Reduction of pathogenic bacteria in ground beef using a *Lactobacillus*-based biological intervention

by

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ABSTRACT

Two pathogenic bacteria that can be found in ground beef are *Escherichia coli* (*E.coli*) O157:H7 and *Salmonella*. Six additional non-O157 Shiga toxin-producing *E.coli* (STEC) serogroups were declared adulterants in raw ground beef by the USDA-FSIS, posing additional concerns to the industry. The objective of this project was to evaluate the efficacy of biological intervention with *Lactobacillus* multi-species strains - *Lactobacillus acidophilus* (NP51 and NP28), *Pediococcus acidilactici* (NP3) and *Lactococcus lactis* subsp. *lactis* (NP7) on the reduction of *E. coli* O157:H7, *Salmonella* and non-O157 STEC populations in ground beef during retail display. Coarse ground beef (22.68 kg) was inoculated with cocktail mixtures of either *E. coli* O157:H7 or *Salmonella* or non-O157 STECs and mixed to assure uniform pathogen distribution. The coarse ground beef was then divided into two equal portions - Control (CON) and *Lactobacillus*-based biological intervention-treated (LAC). The LAC treatment was prepared by adding potable water to each of the aforementioned freeze-dried strains (NP51 and NP28 at 10^9 CFU/mL and NP3 and NP7 at 10^8 CFU/mL) and mixed to make the cocktail to achieve a final desired concentration of 10^7 CFU/g on the meat. After preparation, 50 mL of the LAC treatment was added to the inoculated ground beef and thoroughly mixed before grinding. Similarly, CON ground beef was finely ground without the application of LAC treatment. Finely ground meat from both treatments was portioned on black expanded polystyrene trays before overwrapping with polyvinyl chloride film. Packages were arranged in a retail display case (0 to 4°C) and samples were collected on d - 1, 3, and 5. At each sampling interval, ground beef samples were stomached in buffered peptone water (BPW) before serial dilution. All the dilutions are

plated in duplicates onto MacConkey, Xylose Lysine Deoxycholate (XLD) and deMan Rogosa Sharpe (MRS) agar plates for enumeration of for *E. coli*, *Salmonella* and *Lactobacillus* samples, respectively. On day 1, there were no reduction in microbial count due to LAC treatment with the LAC counts being the same as the CON. Total pathogens in the CON samples changed over time. The total *E. coli* O157:H7 counts in CON samples are not significantly ($P > 0.05$) from day 1 to day 5 of the display, while significant ($P < 0.05$) decrease was observed in the number of *E. coli* O157:H7 in LAC samples on day 3 and day 5 when compared to day 1. Furthermore, LAC samples had significantly ($P < 0.05$) less *E. coli* O157:H7 on days 3 and 5 of retail display than CON. This suggests that LAC was a slight inhibitory toward *E. coli* O157:H7 proliferation in the treated samples with populations being 0.35 and 1.02 log CFU/g lower than the CON samples on days 3 and 5, respectively. A similar trend was observed in ground beef inoculated with *Salmonella*. The populations of LAC samples are 0.9 and 1.44 log CFU/g lower than the CON on days 3 and 5 respectively. Regarding non-O157 STECs, LAC samples had significantly fewer ($P < 0.05$) pathogen populations on day 5 when compared to CON ground beef. STEC populations were 1.5 log cycles less in LAC samples when compared to CON samples on day 5. These results suggest the addition of NP51 + NP28 + NP7 + NP3 strains of *Lactobacillus* to ground beef may not be an effective intervention to eliminate the levels of *E. coli* O157:H7, *Salmonella* and non-O157 STECs but helped to inhibit the proliferation of the aforementioned bacteria in overwrap packaging during extended retail display (145)

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CHAPTER I

INTRODUCTION

The Centers for Disease Control and Prevention (CDC) estimates that every year around 48 million people become sick, 128,000 get hospitalized, and 3,000 die of foodborne illnesses caused by 31 known pathogens and several unspecified agents (UA). These 31 identified pathogens are responsible for approximately 20% of the diseases, 44% of the hospitalizations and deaths in the United States (US). Food contaminated with unspecified agents is responsible for the remainder of the illnesses, hospitalizations, and deaths (30).

According to a surveillance study done by Scallan et al., (2011) each year *Escherichia.coli* (*E.coli*) O157:H7 and non-O157 Shiga toxin-producing *E.coli* (STEC) are responsible for around 3,000 and 400 hospitalizations, respectively. Shiga toxin producing *Escherichia coli* (STEC) are responsible for causing 14,885 illnesses, 4,739 hospitalizations, and 69 deaths in the last 20 years from 1996 to 2016 (31). The total cost associated with *E. coli* O157:H7 cases reported by consuming ground beef is around \$271 million (132). Outbreak data from the CDC during 1982 - 1994 shows that out of 2,334 cases, 49% were linked to ground beef consumption (11). In the US, low-income population consume more beef, mainly ground and processed, when compared with the middle or high-income population (47). Ground beef was responsible for an estimated 21% of *E. coli* O157:H7 cases (123). Eating undercooked hamburgers has been identified as a risk factor for infection from *E. coli* O157:H7 (139). Well-done burgers are not favored by consumers because of the palatability and concerns about carcinogens created on the charred surface (125). The importance of beef safety has risen since the reporting

of the first two outbreaks caused by consumption of ground beef contaminated with *E. coli* O157:H7 (126).

Salmonella is the cause of an estimated 1.4 million illnesses, 20,000 hospitalizations and 400 deaths in the United States each year (132). The most common serotype, *typhimurium*, caused 22% of *Salmonella* infections in 2002 (26). Annual economic burden due to non-typhoidal *Salmonella* was estimated to be \$3.6 billion (132). Studies discovered that the mode of transmission of salmonellosis was through food, and the increase in infections during the 1970s and 1980s was linked to changes in slaughter and food production methods over the years (114). Cattle, swine, chicken carcasses, ground beef and ground pork were the most common foods tied to *Salmonella typhimurium* (133). Reported occurrence, severity, recurrence and persistence of salmonellosis was observed in the immune-compromised population, especially those infected with Human Immunodeficiency Virus (HIV) when compared to the general population (6).

Lactic acid bacteria (LAB) can be used as an antimicrobial intervention in reducing pathogens in raw poultry and meats (170). Smith et al., (2005) reported that addition of LAB to ground beef may act as an effective intervention to control *E. coli* O157:H7 and *Salmonella*. LAB produce several compounds like organic acids, hydrogen peroxide and bacteriocins that cause inhibitory action towards pathogenic bacteria (7). LAB are classified (59) by the Food and Drug Administration (FDA) into Generally Recognized As Safe (GRAS) for use in ground beef, beef patties, and raw whole muscle cut beefs through GRAS Notice No. 000171 (157). When added in beef patties, LAB can

be listed by their common name whereas, for fresh ground beef and whole muscle cuts, they must be labeled descriptively (59).

In beef processing facilities, veterinarians visually inspect cattle presented for slaughter, and animals with visible signs of serious illness are removed (48). When retail cuts are fabricated from beef carcasses in the processing facilities, microbial loads found on carcasses are likely transferred to newly exposed surfaces (57). Microbial quality of the trim and raw materials determine the level of microbial contamination in ground beef (53, 56). In processing plants, meat from one contaminated animal is mixed with meat from hundreds of other carcasses (48). USDA requires food processors adopt Hazard Analysis and Critical Control Point (HACCP) programs to prevent biological, chemical and physical hazards in the food processing. HACCP plans help food processors to monitor and control pathogens in their process by setting critical limits. Corrective actions must be taken if pathogen levels exceed the critical limits set in the HACCP plan. (126). Extensive research has concentrated on the surface application of antimicrobials on beef carcasses (53, 56), whereas the current focus was on the effect of the interventions on ground beef potentially contaminated during processing. Currently, processing plants do not have an effective intervention step to reduce pathogens while making ground beef (Smith *et al.*, 2005)

This study was conducted to investigate the effect of the *Lactobacillus*-based biological intervention on pathogenic bacteria in ground beef. We hypothesized that the addition of a *Lactobacillus*-based biological intervention reduces the number of pathogenic bacteria in traditionally packaged ground beef stored in a retail display case at 4°C.

