tissue pieces, was composited in a sterile stomacher bag. Pre- and post-inoculation samples taken from positive control strip loins were collected at the AgriLife and Research Extension Center in Stephenville, Texas. These stomacher bags were thermally sealed and transferred into insulated coolers with frozen ice packs, then transported to the Food Microbiology Laboratory at Texas A&M University for processing and plating. Bacterial populations were assayed by adding 99 ml phosphate buffered saline (PBS; Sigma Aldrich Co.) to each stomacher bag, pummeling for 1 min in a stomacher apparatus (AES Laboratoire, Combourg, France), then serially diluting in 0.1% peptone water and spread plating. Antibiotic-resistant LAB were enumerated by spreading 0.1 ml aliquots on pre-poured de Man, Rogosa and Sharpe (MRS; Becton, Dickinson and Co.) agar supplemented with streptomycin sulfate (40 µg/ml, Amresco), sodium oxacillin (0.4 µg/ml, Chem-impex Intl), and gentamycin sulfate (5µg/ml, Amresco) and incubating for 48 h at 35 °C, included per LAB supplier instructions. Rif^r *E. coli* were be counted by spreading on TSAR and enumerated following incubation at 35 °C for 24 h.

Meat preparation and inoculation. Pre-rigor beef strip loins were procured from a USDA-FSIS-inspected establishment located in Texas. Strip loins were harvested from carcasses prior to chilling within 2 h of animal death. Once excised, each strip loin was transferred into a zipper polyethylene bag and swathed in a Mylar thermal blanket

(Everready First Aid, Brooklyn, NY) to minimize heat loss. Wrapped beef pieces were collected in insulated coolers containing activated instant hot packs (Dynarex, Orangeburg, NY) for transport to the BSL 2 Laboratory at the Texas A&M AgriLife Research and Extension Center in Stephenville, Texas. Upon receipt, each strip loin was aseptically removed from zippered polyethylene bags and transferred into sterile polypropylene autoclave bags. Next, the external pH and temperature of individual strip loins were measured in triplicate using an ExStik® pH and temperature meter (Extech Instruments Corporation, Nashua, NH). The internal temperature at the geometric center of each strip loin was measured once using a VWR® Total-Range Digital Thermometer and stainless steel probe attachment. At the completion of these readings, strip loins were placed into a biological cabinet for inoculation. The previously prepared inoculum misting bottle was primed by spraying 3 pumps of inoculum into a designated biohazard bag, then held 25 to 31 cm above the meat for application of three pumps of inoculum onto the lean side of the strip loin surface. The biohazard bag containing the inoculated strip loin was sealed with a zip tie and hand tumbled for 1 min to achieve uniform distribution of inoculum. Beef pieces were held at 25 °C for 30 min to allow for bacterial attachment. Following the allotted time, post-inoculation samples were taken from positive control pieces. All strip loins were double-bagged with a second polypropylene autoclave bag sealed around each strip loin to minimize the risk of cross-contamination during transport to the Food Microbiology Laboratory. Strip loins were collected in insulated coolers containing frozen ice packs to begin chilling during transport. Upon arrival to the Food Microbiology Laboratory, strip loins were removed from coolers and

placed in a single layer on foil-lined wire shelving within a walk in refrigerator at 4 °C until a chilling period of 24 h had elapsed.

Preparation of treatment solutions. LactiGuard™ is a commercially available food safety intervention composed of *Lactobacillus acidophilus, Lactococcus lactis* subsp. *lactis,* and *Pediococcus acidilactici.* The isolate sources and identifiers of the isolates included in this product are listed in Table 4. Once received, lyophilized lactic acid bacteria were stored in original packaging at -80 °C. Immediately prior to use, the LactiGuard™ treatment was prepared by combining 7.5 g (11.0 $\log_{10}$ CFU/g) of each strain in 3.0 liters sterile water to a concentration of 8.7 $\log_{10}$ CFU/ml. To verify the concentration, a 10 ml aliquot will be transferred into a sterile conical tube for serial dilution in 0.1% peptone water and plating on MRS supplemented with the previously described antibiotics.

Table 4. LactiGuard™ strains and isolate sources.

Lactic Acid Bacteria Name	**Isolate ID[a]**	**Isolate source**
Lactobacillus acidophilus	NP 51	Cattle isolate
Lactobacillus acidophilus	NP 28	Cattle isolate
Lactococcus lactis subsp. *lactis*	NP 7	Alfalfa seed and sprout isolate
Pediococcus acidilactici	NP 3	Ham and frankfurter isolate

[a]Isolate identifiers are identical to those provided by S. Lerner (Nutrition Physiology Corp., Guymon, OK).

