COMPARISON OF *SALMONELLA* PRESENCE IN BOVINE LYMPH NODES ACROSS FEEDING STAGES

A Thesis

by

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ABSTRACT

Peripheral lymph nodes (LNs) located in the fatty tissues of beef carcasses have been shown to harbor *Salmonella*, and thus are a potential source of contamination in beef trimmings. *Salmonella* prevalence within LNs differs among feedlots, although the sources and routes of transmission have yet to be confirmed. The objective of this study was to determine if *Salmonella* prevalence in bovine LNs varies across feeding stages and feedlot environments in South Texas. Two feedlots with historically different levels of *Salmonella* prevalence within bovine LNs were selected. Twenty steers were to be harvested at each of 4 feeding stages: (1) post-weaning, (2) stocker, (3) 60 d on feed, and (4) 120 d on feed. Four steers did not complete the study. Left and right subiliac and superficial cervical LNs were collected from each carcass (n = 304), and similar node types were pooled by animal (n = 152). *Salmonella*, if present, was isolated from the LNs following the USDA-FSIS Microbiological Laboratory Guidebook (MLG) 4.08. Presumptive positive isolates were confirmed using PCR, and isolates were submitted for serotyping. Results showed a difference ($P < 0.05$) in prevalence of *Salmonella* in bovine lymph nodes between feedlots and among feeding stages.

There was no *Salmonella* isolated from LN samples taken after feeding stage 1 (weaning) or from Feedlot A. Within feedlot B, there was an increase in *Salmonella* prevalence as cattle moved into later stages of feeding, at 22.2% (4/18), 77.8% (14/18), and 94.4% (17/18) for feeding stages 2, 3, and 4, respectively. There was a difference ($P < 0.05$) in LN *Salmonella* prevalence between stages 2 and 3, and a numerical

difference between stages 3 and 4. It appears there is an environmental effect that influences the prevalence of *Salmonella* in LNs. The cause of these differences is unknown, and provides opportunity for future investigation into pre-harvest environmental conditions relating to *Salmonella* exposures.

DEDICATION

This thesis is dedicated to my parents. They have been a constant inspiration for me, and I could never have come as far as I have without them.

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NOMENCLATURE

CDC	Centers for Disease Control and Prevention
DFM	Direct fed microbial
DNA	Deoxyribonucleic acid
FSIS	Food Safety and Inspection Service
GI	Gastrointestinal
LN	Lymph node
PCR	Polymerase Chain Reaction
PMN	Polymorphonuclear leukocytes
Salmonella	Non-typhoidal *Salmonella enterica*
SPI	*Salmonella* Pathogenicity Island
USDA	United States Department of Agriculture

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CHAPTER I

INTRODUCTION

Non-typhoidal *Salmonella enterica* are known contaminants of fresh beef products, particularly in trimmings and ground beef. On average, *Salmonella* is responsible for 1.2 million illnesses a year from food and meat products, pets, and laboratory accidents; 375,000 of these illnesses specifically originate from products inspected by the United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) *(68, 72)*. Recent outbreaks resulting from ground beef include a 2013 outbreak of *Salmonella* ser. Typhimurium that infected 22 people across 6 states, and a 2012 outbreak of *Salmonella* ser. Enteritidis that resulted in 46 infections across 9 states *(19, 20)*.

It has been well-documented that bovine lymph nodes (LNs) can harbor Gram-negative organisms, specifically *Salmonella (4, 12, 13, 37, 38, 40, 50, 51, 55, 66, 67)*. Early research investigated *Salmonella* located in the mesenteric LNs, which could become contaminated from intestinal drainage *(55, 66)*. More recently, it has been discovered that *Salmonella* can also colonize the peripheral LNs, which are embedded in the fat between skeletal muscles *(4)*. The microorganisms within the LNs are protected from carcass surface antimicrobial interventions and are, therefore, a potential source of contamination for beef trim and ground beef products *(11, 51)*. It also has been demonstrated that live animal production conditions can impact the frequency of fecal shedding and prevalence of *Salmonella (39, 51)*. The *Salmonella* prevalence in LNs has

been shown to vary substantially depending on the feedlot of origin and animal type (i.e., fed vs cull) *(4, 40)*.

The objective of this study was to determine if *Salmonella* prevalence in peripheral bovine LN varies across beef cattle feeding stages and feedlot environments in South Texas. Results from this study will provide additional context for future studies to investigating feedlot management practices and interventions to reduce *Salmonella* prevalence in LNs.

CHAPTER II

LITERATURE REVIEW

***Salmonella enterica*.** Salmonellae are Gram-negative, non-spore forming, rod-shaped microorganisms that are widely prevalent in nature *(44)*. The genus is large and varied. The Centers for Disease Control and Prevention (CDC) recognizes two species: *Salmonella enterica* and *Salmonella bongori (10)*. Most foodborne pathogens fall within the *S. enterica* species, which contains six subspecies referred to by a roman numeral and name: I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salame*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *indica (10, 44)*. *S. bongori* was previously referred to as subspecies V but has since been re-categorized. These subspecies are differentiated based on biochemical properties and genomics *(10)*. A more recent study has identified several primers for use in a multiplex polymerase chain reaction (PCR) which were able to identify all six of the subspecies and *S. bongori (49)*. Furthermore, within subspecies I, the CDC uses names, for example, *Salmonella* ser. Enteritidis and *Salmonella* ser. Typhimurium, to identify serotypes, of which there are more than 2,400 currently identified *(10, 44)*.