CHAPTER II

LITERATURE REVIEW

Escherichia coli

Overview

Shiga toxin-producing *E.coli* (STEC) are a group of foodborne pathogenic bacteria that can cause illness ranging from moderate diarrhea to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). It can also lead to death in some severe cases. More than 200 serotypes of STECs have been identified so far, of which, 150 have been isolated from the patients suffering from HC and HUS. The most common STEC strain associated with foodborne illnesses and outbreaks is *E.coli* O157:H7. During the investigation of an outbreak in 1982, *E.coli* O157:H7 was recognized as a pathogen, but it was not until the Jack in the Box multistate outbreak in 1993 (25) that it was considered an essential and threatening pathogen in the food industry (102).

From 1982 to 2002, 940 non-O157 STEC isolates from humans were sent to the CDC by 43 state public health laboratories. The CDC has named 6 STEC groups that have been identified as the cause for 71% of non-O157 STEC outbreaks and illnesses associated with food. The big six STEC serogroups include O26, O45, O103, O111, O121, and O145 (22). In U.S, 67% of EHEC infections are estimated to be caused by *E. coli* O157:H7 whereas the other 33% of cases are credited to the non-O157 STEC population (103).

History and Background

Pathogenic *E. coli* can be classified into five virotypes: Enteroaggregative *E. coli* (EAEC), Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), Enteropathogenic *E. coli* (EPEC) and Enterotoxigenic *E. coli* (ETEC). *E. coli* O157:H7

belongs to the EHEC group (127) and found to produce shiga toxins (stx). Shiga toxins occur in two major antigenic groups, namely stx1 and stx2 (119) and are the main virulence factors linked with causing HC and HUS (87). The name of *E. coli* O157:H7 came from the expression of 157th somatic (O) antigen and the 7th flagellar (H) antigen (102). In 1983, a connection between infection with *E.coli* O157:H7 and post-diarrheal HUS was reported (88, 102).

Pathogenesis

E. coli O157:H7 accounts for more than 90% of all cases of HUS in industrialized countries (138). *E. coli* O157:H7 can be communicated through food and water, directly from person to person, and sometimes through job-related exposure. Most foodborne infections caused by this bacteria were traced to foods derived from cattle, especially ground beef and raw milk (102). Cattle are considered to be the principal reservoirs of *E. coli* O157:H7 (5, 35,73) and non-O157 STEC (67, 135). Recently, leafy greens are also more frequently linked with outbreaks (34, 110). *E.coli* O157:H7 infection in humans causes bloody diarrhea and abdominal cramps that last for 7 to 10 days and in some cases can lead to complications that result in HUS, renal failure and even death (102). HUS is characterized by abdominal cramps and initial watery diarrhea followed by bloody diarrhea with little or no fever (89, 102).

The infectious dose for illness depends on factors like the capability of bacteria to survive ingestion, bacterial virulence profiles, host susceptibility, and immunity. Paton et al., (1998) stated that the infectious dose of both O157 STEC and O111 STEC was less than 100 organisms. Studies estimated that minimum dose for non-O157 STEC

serogroups O111 and O145 was comparable to minimum dose for *E. coli* O157:H7 (153, 155).

EHEC pathogenesis primarily includes colonization and attachment by the bacteria. *E. coli* O157:H7 strains frequently exhibit tolerance to an acidic environment in the gastrointestinal tract (119). Type III secretion systems play an important role in effacing and dispensing virulence factors such as EspB, EspD, EspH, EspF and EspG into the host cell. These virulence factors disrupt actin cytoskeleton and may cause cell death (75). Two classes of shiga toxins were identified, stx₁ and stx₂. Stx₁ is 98% homologous to the stx produced by *Shigella dysenteriae* type 1. The stx₂ is 60% homologous to stx₁ and is antigenically distinct (118). Stx₁ and stx₂ stick to different epitopes on the globotriaosylceramide (Gb3) molecule and vary in binding attraction and kinetics. Stx₁ easily attaches to and detaches from Gb3 whereas stx₂ binds and dissociates very slowly (108). Stx₁ and stx₂ have nephrotoxic, cytotoxic, enterotoxic, and neurotoxic features, which may cause HUS (147). After ingesting orally, shiga toxins (stx) travel into the stomach and cling to the epithelial cells of the gastrointestinal mucosa (52). Fey et al., (2000) reported that stx adhere to endothelial cells at the site of infection. The attachment is facilitated by a 97-kD outer membrane protein named intimin (52). Stx further translocates into the blood circulation through transcellular pathways (2).

In vitro studies revealed that stx can bind to human erythrocytes (15), platelets (41), and activated monocytes (160). Recent studies have found that stx immediately and more efficiently binds to polymorphonuclear leukocytes (PMNL). These leukocytes are thought to play a role in stx transfer into the blood (148). Stx were consistently identified

on circulating PMNL in the blood of patients with HUS (149). Attachment of stx to target cells is reliant mostly on B-pentamer subunits and happens with the assistance of terminal di-galactose moiety of the receptor Gb3 (108).

EHEC caused illness should be suspected in people who have bloody diarrhea or history of bloody diarrhea; also, in people who were exposed to recent outbreaks (64, 139). EHEC screening can be executed in stool with SMAC (Sorbitol-MacConkey) agar and strains identified as O157:H7 should be sent to reference lab for affirmation. CDC recommends that all stools, not only bloody stools, submitted for culture to be screened for *E. coli* O157:H7. Modern diagnostic techniques are concentrating on directly detecting Shiga toxins or using DNA probes for recognizing the toxin genes in feces. Enzyme linked immunosorbent assays (ELISA) detects stx1 and stx2 in the stool (91, 99). Because these assays cannot identify the strain serotype, CDC recommends strain isolation using cultures and direct detection of shiga toxins in the stool samples.

Stx related HUS is the cause of 90% of all cases in children (62, 159). Symptoms usually start with abdominal cramps and non-bloody diarrhea, which may become hemorrhagic in 70% of cases within 1 or 2 days (33). Severe HUS leads to acute renal injury, thrombocytopenia, and microangiopathic hemolytic anemia (147). The average interval between exposure to *E. coli* and occurrence of symptoms is around three days (33). The treatment for illness caused by *E. coli* O157:H7 is restricted to non-specific supportive therapy. Salt and water management is vital in patients desiccated from diarrhea and vomiting (146). Antibiotics may cause the bacteria to release toxins, and are

not usually recommended to treat STEC infections (101). Directly targeting the toxin is an alternative intervention which remains unproven (154, 167).

Outbreaks and Recalls

The most common serotype of EHEC, in the US, is O157:H7 (113). According to the CDC, the most common non-O157 STEC serogroups between 1983 and 2002 were O26, O111, O103, O121, O45 and O145 (22). According to the 2015 FoodNet survey conducted by the CDC, there were 2.6 STEC infections overall reported per 100,000 people, approximately 40 percent due to *E. coli* O157:H7 and the other 60 percent due to other STEC strains (42, 81). In the US, STECs causes approximately 75,000 illnesses in patients every year (17). *E.coli* O157:H7 was first identified in 1982 in two outbreaks of severe bloody diarrhea among 47 individuals in Oregon and Michigan. Epidemiologic investigation of these outbreaks by the CDC established that all the patients had ingested ground beef from the same hamburger meat source before the onset of illness (126).