Treatments to disinfect. Following an elapsed chilling period of 24 h, inoculated beef subprimals were removed from refrigerated storage and placed into sanitized coolers containing frozen ice packs for transport to the Texas A&M Center for Food Safety (College Station, Texas) for further processing. Treatment application and vacuum packaging of strip loins proceeded within a bioBUBBLE enclosure (46.5 sq. m) equipped with high efficiency particulate air (HEPA) filtration under negative pressure, located within a BSL 2 pilot processing area. Once received, bagged strip loins were placed into refrigerated storage at 4 °C. Each strip loin was removed from refrigerated storage immediately prior to treatment and subjected to pre-treatment sampling and measurement of internal temperature, surface temperature, and surface pH in the previously described manner. Next, a sterile meat hook was be inserted into the distal end of the strip loin and hung with lean side facing outward in a model spray cabinet (Birko Corp., Centennial, CO) within the bioBUBBLE for treatment. Each piece was randomly assigned a treatment consisting of: a) conventionally applied LactiGuard™ solution at 25 °C for 100 s at 310 kPa; or b) electrostatically applied LactiGuard™ solution at 25 °C for 120 s at 207 kPa. Electrostatic application was performed using an ESS model XT-3 air-assisted electrostatic sprayer (Electrostatic Spraying Solutions, Inc., Watkinsville, GA) charged to ≤ -10 amps at a flow rate of 2.1 ml/s, while conventional application was achieved using a hand-held, pressurized tank air sprayer (Roundup, Marysville, OH) at a flow rate of 1.7 ml/s. Interventions were sprayed approximately 90 cm away from the strip loin surface with sweeping horizontal zig-zag motion. Following treatment, strip loins were removed from the model spray cabinet and placed on a new

absorbent bench under pad for post-treatment excision, internal temperature measurement, and triplicate readings of external pH and temperature. The strip loins were then inserted into commercial-grade vacuum bags (oxygen transmission rate of ≤50 $cm^3/m^2 \cdot 24\ h \cdot 0.1$ MPa; Weston, Strongville, OH) and packaged in a vacuum sealer (Model X180, KOCH Supplies Inc.). Strip loins were then arranged in a single layer and aged at 4 °C for 14 or 28 days prior to sampling as previously described.

Statistical analysis. The experiment was performed as triplicate identically prepared samples over two replications (n=6). All statistical analyses were performed using JMP Pro v. 11.0 (SAS Institute, Inc., Cary, NC). Colony counts were transformed and expressed as $\log_{10}$ CFU/cm^2, with $\log_{10}$ reductions calculated by subtracting $\log_{10}$ counts enumerated at sampling times for each beef piece. Samples with bacterial counts that were below the limit of detection were assigned a value of 0.25 $\log_{10}$ because this is the number between 0 and the minimum detection level (0.5 $\log_{10}/cm^2$). The following variables were included as main effects: replication, post-treatment storage period (0, 14, and 28 days), and treatment application method (conventional spray vs. electrostatic spray). Differences in bacterial populations and $\log_{10}$ reductions among main effects and/or the interactions of these main effects were identified using analysis of variance (ANOVA) at a p-value of 0.05. Statistically significant differences among means ($p<0.05$) were further analyzed and separated with Tukey's Honestly Significant Differences (HSD) test.

Results and Discussion

To simulate cross-contamination at slaughter prior to intervention application, strip loins were harvested and inoculated with a STEC cocktail within 2 h of animal death. The mean internal and surface temperatures of beef strip loins at the point of inoculation were 30.5 ± 1.79 °C and 27.4 ± 1.24 °C, respectively. As shown in Figure 3, the mean surface pH was 6.15 ± 0.06 immediately prior to pathogen inoculation. It may be deduced that the strip loins used in the present study were inoculated prior to the completion of rigor *(191, 245, 396)*.