Salmonella enterica subsp. *enterica* is an organism of significance to food production industries, as it is the primary cause of bacterial foodborne illness in the United States *(72)*. On average, salmonellae are responsible for 1.2 million illnesses per year from food and meat products, pets, and laboratory accidents, with 375,000 of these

resulting from USDA-FSIS regulated products including meat and poultry *(21)*. *Salmonella* is generally considered a fecal contaminant, as it inhabits the intestinal tracts of animals, although it has also been isolated from other body tissues and environmental samples *(44, 50, 55)*.

In humans, *Salmonella* can cause several clinical conditions: enteric or typhoid fever, uncomplicated enterocolitis, and systemic infections *(54)*. In general, the food production industries are concerned with non-typhoidal *Salmonella*. The infectious dose of *Salmonella* is generally thought to be 10^5 cells, although there have been reports of lower doses causing illness when consumed with protective foods, including water, hamburger, chocolate, and cheddar cheese *(7, 22, 77)*. The incubation period varies between reports, but generally appears to be 6 to 72 h *(22, 44, 54)*. Reported disease symptoms consist of nausea, vomiting, cramping abdominal pain, headache, chills, and diarrhea *(22, 44)*. The disease is generally self-limiting, and resolves in 5 to 7 days *(22)*. An entero-colitic infection also can occur, especially in young children, which is characterized by increased inflammatory response, bloody diarrhea, and an increased duration *(22)*. Chronic conditions caused by *Salmonella* also have been reported, including aseptic reactive arthritis, Reiter's syndrome, and ankylosing spondylitis *(54)*.

The large number of *Salmonella* serotypes allows for host-specificity. For instance, *Salmonella* ser. Typhi, the causative organism of typhoid fever, causes illness only in humans *(43)*, while *Salmonella* ser. Dublin, though occasionally found in other species, is generally specific to cattle *(59)*. Paulin et al. *(59)* compared the degrees of virulence of different *Salmonella* serotypes at various stages of pathogenesis in cattle,

using serotypes that were generally host-specific for several different species. Previous findings suggested the ability of a serotype to cause disease in cattle was not based on invasion, as many serotypes were able to invade intestinal tissue, without resulting in disease symptoms. Further study revealed that the mesenteric LNs played a role in the movement of specific serotypes to systemic tissues and thus caused more severe disease symptoms. The serotypes that did not remain in the LN did not cause the same level of virulence as the serotypes that continued to colonize the LNs up to 10 h after inoculation *(59)*.

The mechanism of *Salmonella* invasion and pathogenesis is well understood. Although the high acidity of the human stomach is generally sufficient to destroy acid-sensitive pathogens, including *Salmonella*, a study by Waterman and Small *(77)* indicated the food product contaminated with bacteria may provide a protective effect to the microorganisms. Researchers found that a *Salmonella* cocktail inoculated onto ground beef and boiled egg whites was able to survive a pH of 2.5, but a *Salmonella* cocktail inoculated onto boiled rice was not *(77)*. This led to the conclusion that the protective effect was possibly due to the fat or protein content of a food product, not the carbohydrate content *(77)*. Once the *Salmonella* survive the stomach, they colonize the intestine and localize to the apical epithelium in order to invade the host cells and elicit an inflammatory response *(22)*.

In 1989, Finlay et al. *(31)* demonstrated that there are several bacterial proteins required for *Salmonella* internalization into host cells, which are induced by the contact between host epithelial cells and bacterial cells. In more recent literature, the induction

of these proteins is referred to as a type III-secretion system, which is encoded by several chromosomal genes including *invA* and *spa*. These genes are located on *Salmonella* pathogenicity island 1 (SPI-1) *(36)*. The *InvA* gene, specifically, is critical to *Salmonella* invasion of host cells. Galán et al *(34)* created *InvA* knockout *Salmonella* that was unable to invade the host cells, but, interestingly, could be induced to invade if cultured with wild-type *Salmonella*. In 1997, Richter-Dahlfors et al. *(64)* were able to demonstrate, using confocal microscopy, that most *S.* Typhimurium cells resided in, and exerted a cytotoxic effect on, macrophages at late stages of infection, which has implications for the ability of *Salmonella* to evade the host immune system.