Table 2.1 illustrates the outbreaks, illnesses, and hospitalizations cause by STECs and ground beef from 1998 to 2016. In 2000, 50 people were infected, and 30 were hospitalized with O157:H7 in multiple states; the food vehicle is confirmed to be ground beef from a grocery store. In 2002, a plant recalled 354,200 pounds of ground beef based on positive O157:H7 cultures (26). Despite the recall, an outbreak involving 28 patients in seven states occurred. Seven patients were hospitalized, and five had HUS. The recall was expanded to include 18.6 million pounds of fresh and frozen ground beef. In April 2004, O157:H7 from ground beef was confirmed to be the cause of 59 infections and 18

hospitalizations. In April 2005, O157:H7 from ground beef had caused 11 hospitalizations and a total of 60 persons infected.

In a Canadian review of cases, non-O157 strains caused severe illness comparable to illness caused by O157 including HUS (168). In a 2008 Oklahoma outbreak, 341 individuals were affected by *E. coli* O111; in which the rate of HUS was similar to that reported for O157 infection (121). In 2010, there was an outbreak of *E. coli* O111 among inmates of a Colorado prison (29). In a 2011 outbreak of 3842 reported cases, O104:H4 serotype caused bloody diarrhea and HUS in travelers returning from northern Germany and 15 other European countries. This strain has shown 22% higher incidence of HUS than previous outbreaks (64). In April 2012, O145 from ground beef caused 5 infections, 3 hospitalizations and one death in Louisiana. In May 2014, O145 from ground beef caused 8 infections and 3 hospitalizations in multiple states.

Table 2.1 Outbreaks, illnesses, hospitalizations and deaths caused by STECs due to consumption of ground beef from 1998 to 2016.

Year	Outbreaks	Illness	Hospitalizations	Deaths
1998	5	64	13	0
1999	12	84	11	0
2000	13	198	48	1
2001	4	43	13	0
2002	8	157	38	1
2003	4	13	5	0
2004	4	74	22	0
2005	5	78	17	0
2006	5	72	38	1
2007	12	163	27	0
2008	8	216	72	0
2009	9	90	34	2
2010	4	27	5	0
2011	4	20	10	0
2012	3	39	5	0
2013	2	27	8	0
2014	5	40	16	1
2015	3	28	2	0
2016	2	25	12	0

Adapted from CDC. 2016. Foodborne Outbreak Online Database (FOOD Tool). Available at <http://wwwn.cdc.gov/foodborneoutbreaks/>. Accessed Oct. 28, 2017.

Salmonella

Overview

The CDC estimates that every year in the U.S about 1 million people get sick, 19,000 people get hospitalized, and 360 people die because of non-typhoidal *Salmonella* (132). In 2014 FoodNet survey, *Salmonella* remained the most commonly isolated bacterial pathogen, constituting for approximately 40% of the cases (43). *S. enterica* and *S. bongorii* are two species in genus *Salmonella*. Serovars of *S. enterica* species are the greatest threat as foodborne pathogens. The most common serovars present in both animals and foods include *typhimurium* (all species), *enteritidis*, *pullorum*, and *gallinarum* in poultry, *dublin* in cattle (124, 142). The most common serotypes are *S. enteritidis*, *S. newport*, and *S. typhimurium* (43).

History and Background

In 1885, *Salmonella* was discovered by Theobald Smith while working as a research assistant under USDA veterinary pathologist, Daniel Elmer Salmon. Smith accidentally isolated a new species of bacteria while he was trying to find a cause for hog cholera. However, Salmon claimed credit for Smith's discovery, and this new species of bacteria was later named after him (134).

Salmonellae are rod-shaped, gram-negative facultative anaerobes with peritrichous mobility belonging to the *Enterobacteriaceae* family. They range around 0.7 to 1.5 μm in diameter and 2 to 5 μm in length (39, 107). The classification of the genus *Salmonella* is defined and based on the Kauffmann-White scheme. *S. enterica* can be divided into six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* (71). *S. enterica* strains can also be classified based on the O lipopolysaccharide (LPS) surface antigen into 67 serogroups and 2,557 serovars when both their O and H

(flagellar) antigens differentiate strains. Among them, 1,531 serovars are recognized to belong to *S. enterica* subspecies *enterica*. Almost all *Salmonella* organisms that cause disease in humans and domestic animals belong to *S. enterica* subspecies *enterica* (61).

Pathogenesis

Salmonella is considered a global human pathogen because of its presence in the environment. *Salmonella* is predominantly present in poultry, meat, and eggs (68).

Farming practices in the meat, fish, and poultry industries can spread *Salmonella* among animals (68). *Salmonella enterica* species are typically acquired in humans by oral ingestion (61). Ingestion of *S. enterica* serovar occurs through contaminated animal products or water or close contact with an infected carrier or individual (79). The most common culprits for the *salmonella* outbreaks were fruits, seeded vegetables, sprouts, and nuts/seeds, but the three largest outbreaks were traced to eggs, poultry, and a raw tuna product. In adults, the infective dose for salmonellosis is estimated to be in the range of 10^4 to 10^6 cells or higher. The dose can be as low as 10^1 to 10^2 cells in immune-compromised individuals or if contained in high-fat matrix foods such as chocolate, cheese, salami, or peanut butter (10).

To establish a gastrointestinal infection, *Salmonella* must pass through few steps such as crossing the stomachs' acidic barrier, enduring the normal intestinal flora and bearing the enteric defenses enzymes, bile salts and antibodies (63, 104). The pathogenesis of *Salmonella* infection primarily consists of adherence, invasion and intracellular replication of the bacteria (165). Attachment to the tissues is one of the first crucial events in successful colonization by *Salmonella*. A reversible initial attachment is followed by an irreversible tight adhesion which depends on bacterial factors (32) such as

SipA, SipC, and SopB (117). The occurrence of *Salmonellosis* depends on the capability of the bacterium to colonize and attack the intestinal cells. During colonization, *Salmonella* competes with the intestinal microflora for attachment sites on the intestinal cell walls (122). The bacteria adheres to microfold (M) cells of the intestine (165). Adherence is a complex process which is mediated by multiple genes (98). *Salmonella* develops proteinaceous appendages on the surface when in contact with the epithelial cells. After attachment, *Salmonella* invades the intestinal cells by ruffling the membrane of the epithelial cells and causes the death of epithelial cells (122). Once a cell has been invaded, bacteria can remain within an altered phagosome known as *Salmonella*-containing vacuole (SCV) in which they can survive and replicate. SCV replicates when enough nutrients are available (98). Bacterial effector proteins are transferred into the host cell via type III secretion systems, mediating both invasion and vacuole formation. *Salmonella* is known to remain within the reticuloendothelial system, especially, macrophages (4, 9).

The pathogenicity island of *Salmonella* is multi-locus with 30 genes. Most of the genes are involved in the creation of enzymes and transcriptional activators that are accountable for the regulation, expression, and translocation of effectors to the surface of host cells. These genes are the usual targets for the polymerase chain reaction (PCR) for finding *Salmonella* in foods (3). Primary indications of salmonellosis include diarrhea, nausea, vomiting, fever and abdominal cramping which occur between 8 to 72 hours of exposure. Some other symptoms include fatigue, malaise, chills, weight loss, and headaches. Fever usually resolves in 48 to 72 hours and diarrhea within 4 to 10 days (105, 129). Intestinal *Salmonella* illnesses usually last about 5 to 7 days, and most do not

require treatment other than oral fluids (39). The rate of extra-intestinal infections in young children and infants is 10-fold higher than in the normal population (137).