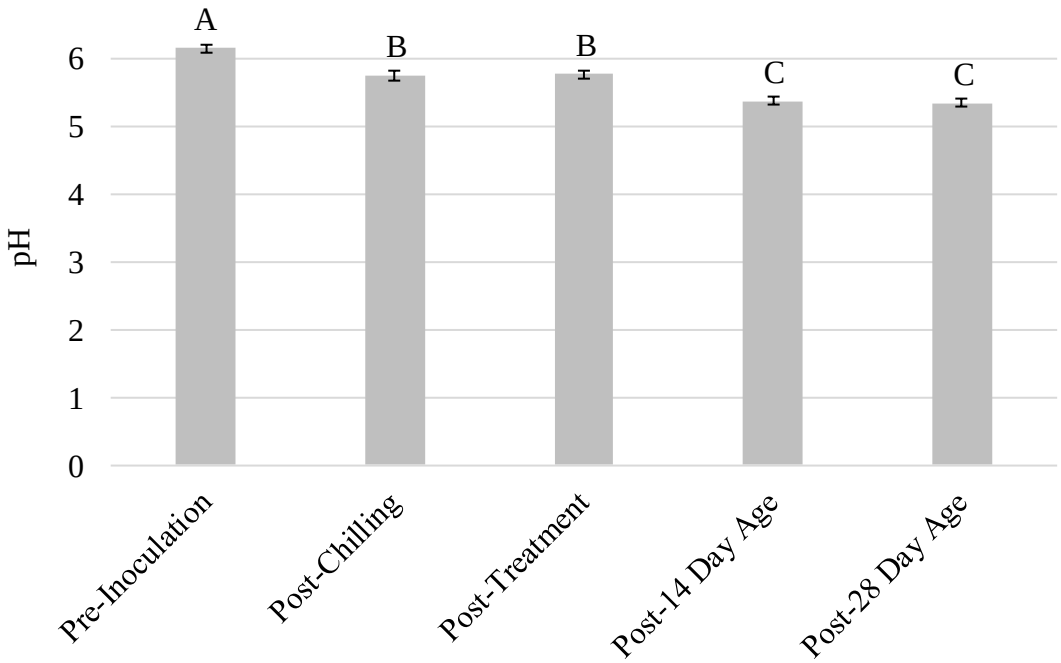

Figure 3. Least squares means of beef strip loin pH over time. Columns depict least square means from two replications with triplicate samples in each replication (n = 6), with error bars representing the standard error (0.06). Columns lacking common letters (A, B, C) are significantly different ($p<0.05$).

To determine whether transport and chilling influenced the *E. coli* populations after inoculation, pathogen numbers in non-treated positive control strip loins were enumerated immediately following inoculation and again after chilling. The concentrations of *E. coli* assayed post-inoculation were not different ($p \geq 0.05$) from those present after chilling (data not shown). This is not surprising, as previous research has demonstrated that the process of chilling exerts no bactericidal effect on *E. coli (246)*. In addition, this data confirms that the temperature controls implemented during transport and chilling were adequate to prevent pathogen growth. As depicted in Table 5, mean STEC populations on beef strip loins after chilling at 4 °C for 24 h (pre-treatment) were 7.2 ± 0.1 $\log_{10}$ CFU/cm^2. A significant reduction ($p<0.05$) in pH was observed after chilling (Figure 3). The pH value at this sampling point was 5.77 ± 0.06, which was likely due to the completion of rigor mortis *(191)*. Application of the lactic acid bacteria intervention did not exert an effect on surface pH ($p \geq 0.05$). No differences in numbers of STEC, reductions of STEC, or numbers of LAB were observed between conventional and electrostatic sprays ($p \geq 0.05$). As shown in Table 5, the spray treatment of beef strip loins applied 6.5 ± 0.1 $\log_{10}$ CFU/cm^2 of LAB. Although treatment application significantly decreased ($p<0.05$) the mean pathogen loads to 6.8 ± 0.1 $\log_{10}$ CFU/cm^2, post-treatment STEC numbers varied, with significant differences observed between replications ($p<0.05$). This variability did not influence treatment efficacy, as reductions in STEC populations were consistent between replications ($p \geq 0.05$) at a mean of 0.4 ± 0.1 $\log_{10}$ CFU/cm^2.

Table 5. Bacterial populations on beef strip loins treated with LAB and vacuum aged at 4 °C.

Target Organisms	Pre-Treatment[1]	Post-Chilling[1]	Post-14 Day Age[1]	Post-28 Day Age[1]	P-value
STEC	$7.2^{a} \pm 0.1$	$6.8^{b} \pm 0.1$	$7.1^{ab} \pm 0.1$	$6.7^{b} \pm 0.1$	0.0151
LAB	-	$6.5^{a} \pm 0.1$	$6.2^{b} \pm 0.1$	$6.1^{b} \pm 0.1$	0.0124

[1]Values depict least square means from two replications with triplicate samples in each replication (n = 6). [ab]Least squares means within a row lacking the same superscript differ ($p < 0.05$), determined by one-way analysis of variance (ANOVA), with differing means being separated by Tukey's Honestly Significant Differences test.