Children are not treated for mild to moderate salmonellosis, unless otherwise. Antibiotics are not suggested especially for infants, elderly and immune-compromised individuals to treat gastroenteritis caused by *Salmonella* unless the illness is severe. Chloramphenicol, fluoroquinolones, 3rd generation cephalosporins, ampicillin, and azithromycin are some of the antibiotics that may be used to treat the illness (37, 115).

Outbreaks and Recalls

Table 2.1 illustrates the outbreaks, illnesses, and hospitalizations caused due to *Salmonella* and ground beef from 1998 to 2016. In March 2001, 93 persons were infected by *Salmonella heidelberg* at a restaurant in Louisiana, and the food vehicle is confirmed to be ground beef. In October 2003, 56 people were infected by *Salmonella typhimurium*, and 11 were hospitalized in multiple states. Ground beef, hamburger, and meatballs were confirmed to be the food vehicles (27). In December 2011, a Maine-based grocery chain had to recall an undisclosed amount of fresh ground beef products after 20 people were infected with a strain of *S. typhimurium* in seven states (28). In July 2012, Cargill Meat Solutions recalled 29,339 pounds of fresh ground beef products after 46 people were infected in nine states with the strain of *S. enteritidis* (29). In January 2013, Jouni Meats, Inc., and Gab halal foods recalled 500 and 550 pounds of ground beef products, respectively after 22 people were infected with *S. typhimurium* in six states (CDC 2013). In July 2014, 47 people from multiple states were infected with *S. typhimurium* from ground beef (30).

Table 2.2 Outbreaks, illnesses, hospitalizations and deaths caused by *Salmonella* due to consumption of ground beef from 1998 to 2016.

Year	Outbreaks	Illness	Hospitalizations	Deaths
1998	2	11	3	0
1999	0	0	0	0
2000	2	8	0	0
2001	3	100	21	1
2002	4	86	14	1
2003	6	173	22	0
2004	1	34	0	0
2005	5	103	18	1
2006	1	9	0	0
2007	4	75	30	0
2008	1	3	0	0
2009	1	68	4	0
2010	0	0	0	0
2011	2	25	10	0
2012	3	97	9	0
2013	2	41	9	0
2014	2	51	11	0
2015	1	10	1	0
2016	2	37	4	0

Adapted from CDC. 2016. Foodborne Outbreak Online Database (FOOD Tool).
 Available at <http://wwwn.cdc.gov/foodborneoutbreaks/>. Accessed Oct. 28, 2017.

Lactic Acid Bacteria

Overview

Lactic acid bacteria (LAB) are a group of gram-positive, mesophilic bacteria united by certain morphological, metabolic, and physiological characteristics. Lactic acid is one of the main fermentation products in this non-sporulating, non-respiring but aero-tolerant rod or coccus shaped bacteria. *Carnobacteriaceae*, *Enterococcaceae*, *Lactococcaceae*, *Lactobacillales*, *Lactosphaera*, *Leuconostocaceae*, *Oenococcaceae*, *Pediococcaceae*, *Streptococcaceae*, *Tetragenococcaceae*, *Vagococcaceae*, *Weissella* are the 12 known genera of the LAB. They obtain their energy by substrate-level phosphorylation due to the absence of functional respiratory system. Based on the byproducts of the hexose metabolism, LAB are classified into two groups: homofermentative bacteria and heterofermentative bacteria. Homo-fermentative or homolactics produce lactic acid as a major product of hexose fermentation. Heterofermentative bacteria can produce lactic acid, carbon dioxide, and ethanol in equal molar quantities from the glucose metabolism (95).

Lactobacillus is sub-classified into three subgenera: *Betabacterium*, *Streptobacterium*, and *Thermobacterium*. *Betabacterium* are heterofermentative bacteria. *Streptobacterium* can produce up to 1.5% lactic acid at an optimal temperature of 30 °C whereas *Thermobacterium* can produce up to 3% lactic acid at an optimal temperature of 40 °C (90). LAB are probably the most abundant and widespread in nature because of their ability to grow in a variety of substrates and biological conditions. Among the LAB, *Lactobacillus* is the most important and diverse species with very different biochemical and physiological properties. LAB do not require oxygen for growth, are tolerant to the

presence of CO₂ and nitrites. They also withstand relatively high concentrations of salt and low pH values. The environment in vacuum packaged meats is favorable and boosts the growth of these microorganisms. Microbial antagonism in the LAB is achieved by the use of available carbohydrates and pH reduction in the food. LAB also produce other antagonistic substances which include diacetyl, hydrogen peroxide, acetaldehyde, non-proteinaceous low molecular weight compounds and bacteriocins (36).

History and Background

The anti-bacterial effect of *Lactobacillus* was first published in 1896 by Ernest Hankin who reported an unknown substance in water that instigated bacterial death. Frederick Twort revealed a similar effect and hypothesized that lysis might be due to a virus. Felix d'Herelle discovered the actual phage and allocated them the name bacteriophage (50). Soon after the discovery of *Lactobacillus*, therapeutic phages were prepared and applied to regulate several bacterial infections (144). d'Herelle created a series of phages with a former Soviet microbiologist, Giorgi Eliava (152). Eli Lilly and Company manufactured *Lactobacillus*-based interventions in the U.S. before the discovery of antibiotics (50, 144).

LAB belong to the phylum *Firmicutes* and class *Bacilli*. The common ancestor has a coding potential for about 3,000 genes, but the LAB have a smaller genome (3.5Mb) that can code for 2,000 genes only. Analysis of the evolution shows LAB have lost 1,000 genes compared to the common ancestor of the family *bacilli*. This loss might have occurred due to the loss of a set of genes involved in sporulation, catalase genes, and some cofactor pathway genes. Some LAB have pseudo-genes possibly due to the specific ecological niche adapted (Brussow, 2014).

LAB was used in fermentations to protect the nutritive qualities of several foods (8). The antimicrobial effect by the LAB is mainly associated with the production of lactic acid and reduction of pH (45). Besides lactic acid, LAB also produce several antimicrobial compounds like bacteriocins, hydrogen peroxide (H₂O₂), carbon dioxide (CO₂), diacetyl (2,3-butanedione), and uncharacterized compounds (85, 92). The range of inhibitory activity of LAB can be either narrow - inhibiting strains that are closely related or wide - inhibiting a diverse group of gram-positive microorganisms (84, 92).

Strains used in this project

Lactococcus (Lc) lactis

The subspecies of *Lactococcus lactis* are non-motile, coccus shaped, gram-positive and homo-fermentative bacteria that produce lactic acid from glucose (143). Due to their effect on foods and their biochemical and physiological characteristics, *Lc. Lactis* was extensively studied (150). The subspecies of *Lc. lactis* are the most vital of the commercially used LAB (143) and are isolated from plant material (130) although dairy is the most recognized habitat.

Pediococcus (Pc) acidilactici

Pediococci species are homo-fermentative LAB which are typically used for rapid and strong acidification in meats. They are naturally present in the intestinal tracts of animals and humans. *Pc. acidilactici* and *Pc. pentosaceus* are among the most common starter cultures in the meat sector (40, 72). *Pc. acidilactici* produces a bacteriocin called pediocin which is usually used in the spanish meat industry as a starter culture (112). *Pc. acidilactici* has been intensively studied for its ecological significance and biotechnological potential (78). *Pc. acidilactici* is heat tolerant and grows at 50°C (143).