As depicted in Table 5, mean STEC populations on beef strip loins after chilling at 4 °C for 24 h (pre-treatment) were 7.2 ± 0.1 $\log_{10}$ CFU/cm^2. A significant reduction ($p<0.05$) in pH was observed after chilling (Figure 3). The pH value at this sampling point was 5.77 ± 0.06, which was likely due to the completion of rigor mortis *(191).* Application of the lactic acid bacteria intervention did not exert an effect on surface pH ($p\geq0.05$). No differences in numbers of STEC, reductions of STEC, or numbers of LAB were observed between conventional and electrostatic sprays ($p\geq0.05$). As shown in Table 5, the spray treatment of beef strip loins applied 6.5 ± 0.1 $\log_{10}$ CFU/cm^2 of LAB. Although treatment application significantly decreased ($p<0.05$) the mean pathogen loads to 6.8 ± 0.1 $\log_{10}$ CFU/cm^2, post-treatment STEC numbers varied, with significant differences observed between replications ($p<0.05$). This variability did not influence treatment efficacy, as reductions in STEC populations were consistent between replications ($p\geq0.05$) at a mean of 0.4 ± 0.1 $\log_{10}$ CFU/cm^2. The inhibitory potential of lactic acid bacteria in vacuum packaged lean beef tissue during storage at 4 °C was also investigated (Table 5). After 14 days of aging, STEC populations increased to 7.1 $\log_{10}$

CFU/cm^2, which was not different ($p \geq 0.05$) from enumerated pathogens immediately prior to or after treatment. In the same period, numbers of LAB were significantly reduced ($p < 0.05$) to 6.2 ± 0.1 $\log_{10}$ CFU/cm^2. The beef surface pH also decreased significantly ($p < 0.05$) from post-treatment values to 5.37 ± 0.07 (Figure 3). Populations of LAB and beef surface pH values did not deviate significantly ($p \geq 0.05$) from day 14 levels following refrigerated storage for 28 days. In the same period, mean STEC numbers were reduced to 6.7 ± 0.1 $\log_{10}$ CFU/cm^2 which was lower ($p < 0.05$) from post-treatment populations, but not different ($p \geq 0.05$) than those enumerated at the 14 day sampling point (Table 5).

The lack of inhibition observed in the present study may also be attributed to the inoculation protocol. Bacterial attachment to beef surfaces increases with time *(248)* and contamination of product prior to chilling may have a protective effect against subsequent interventions. In a study by King et al. *(246)*, hot-boned beef carcass surfaces were inoculated with *E. coli* O157:H7 and then chilled at 4 °C for 24 h. Subsequent application of peroxyacetic acid (200 ppm at 43 °C and 0.85 MPa for 15 s) produced no significant reductions ($p \geq 0.05$). However, this treatment significant reduced *E. coli* O157:H7 (0.7 $\log_{10}$ CFU/cm^2) when applied to carcass surfaces prior chilling. To determine whether the physiological state of beef influenced the efficacy of decontamination treatments, Cutter et al. *(101)* inoculated pre-rigor, post-rigor (chilled at 5 °C for 24 h), and previously frozen (thawed at 5 °C for 18 h, then warmed to 25 °C) lean beef carcass tissues with *E. coli* O157:H7 (5.0 to 5.4 $\log_{10}$ CFU/cm^2), then treated each piece with a water spray (52 °C for 15 s at 552 kPa). While no significant

differences ($p \geq 0.05$) were observed in pathogen numbers on untreated beef, remaining populations of *E. coli* on previously frozen carcass surfaces (2.8 $\log_{10}$ CFU/cm^2) were significantly ($p < 0.05$) lower than on samples inoculated pre-rigor (3.5 $\log_{10}$ CFU/cm^2) or post-rigor (3.3 $\log_{10}$ CFU/cm^2) following treatment. However, no post-inoculation chilling was used in this study and treatments were applied after only 15 min of attachment (25 °C). Dickson and Koohmaraie *(114)* evaluated the relative propensity of *E. coli* O157:H7 to adhere strongly to previously frozen lean beef tissue (thawed at room temperature) submerged in diluted culture 5 min. The resulting S_r value (strongly attached bacteria/strongly + loosely attached bacteria) was low at 0.118, which showed that *E. coli* tended to adhere loosely to previously frozen beef muscle and was readily removed.