Lactobacillus (Lb) acidophilus

Lactobacilli are sternly fermentative, have complex nutritional necessities and grow in various diverse habitats. They are acidophilic and yield pH 4.0 in foods comprising a fermentable carbohydrate and often suppress the development or kill other bacteria. *Lb. acidophilus* produce antimicrobial agents that can inhibit the growth of unwanted intestinal flora (69, 136, 158, 162). With few substrate and strain exceptions, *Lactobacilli* grow up to pH of 7.2. *Lactobacilli* are used as starter cultures for several varieties of cheese, fermented plant foods, fermented meats, wine and beer production, sourdough bread and silage (143).

Lactobacillus acidophilus is a probiotic aide culture used in yogurt, cheese, fermented cream and milk beverages (23). They can survive in high temperatures and are known to produce exopolysaccharides that increase viscosity, stability and water binding capacity in cheeses (21). *Lb. acidophilus* delivers vitamin K and lactase that is found in the upper gastrointestinal (GI) tract and is a standout amongst the most utilized probiotics used to advance human well-being (36).

Bacteriocins

Bacteriocins in the LAB are divided into four classes. Class I are small (< 5 kDa) peptides that are also called lantibiotics for having lanthionine-containing peptides with antibiotic activity. Nisin, produced by *Lactococcus lactis subsp. lactis* strain belongs to the class I bacteriocins (51, 84) and is isolated from milk and vegetable-based products (74, 83). Nisin is the most intensely defined and studied bacteriocin to date (49) and is active against many gram-positive bacteria including *Listeria monocytogenes* and *Staphylococcus aureus* (38, 83). Nisin inhibits the development of germinating *Bacillus*

and *Clostridium* spores (1,83). With the addition of calcium chelation, nisin was found to be effective against some gram-negative bacteria (141). Nisin spray used as an intervention resulted in 2 to 3.5 log CFU/cm² bacterial reduction in both lean and adipose tissue of the beef carcass (44). Class I and II bacteriocins are by far the most studied because of their abundance and prominence for industrial applications (63, 64).

Class II bacteriocins are medium (< 10 kDa) peptides that are heat-stable non-lantibiotics. Class II bacteriocins are subdivided into four different subclasses: a, b, c and d. Class IIa comprises single peptide bacteriocins, which include the pediocin-like group. Pediocin PA-1 is the most intense bacteriocin within the Class IIa group (70, 76, 100, 106, 111) and is produced by *Pc. acidilactici*. Pediocin displayed antimicrobial effect against a broad spectrum of gram-positive bacteria and showed potent activity against *L. monocytogenes* (128). Pediocin AcH, produced by *Pc. acidilactici* inhibited the growth of *L. monocytogenes*, *Staph. aureus* and *Clostridium perfringens*. Pediocin A, produced by *Pc. pentosaceus* can be used as a preservative in foods (13).

Class IIb are double-peptide bacteriocins and need equal peptide ratio to employ best antimicrobial activity (66). Lactococcin G and lactacin F produced by *L. lactis* and *L. johnsonii* are the first reported class IIb bacteriocins with a narrow inhibition spectrum against *Clostridium* and *E. faecalis* (169). Lactocidin is a broad-spectrum inhibitor bacteriocin extracted from aged liver veal agar cultures of intestinal isolates of *Lb. acidophilus*. Lactocidin was non-dialyzable and active against both gram-negative and positive cultures (162). Class IIc are sec-dependent secreted bacteriocins and have a different head-to-tail cyclization of their backbones (65). Class IId bacteriocins include

the remaining well-characterized bacteriocins, non-pediocin like single linear peptides, *sec*-dependent bacteriocins and N-terminal leaderless bacteriocins (93).

Class III are large (> 30 kDa) peptides that are heat-labile and include many bacteriolytic extracellular enzymes that may mimic the physiological activities of bacteriocins. Class IV are complex bacteriocins that contain essential lipid or carbohydrates moieties in addition to protein (93).

Various aspects impact the efficacy of bacteriocins in food environment such as the capability of the food matrix for bacteriocin production; antagonism with other bacteria; inhibition by bacteriophage; development of a bacteriocin-resistant microflora; and inactivation of the bacteriocin by enzymes or binding to fats or proteins (46). Studies have shown the application of bacteriocins can reduce pathogen levels in different food matrices; however, the direct application of bacteriocins may result in the reduction or loss of antimicrobial activity due to problems related to the interaction with food components. *Lactobacillus* species application in the food industry has no negative impact on the taste or quality of the product. Some of the most studied bacteriocins in foods include nisin, leucocina, pediocin, and enterocin. Enterocin A and B inhibit *E. coli* in sausage and improve bactericidal effect at temperatures 15°C to 22°C (120).

Organic Acids

Lactic acid produced through carbohydrate fermentation is known to inhibit bacterial spoilage in food (109). Lactic acid and acetic acid in food promote and maintain the acidic pH of metabolites (20). The capability of the LAB to harvest organic acid differs from species to species, and this is a crucial feature in classification. Lactic, citric, and acetic acids are some of the extensively researched organic acids for their

antimicrobial properties (14). Concentration, pH, pKa of the acid and the concentration of the undissociated molecule determine the efficacy of the antimicrobial agents (12).

LAB strains, culture mix, and growth environments determine the amount and forms of organic acids released during the fermentation process. Lactic acid can be in its undissociated or dissociated forms, and the level of the dissociation relies on pH (97). Fermented foods contain a relatively high amount of organic acids that reduce the pH of the food. The un-dissociated acid causes an antimicrobial effect because of its lipophilic nature. Un-dissociated acid can passively cross the cell membrane and dissociate into the cell cytoplasm. Un-dissociated acids fluctuate the cell membrane permeability, which results in disruption of substrate translocation and oxidative phosphorylation (140). LAB can survive and grow in the presence of relatively low pH unlike other microbial groups with respiratory metabolism (120).

Other metabolites

LAB can produce hydrogen peroxide (H_2O_2) in the presence of oxygen. H_2O_2 is responsible for the oxidation of the sulfhydryl groups initiating enzyme denaturation. It is also accountable for the oxidative degradation of lipids consequently increasing membrane permeability and causing anti-microbial activity (94). H_2O_2 may act as a precursor in the production of superoxide and hydroxyl, which are bactericidal free radicals (24). Heterofermentative LAB produces carbon dioxide (CO_2) that creates an anaerobic environment which hinders enzymatic decarboxylations. The buildup of CO_2 in the lipid bilayer impairs permeability of the membrane (55). CO_2 can efficiently reduce the growth of gram-negative psychrotrophic bacteria (58). All LAB, through citrate

fermentation, produce diacetyl that prevents the growth of gram-negative bacteria. Jay (1982) studied the antimicrobial effect of diacetyl and reported that the growth of *Listeria*, *Salmonella*, *Yersinia*, *E. coli* and *Aeromonas* strains can be prevented.

Anti-microbial activity

Several factors influence the microbial growth and metabolic production in meat products. Bio-preservation use should be considered in addition to good manufacturing practices (GMPs) during processing, storage, and distribution of meat (36). LAB in fresh meat aid in a mild fermentation process without creating any variations in the sensory features due to the low carbohydrate content and the strong buffering capability of meat. LAB in meat can cause microbial disturbance to spoilage and pathogenic bacteria through various mechanisms like nutrient and oxygen competition, competition for attachment/adhesion sites. LAB also produce a wide range of inhibitory substances primarily lactic acid, acetic acid, diacetyl, hydrogen peroxide, reuterin and bacteriocins (82). Metabolites produced by LAB species display inhibitory activity against pathogens such as *E. coli*, *S.typhimurium* and *Pseudomonas.aeruginosa* (131, 151). It was reported that LAB shows a strong antagonistic activity against both spoilage and pathogenic bacteria in food (77).

Vescovo et al., (1996) isolated LAB strains of *Lactobacillus casei*, *L. plantarum*, *Pediococcus species* in commercially available RTE salads. These LAB cultures were efficient in inhibiting the growth of *Aeromonas hydrophila*, *S.typhimurium* and *Staphylococcus aureus* on salads. *Lb.casei* was most effective in inhibiting the growth of the aforementioned pathogenic bacteria in RTE salads during a six-day storage when compared to other LAB strains.

Lactobacillus-based direct fed microbials were highly effective for reducing *E. coli* O157:H7 shedding in cattle (19). Pascual et al., (1999) reported that *S. enteritidis* could be eliminated from the gastrointestinal tract of one-day old chickens utilizing a single strain of *Lb. salivarius* when combined with *Salmonella* specific avian antibodies. Lema et al., (2001) studied the decrease of fecal shedding of *E.coli* O157:H7 in lambs by utilizing LAB. In a seven-week study, the researchers fed a combination of multiple LAB strains (*Lb. acidophilus*, *Enterococcus faecium*, *L. casei*, *L. fermentum*, and *L. plantarum*) to the lambs inoculated with *E. coli* O157:H7. They observed a 4-log CFU/gm reduction in the fecal shedding. Echeverry et al., 2010 reported reduced *E.coli* O157:H7 counts of 0.66 log CFU/cm² (day 14), 0.89 and 1.33 CFU/cm² (day 21) when LAB spray was used as an intervention on strip loins. Reduction in *Salmonella* counts of 0.7 log CFU/cm² (day 14) were also reported.

CHAPTER III

MATERIALS AND METHODS

Bacterial Strains

A cocktail of four *E. coli* O157:H7 strains were used for this study and included: A4 966, A5 528, 966 and A1 920. All strains were originally isolated from cattle and were each connected to the outbreaks that were documented by the CDC. A cocktail of four *Salmonella* strains was also used for this study and included: Typhimurium ATCC® 14028™, Anatum ATCC® 9270™, Muenchen ATCC® 8388™, Abaetetuba ATCC® 35460™. A cocktail containing six *E. coli* non-O157:H7 serogroups (O26, O45, O103, O111, O121, and O145) was used for this study. Strains were isolated from both bovine and ground beef sources (Table 2.1). All cocktails were prepared by making frozen concentrated cultures of each strain as described by Brashears et al., (1998). Each strain was attained from the -80 °C freezer of Texas Tech University stock culture and was added to separate tubes of Brain Heart Infusion Broth (BHI; EMD, Gibbstown, NJ) using a sterile loop. The strains were incubated overnight at 37 °C, transferred into fresh BHI tubes and incubated an additional night at 37 °C. Each strain was plated on Tryptic Soy Agar (TSA; EMD, Gibbstown, NJ) and incubated for 24 hours at 37 °C to determine the concentration. All strains were to transferred BHI, allowed to grow at 37 °C overnight and then centrifuged for 10 minutes at 4,000 x g. The concentrated pellet was suspended in BHI containing 10% glycerol. Equal portions of strains were combined and stored as a frozen culture at -80 °C in 1 mL portions at a concentration of 1.0×10^9 CFU/mL at the Texas Tech University stock culture collection.

The LAB cultures for this project was commercially prepared by Cultures System Inc., (Mishawaka, IN) and packaged in freeze-dried 100-g portions before shipping to Texas Tech University and stored at -20°C. The Lactic Acid Cocktail (LAC) product was comprised of four LAB strains. *Lactobacillus acidophilus* NP51 was originally isolated from calf whereas *Lactobacillus acidophilus* NP28 was originally isolated from cattle. *Pediococcus acidilactici* NP3 was originally isolated from ham and frankfurters whereas *Lactococcus lactis* subsp. *lactis* NP7 was originally isolated from alfalfa seeds and sprouts (Smith et al., 2005). A LAB cocktail with a concentration of 1.0×10^9 CFU/mL was prepared by combining 6 g of NP51, NP28 (3 g each) and 0.6 g of NP3, NP7 (0.3 g each) to 87 mL of potable tap water. The concentration of LAB was determined by making serial dilutions in BPW and plating on Lactobacilli MRS Agar (EMD, Gibbstown, NJ). The MRS agar plates were incubated at 37 °C for 24 to 48 h.

Table 3.1 Sources and virulence factors of non-O157 STECs used in this project

O type	Stx ₁	Stx ₂	Source
26	-	-	ground beef
45	-	-	bovine
103	+	-	bovine
111	+	+	bovine
121	-	-	bovine
145	-	+	ground beef

Pathogen reduction study

All inoculations and packaging were conducted in BSL II Pathogen Processing Laboratory located in food technology building of Texas Tech University. Three days before the project, every day, whole pathogen lab including, walls, ceiling and equipment were cleaned and sanitized (Bi-Quat, Birko Corp., Henderson, CO). Also, all equipment was cleaned and sanitized in between each treatment.

The same inoculation procedure for the ground beef was followed for all three bacterial cocktails (*E. coli* O157:H7, non-O157 STEC, *Salmonella*). For each bacterial cocktail, 22.38 kg of coarse ground beef (80% lean/20% fat) obtained from Cargill Inc., Plainview, TX was obtained after released from company's test and hold process. Coarse ground beef (meat block) was immediately transferred to Texas Tech University pathogen processing lab. For each bacteria, 240 mL of each pathogen cocktail was re-suspended in buffered peptone water (BPW; OXOID, Basingstoke, Hampshire, England) and was subsequently added to 22.68 kg of ground beef at the concentration of 1.0×10^5 CFU/mL to obtain a final concentration of 1.0×10^3 CFU/g. The meat block was thoroughly mixed by hand for two minutes and transferred to a mixer/grinder (Grinder MGR-101) and mixed for 10 minutes for even distribution. Out of the 22.68 kg meat block, 11.34 kg of inoculated, non-treated control (CON) ground beef was finely ground and portioned onto black expanded polystyrene trays and overwrapped with poly-vinyl chloride film ($\sim 21,700$ cc oxygen/m²/24hr).

A total of 50 mL of the LAB cocktail (NP51 and NP28 at 1.0×10^9 CFU/mL and NP3 and NP7 at 1.0×10^8 CFU/mL) was added to the remaining 11.34 kg of inoculated meat, mixed by hand for two minutes to achieve a target concentration of 1.0×10^7 CFU/g

of ground beef. The treated ground beef was transferred back to the mixer (10 minutes) for even distribution, finely ground and portioned onto black expanded polystyrene trays and overwrapped with poly-vinyl chloride film. Initially, 10g of the ground beef sample was taken from treated samples to determine LAB concentration at the beginning of the experiment.

All packages were labeled by sampling interval (day 1, day 3, and day 5) for each pathogen. Inoculated control samples with no LAB treatment were labeled CON. Samples inoculated and treated with LAB were labeled LAC. All samples were stored in a multi-deck retail style display case at 0 to 4°C with continuous high output 1,900 lux fluorescent lighting bulbs, 3,500 color temperature rating and a 70-color rendering index. The retail display case was in the Experimental Sciences Building of Texas Tech University.