Treatment efficacy is influenced by a number of intrinsic, extrinsic, and microbial factors *(105)*. The STEC cocktail used in the present study consisted of organisms grown to stationary phase. During exponential growth in non-stress conditions, the σ^{70} controls expression of housekeeping genes *(203)*. However, gene expression in the stationary phase of growth is directed by the σ^S. This sigma factor facilitates the transcription of genes involved in the general stress response of *E. coli*. Cross-protection from various stresses results from the induction of this sigma factor. Stationary phase cells convert membrane-bound unsaturated fatty acids into cyclopropane fatty acids, which provide increased protection from acid stress *(85, 97)*. In addition, the cultures may have been exposed to cold stress during transport. Sub-optimal temperatures cause cells to express mRNA and DNA supercoiling stabilizing

cold shock proteins (CSPs) *(177, 236)*. Exposure to cold shock has been shown to increase *E. coli* tolerance to subsequent acid stress *(44)*.

One factor which may have reduced the efficacy of the LAB interventions is the high pathogen load inoculated on the beef strip loins, which would not be representative of expected STEC concentrations at slaughter facilities. In addition, the greater number of STEC applied to be beef tissue relative to the quantity of LAB may have overwhelmed the inhibitory capacities of these organisms. However, LAB organic acid production in post-rigor meats held in refrigerated conditions is unlikely to occur and would not be sufficient to reduce the muscle pH regardless of microbial loads. Hoyle et al. *(216)* reported no differences ($p \geq 0.05$) in the rates of inactivation of *E. coli* O157:H7 in ground beef (5.5 to 5.8 $\log_{10}$ CFU/g) during storage at 5 °C (traditional overwrapped packaging) between LAB treatments consisting of 6.0, 7.0, or 8.0 $\log_{10}$ CFU/g. Muthukumarasamy et al. *(333)* also found that treatment of ground beef with 3.0 $\log_{10}$ CFU/g or 6.0 $\log_{10}$ CFU/g *Lactobacillus reuteri* and glycerol (250 mM/kg) displayed similar efficacies against *E. coli* O157:H7 during storage at 4 °C in vacuum packaging. Combined, these studies demonstrate that pathogen to LAB ratio does not markedly influence the efficacy of biological interventions against *E. coli* in beef systems.

The order in which pathogenic and protective cultures were applied to the beef surface may have influenced the treatment efficacy. For example, greater inhibition has been reported during storage at 5 °C in ground beef treated with *Enterococcus faecalis* (4.0 $\log_{10}$ CFU/g) prior to the addition of *E. coli* O157:H7 (3.0 $\log_{10}$ CFU/g), compared to ground beef treated in conjunction with, or after pathogen contamination *(444)*. The

lactic acid bacteria in the present study were rehydrated from a lyophilized state immediately prior to application to beef strip loin surfaces. As these organisms were in a stressed state, the metabolic functions were possibly impaired. In addition, the LAB may have been exposed to the rifampicin present in the STEC inoculum that was sprayed on the beef surface. The lactic acid bacteria may have been inhibited in the presence of rifampicin. This antibiotic changes the RNA polymerase structure and inhibits mRNA synthesis *(74)*. The reduction in lactic acid bacteria populations observed at the 14 day sampling point of the present study may also be due in part to the presence of this antibiotic. In addition, the production of antimicrobial metabolites by LAB is markedly reduced in refrigeration temperatures *(2)*. For example, these organisms do not usually generate organic acids in in refrigeration *(328)*. While the beef surface pH decreased significantly ($p<0.05$) from post-treatment values following aging for 14 days, the gradual reduction in pH may have allowed pathogens to become habituated to the acid and thus have increased tolerance. These organisms can survive in pH 2.5, and remain acid tolerant following storage in neutral conditions at 4 °C for longer than 28 days *(288)*. Further, wild type isolates of EHEC display greater susceptibility to acid stress than outbreak-associated strains *(312)*. The influence of storage temperature on the antagonistic activities of LAB metabolites was demonstrated by McDonnel et al. *(311)*. They observed no inhibition of *E. coli* O157:H7 by *Lactobacillus acidophilus* antimicrobial peptides (1 mg/ml caseicin A or 2 mg/ml caseicin B) throughout incubation at 4 °C (diluted tryptic soy broth), though pathogen numbers were significantly ($p<0.05$) reduced by 2.0 to 4.0 $\log_{10}$ CFU/ml when temperatures were