Microbiological Analysis

At each time point (1, 3 and 5 days), 10 g of ground beef from both LAC and CON trays was aseptically opened and weighed into a filtered stomacher bag (Stomacher® 80 Biomaster), and 90 mL of BPW was added. The samples were stomached (Seward Model 400, Bohemia, NY) for 2 minutes at 230 RPM and serially diluted in 90 mL BPW. Treated LAC samples were also plated onto MRS agar plates to determine the growth and concentration of LAB strains. Homogenized samples were serially diluted, spiral plated (Autoplate® 4000, Spiral Biotech Inc., Norwood, MA) and analyzed for total *E. coli* O157:H7 and non-O157 STECs by plating on MacConkey agar. MacConkey plates were incubated at 37 °C for 24 ± 2 hours. Pink colonies were counted on a colony counter (QCount® 530, Advanced Inst., Inc. Norwood, MA) as presumptive positive for

E.coli O157:H7 and non-O157 STECs and agglutinated at random for confirmation using a latex agglutination kit (Remel, Lenexa, KS). Samples inoculated with *Salmonella* cocktail were homogenized, serial diluted and spiral plated on Xylose Lysine Deoxycholate (XLD) agar. XLD plates were incubated at 37°C for 48 ± 2 hours. Black colonies were counted as presumptive positive for *Salmonella* and agglutinated at random for confirmation using a latex agglutination kit (Remel, Lenexa, KS). All treated LAC samples were also homogenized, serial diluted and spiral plated on MRS agar. MRS plates were incubated at 37°C for 48 ± 2 hours. White colonies were counted as presumptive positive for Lactic acid bacteria.

Statistical Analysis

All tests were performed in triplicate for each of the bacteria, and the averages of the samples were used for statistical analysis. Once microbial counts were determined, the CFU/g of the microbial counts of all bacteria were converted into log₁₀ units. The Mixed Model of the Statistical Analysis System (SAS; version 9.1, SAS Institute, Inc., Cary NC) was used to evaluate the effect of *Lactobacillus*-based biological intervention on microbial loads of all 3 bacteria with replication as a random effect. The dependent variable in this model is the population of bacteria. Independent variables were treatments and sampling time. Significant main effects and interactions were calculated using least square means method. All tests were conducted with a significance level of 0.05.

CHAPTER IV

RESULTS

Table 4.1 shows the effect of the *Lactobacillus*-based biological intervention on *E.coli* O157:H7 populations in ground beef that was traditionally packaged (overwrap) and placed in a retail display case for 5 days. There was a significant ($P<0.0001$) effect with treatment, a significant ($P=0.0017$) effect with display length duration and a significant ($P<0.0001$) effect with treatment x display duration length interaction. No significant ($P>0.05$) difference in pathogen counts were observed in CON and LAC samples on day 1 with *E.coli* O157:H7 counts ranging from 3.43 to 3.49 log CFU/g. On day 3 the concentration of *E.coli* O157:H7 in CON samples was 3.74 log CFU/g which was significantly ($P<0.05$) higher than the counts (3.39 log CFU/g) in LAC samples. Similarly, on day 5, the counts of *E.coli* O157:H7 were significantly ($P<0.05$) higher in CON samples at 4.25 log CFU/g when compared to the population (3.27 log CFU/g) on LAC samples. The concentration of *E.coli* O157:H7 significantly ($P<0.05$) decreased on days 3 and 5 when compared to day 1 in LAC samples. The populations of *E.coli* O157:H7 were not significantly ($P<0.05$) different on days 1,3 and 5 ranging from 3.49 to 4.25 log CFU/g. The LAC samples exhibited no inhibition of the pathogens on day 1. This suggests that our intervention started to inhibit the growth *E.coli* O157:H7 from day 3 in overwrap packaging at retail display temperature. This indicated that LAB cocktail inhibited further growth of *E. coli* O157:H7 in ground beef under simulated retail conditions in overwrap packages.

Table 4.2 illustrated the effect of the *Lactobacillus*-based biological intervention on *Salmonella* counts in ground beef that was traditionally packaged (overwrap) and

placed in a retail display case for 5 days. There was a significant ($P<0.0001$) effect with treatment, a significant ($P<0.0001$) effect with display length duration and a significant ($P<0.0001$) effect with treatment and display duration length. Similar to *E.coli* O157:H7, on day 1 there was no significant ($P>0.05$) difference between CON and LAC ground beef samples in *Salmonella* populations. The counts ranged from 3.11 to 3.20 log CFU/g. On day 3 the counts of *Salmonella* were significantly ($P>0.05$) higher in CON samples at 3.95 log CFU/g when compared to the counts in LAC samples at 3.06 log CFU/g. Similarly, the same effect was seen on day 5, the counts of *Salmonella* were significantly ($P<0.05$) higher in CON samples at 3.90 log CFU/g when compared to the population on LAC samples at 3.11 log CFU/g. The concentration of bacteria was significantly ($P>0.05$) different on days 3 and 5 LAC samples when compared to day 1. Above results suggest that our intervention started to inhibit the further growth *Salmonella* from day 3 in overwrap packaging at retail display temperature. This indicated that the *Lactobacillus*-based biological intervention inhibited further growth of *Salmonella* in ground beef under simulated retail conditions.

Table 4.3 demonstrated the effect of the *Lactobacillus*-based biological intervention on non-O157 STECs in ground beef that was traditionally packaged (overwrap) and placed in a retail display case for 5 days. There was a significant ($P=0.0039$) effect with treatment, a significant ($P<0.0001$) effect with display length duration and a significant ($P=0.0228$) effect with treatment and display duration length. No significant ($P>0.05$) difference was found in populations of non-O157 STECs in CON and LAC samples on both day1 and day 3. The concentrations ranged from 3.19 to 3.83 log CFU/g. In LAC samples, non-O157 STECs significantly ($P<0.05$) increased to

3.95 log CFU/g on day 5 when compared to day 1 and 3 that ranged from 3.19 to 3.39 log CFU/g. On day 5, non-O157 STECs were 5.45 log CFU/g in CON samples and significantly ($P<0.05$) higher when compared to LAC samples at 3.95 log CFU/g. This suggests that our intervention started to inhibit the further growth non-O157 STECs from day 5 in overwrap packaging at retail display temperature.

Table 4.1 *E.coli* O157:H7 concentrations in traditionally overwrapped ground beef after the addition of a *Lactobacillus*-based biological intervention and stored in retail display case

Treatment	Retail Display Duration, d ¹			<i>Main Effect</i> ²
	1	3	5	
CON	3.49 ^{az}	3.74 ^{bz}	4.25 ^{cz}	3.83 ^z
LAC	3.43 ^{az}	3.39 ^{ay}	3.27 ^{ay}	3.36 ^y
<i>Main Effect</i> ³	3.46 ^a	3.57 ^a	3.76 ^b	

¹ Treatment x Retail Display Duration, d(day): $P < 0.0001$; SEM = 0.19.

² Treatment: $P < 0.0001$; SEM = 0.18.

³ Retail Display Duration: $P = 0.0017$; SEM = 0.18.

CON Ground beef control samples inoculated with *E.coli* O157:H7.

LAC Samples inoculated with *E.coli* O157:H7 and treated with *Lactobacillus* intervention.

SEM Standard error mean

^{a, b, c} Means within rows having different letter are significantly different ($P < 0.05$)

^{y, z} Means within columns having different letter are significantly different ($P < 0.05$)

Table 4.2 *Salmonella* concentrations in traditionally overwrapped ground beef after the addition of a *Lactobacillus* intervention and stored in retail display case

Treatment	Retail Display Duration, d ¹			<i>Main Effect</i> ²
	1	3	5	
CON	3.20 ^{az}	3.95 ^{bz}	4.55 ^{cz}	3.90 ^z
LAC	3.11 ^{az}	3.06 ^{ay}	3.11 ^{ay}	3.09 ^y
<i>Main Effect</i> ³	3.15 ^a	3.51 ^b	3.83 ^c	

¹ Treatment x Retail Display Duration, d: $P < 0.0001$; SEM = 0.10.

² Treatment: $P < 0.0001$; SEM = 0.08.

³ Retail Display Duration: $P < 0.0001$; SEM = 0.09.

CON Ground beef control samples inoculated with *Salmonella*

LAC Samples inoculated with *Salmonella* and treated with *Lactobacillus* intervention.

SEM Standard error mean

^{a, b, c} Means within rows having different letter are significantly different ($P < 0.05$)

^{y, z} Means within columns having different letter are significantly different ($P < 0.05$)

Table 4.3 Non-O157 STEC concentrations in traditionally overwrapped ground beef after the addition of a *Lactobacillus* intervention and stored in retail display case

Treatment	Retail Display Duration, d ¹			<i>Main Effect</i> ²
	1	3	5	
CON	3.21 ^{az}	3.83 ^{az}	5.45 ^{bz}	4.16 ^z
LAC	3.19 ^{az}	3.39 ^{az}	3.95 ^{ay}	3.51 ^y
<i>Main Effect</i> ³	3.20 ^a	3.61 ^a	4.70 ^b	

¹ Treatment x Retail Display Duration, d: $P = 0.0228$; SEM = 0.31.

² Treatment: $P = 0.0039$; SEM = 0.23.

³ Retail Display Duration: $P < 0.0001$; SEM = 0.25.

CON Ground beef control samples inoculated with non-O157 STECs.

LAC Samples inoculated with non-O157 STECs and treated with *Lactobacillus* intervention.

SEM Standard error mean

^{a, b, c} Means within rows having different letter are significantly different ($P < 0.05$)

^{y, z} Means within columns having different letter are significantly different ($P < 0.05$)

CHAPTER V

DISCUSSION

In addition to O157:H7, USDA-FSIS declared raw nonintact beef and its components as well as intact beef intended to be processed into nonintact beef to be adulterated if found positive for non-O157 STECs belonging to serogroups O26, O45, O103, O111, O121, O145 (156). *Salmonella* contamination is linked with various foods such as poultry, pork, beef products and raw milk. *Salmonella enterica* is associated with 17% of all foodborne outbreaks in the United States (166).

Smith et al., (2005) experimented to study the effect of four strains of LAB both individually and as a cocktail on the counts of *E.coli* O157:H7 and *Salmonella* in ground beef at 5°C. They observed a 3 to 5 log cycle reduction from individual LAB strains. With the cocktail, they reported a 2 log reduction of *E.coli* O157:H7 after 2 days and a 3 log reduction after 5 days storage. After day 5 day storage, *Salmonella* counts were reduced to non-detectable levels. Sensory studies done on the non-inoculated, LAB treated ground beef samples revealed no adverse effects on the sensory properties.

Hoyle et al., (2009) studied the effect of the LAB on *E.coli* O157:H7 and *Salmonella* counts present in ground beef while using traditional overwrap packaging and Modified atmosphere packaging (MAP) at retail display temperature (0°C) and abusive temperature (10°C). Three concentrations (10^6 , 10^7 and 10^7) of LAB were used to determine the dose levels needed for pathogenic bacteria inhibition. After 3 days of storage, 2.6 log CFU/g and 5 days of storage, 3.4 log CFU/g reduction in *E.coli* O157:H7 counts were observed.

Echeverry et al., (2010) studied the effect of LAB spray on the surface and internal counts of pathogenic bacteria in USDA choice strip loins when mechanical blade-tenderizer (MN) and injection – enhancement (EN) was used. A reduction of 0.66 log CFU/cm² (day 14) and >1.33 CFU/cm² (day 21) of *E.coli* O157:H7 was observed on surfaces that used MN. Also, a reduction of 0.89 log CFU/cm² (day 21) of *E.coli* O157:H7 was observed on surfaces that used EN. Also, reduction of 0.7 log CFU/cm² (day 14) of *Salmonella* counts were observed on the surfaces of sub-primals that used EN.

The target and achieved (results not shown) concentration of *Lactobacillus*-based biological intervention used in our project is 1×10^7 CFU/g. The four strain LAB used are *Lactobacillus acidophilus* NP51 and NP28, *Pediococcus acidilactici* NP3 and *Lactococcus lactis* subsp. *lactis* NP7. Zhang et al., (in press) studied the inhibition effect of four combinations of LAB cocktails (NP28+NP51, NP28+NP51+NP3, NP28+NP51+NP3+NP7, and NP35+NP51+NP3+NP7). The LAB cocktail containing NP28+NP51+NP3+NP7 strains showed best inhibition results when compared to other LAB cocktails. Significant reductions with 1.5 log CFU/g of *E.coli* O157:H7 and 1.7 log CFU/g of non-O157 STECs were recorded on day 5 when the four strain (NP28+NP51+NP3+NP7) LAB cocktail was directly added to ground beef and stored at 4°C. Further research was conducted on the four strains (NP28+NP51+NP3+NP7) LAB cocktail to observe their effect on pathogens at different LAB concentrations (10^7 , 10^8 , 10^9 CFU/g). Out of the three concentrations, 10^8 and 10^9 CFU/g showed stronger inhibition on *E.coli* O157:H7 when compared to 10^7 CFU/g. All three concentrations showed 0.5 to 1.6 log CFU/g reduction in non-O157 STECs. Also, a significant

reduction (1.4 log CFU/g) was observed in *Salmonella* counts on day 5 when the four strain (NP28+NP51+NP3+NP7) LAB cocktail was directly added to ground beef and stored at 4°C.

Our results suggest that direct addition of *Lactobacillus*-based biological intervention to ground beef slightly inhibited the further growth of *E. coli* O157:H7 with counts being 0.35 and 1.02 log CFU/g lower than the inoculated control samples on days 3 and 5, respectively. Our reports indicated that the inhibition of *E. coli* non-O157 in ground beef by applying a *Lactobacillus*-based biological intervention is comparable to the inhibition of *E. coli* O157:H7. Our experiment revealed that the LAB cocktail can only inhibit the further growth of pathogens but may not completely kill them over a 5-day period. In our project, the inhibition effect of the LAB was seen on day 3 and day 5 for *E. coli* O157:H7 and *Salmonella* whereas the inhibition was observed on day 5 for non-O157 STECs. Since the turnaround time for ground beef in the retail stores can be as low as 12 h, further research should be focused on adding the intervention to beef trims before coarse ground beef is made in the processing plants. Retail supermarkets purchase coarse ground beef in bulk from the processing plants and make their individual finely ground beef trays for retail display. Commercial ground beef is formulated from varied beef source materials, including finely textured beef (FTB) and imported frozen lean boneless beef trim (16). Since beef trim is the raw material for ground beef, interventions that target trim before grinding may be a practical point of treatment (86).

There were several limitations to this project. pH of the meat of was never recorded. The temperature of the meat after placed in the retail display cases was not recorded. Background aerobic plate counts (APC) were not taken into consideration.

Further research is needed to study the effect of LAB interventions with vacuum and MAP packaging. *Lactobacillus*-based biological interventions may be used in to inhibit further growth of O157:H7, non-O157 STECs, and *Salmonella* in a retail display case.

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