increased to 10, 15, or 37 °C. Aguilar and Klotz *(2)* reported no inhibitory action *in vivo* by *Lactobacillus plantarum* on *E. coli* when inoculated at equivalent levels (2.6 $\log_{10}$ CFU/ml) in MRS broth during storage at 5 °C for up to 250 h. Echeverry et al. *(126)* found that numbers of *E. coli* O157:H7 inoculated on refrigerated choice-grade strip loins (5.0 $\log_{10}$ CFU/cm^2) and treated with a spray consisting of 7.7 $\log_{10}$ CFU/ml LAB did not change significantly ($p \geq 0.05$) from day 0 levels after refrigerated aging in vacuum packaging for 14 or 21 days. Senne and Gilliland *(427)* inoculated beef steaks with 2.8 to 3.1 $\log_{10}$ CFU/g of *E. coli* O157:H7 and treated each piece with a relatively high level of *Lactobacillus delbreuckii* subsp. *lactis* (7.0 $\log_{10}$ CFU/g). Pathogen numbers on treated beef were not significantly different ($p \geq 0.05$) than untreated beef after 3, 6, and 9 days of aging at 5 °C. Finally, intact post-rigor beef is not a suitable substrate for organic acid production by LAB. The level of available glucose (4.5 to 7 µmol/g) is possibly insufficient for organic acid production sufficient to appreciably change the pH *(328, 387)*.

CHAPTER VIII

CONCLUSIONS

Growth of Shiga Toxin-Producing *Escherichia coli* (STEC) and Impacts of Chilling and Post-Inoculation Storage on STEC Attachment to Beef Surfaces

This study investigated the growth of eight STEC isolates from differing serogroups *in vitro* over a period of 24 h during static incubation at 35 °C in tryptic soy broth. Results from this study showed the mean generation times of STEC isolates differed, ranging from 22.8 min to 33.1 min. The STEC serotypes were classified as having low (O104 and O157), moderate (O26, O45, O103, O111, and O145), or high (O121) growth rates according to statistically differences in mean generation times. Thus, this research established a quantification of optimum growth capacity of *E. coli* O157:H7 and non-O157:H7 STEC. The second research component of this study evaluated the effects of chilling (non-chilled, chilled to surface temperature of ≤5 °C) on STEC attachment to brisket surfaces, and the effects of post-inoculation storage (5 or 25 °C) on STEC recovery over a period of 2 h. Significant effects on cell attachment by the interaction of chilling and post-inoculation storage period, or chilling and post-inoculation storage temperature, were identified ($p<0.05$). Results indicate beef chilling and post-inoculation storage conditions influenced STEC attachment to beef. Therefore, inoculation procedures should be representative of contamination that would occur in the slaughter and/or processing environment so that experimental reductions of food safety interventions are applicable to the beef industry.

Lactic Acid Bacteria as an Intervention Against STEC on Beef During Vacuum Aging

This study investigated the efficacy of a commercial LAB-containing food safety intervention for the reduction of eight STEC isolates on beef strip loins during refrigerated aging in vacuum packaging for 14 or 28 days. Mean STEC numbers were significantly decreased immediately after treatment application, with no significant differences observed in STEC reductions or applied LAB between electrostatic and conventional sprays. Remaining populations of STEC on beef strip loins after 28 days of aging were significantly lower than those present prior to treatment. However, the pathogen levels enumerated after 14 days of aging were not different than pre-treatment and numerical reductions were small at both aging times. While biological interventions consisting of LAB have been shown to be effective in reducing *E. coli* O157:H7 in ground beef products, the utility of such treatments in reducing STEC on intact muscle is decreased. In addition, the findings of this study suggest that food safety interventions consisting of LAB may be less effective in reducing non-O157 STEC in beef.

Application of Findings

The findings of this research provide information that may aid in the development of STEC inoculation protocols and biopreservative-using food safety interventions for preservation of fresh beef safety. In addition, this study provides a new perspective regarding the relationship between beef physico-chemical state and the efficacy of biological preservative treatments as food safety interventions for the disinfection of STEC. Although biopreservatives such as lactic acid bacteria may be

useful for reducing specific pathogens on food products in certain conditions, the reductions achieved in this investigation demonstrate that this treatment would not be sufficiently effective in reducing STEC to justify its implementation when applied alone in these conditions. To improve the efficacy of this treatment as an intervention against STEC in beef, future research should include chemical interventions used in tandem with LAB, membrane-disrupting compounds such as chelators, and/or factors to promote the production of antagonistic metabolites (e.g. supplementation with fermentable carbohydrates, pre-treatment activation of LAB strains, or modulation of post-treatment storage temperature).

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