After attachment and invasion, *Salmonella* is able to secrete pathogenesis factors, or effectors. SPI-1 is critical in *Salmonella* pathogenicity; the effectors encoded by SPI-1 are important in *Salmonella* invasion of the host cell, host inflammatory response, and the disruption of host cellular tight junctions. As previously mentioned, the type III-secretion system is necessary for cell invasion. The system encodes several proteins that promote massive host cytoskeletal restructuring *(22, 36)*. One more recently understood protein, sptP, is translocated into the host epithelial cell and modifies the host actin cytoskeleton through host cell tyrosine phosphatase activity *(36)*. This then induces membrane ruffling that allows for *Salmonella* uptake *(36)*. The intestinal inflammatory response is a reaction by the innate immune system of the host in response to the microbial invasion of the mucosa *(32)*. Polymorphonuclear leukocytes (PMN), also known as neutrophils, are recruited and cross the intestinal epithelia due to SipA, an effector secreted by SPI-1 *(22)*. PMN then selectively release monocyte

chemoattractants, which recruit macrophages, resulting in a secondary inflammation which lasts for several days and causes the 5 to 7 day illness *(32)*. In addition, SPI-1 effector SipB activates caspase-1 mediated proinflammatory cell death. Finally, it has been reported that other effectors secreted by SPI-1, termed SopB, SopE, SopE2, and SipA, are necessary for *Salmonella* to cause diarrhea in the host. These effectors disrupt the tight junctions between epithelial cells *(8)*. SopE possesses guanidine exchange activity, which is responsible for the stimulation of Cdc42, a host signaling factor that is necessary for membrane ruffling *(36)*. Boyle et al. *(8)* indicated that these effectors are able to signal through Rho family GTPases, which modulate the actin cytoskeleton. In addition to the structure, the modulation disrupts the function of the tight junctions and modifies the membrane polarity by altering the host cell calcium levels *(8, 36)*. As a result, more water is recruited into the intestines to balance the polarity, thus initiating diarrhea in the host *(8)*. Curiously, in a study of host-specific *Salmonella* serotype Dublin in cattle, Pullinger et al. *(61)* found that the isolate translocated from the ileum to the draining lymphatics using a cell-free system and type III-secretion system -2, not system -1 as described above, though this publication was the first to describe this system.

The signaling milieu initiated by the contact between bacterium and host cell, which leads to the inflammatory response, is mediated by cytokine signaling *(22)*. Cytokines have various effects. During *Salmonella* infection, interferon -λ, interleukin -12, tumor necrosis factor -α, interleukin -18, transforming growth factor -β, and CCL2 have protective functions for the host *(25)*. Conversely, interleukin -4 and interleukin -10

interfere with the host defenses *(25)*. Study of the role of cytokines in *Salmonella* control has been conducted in mice, where the role of the aforementioned cytokines is understood and reported by Coburn et al. *(22)*. After the recruitment of PMN and, subsequently, macrophages, interferon -λ and tumor necrosis factor -α are generated, although other cells also have been implicated in their production. Interferon -λ is necessary to control bacterial replication early in the infection, though this action is not sufficient for destruction of the microorganism. Tumor necrosis factor -α is, therefore, functional synergistically with interferon -λ to enhance microbial death by producing nitric oxide. Interestingly, the production of interferon -λ is upregulated in lymphatic tissues when gut-associated lymphoid tissue and spleen tissue were infected with *Salmonella* ser. Typhimurium which, though in murine models is more similar to typhoidal *Salmonella* infections in humans, underscores the cytotoxic attributes of these organs *(22, 33)*. The pathological effects of cytokine production also have been evaluated in murine models, though they are less understood. Cytokines interleukin -4 and interleukin -10, as well as the similar chemokines MCP-1, CCL2, CCL20, and CCL3, generate a massive inflammatory response that is substantial enough to cause tissue destruction. Therefore, the induction of this cytokine storm is a crucial part of *Salmonella* pathogenicity.

The nutritional state of the infecting *Salmonella* has been thought to play a role in the ability of the pathogen to infect and cause disease in the host. As reported by Yurist-Doutsch et al. *(81)*, if *Salmonella* Typhimurium cells are deprived of nutrients before and during infection *in vitro*, the ability of *Salmonella* to invade host cells and

trigger human illness is reduced, which would imply that nutritional stress can lower the virulence of the organism. However, once applied *in vivo*, this finding was not repeated. The *Salmonella* burden in the GI tract, host inflammation response, and systemic bacterial transfer were all similar between mice given control *Salmonella* and those given short term nutrient-deprived *Salmonella*. However, there was a difference in post-infection GI microbiome composition between the two models. Researchers used these data to suggest a non-nutrient deprived *Salmonella* inoculation more closely resembles the status of cells causing human infection, and therefore creates a better model for human study. Overall, these results led to the conclusions that *Salmonella* Typhimurium is able to overcome nutritional deprivation to colonize the host and that the metabolic state of *Salmonella* Typhimurium upon entering the GI tract influences the interaction between the pathogen and gut microbiome *(81)*.

Proposed mechanisms for *Salmonella* to enter feedlot. *Salmonella* has been isolated from animals in feedlots: in fecal material, on hides, and in LNs *(1, 2, 13, 37, 39, 40)*. Though many mechanisms have been proposed, it is unclear exactly how *Salmonella* enters the feedlot. It has been suggested that mechanical movement by birds, specifically starlings that roost in feedlots for the winter, is a mechanism for spreading the microorganism *(16-18, 35)*. Gaulker et al. *(35)* evaluated the prevalence and antimicrobial resistance of several microorganisms, including *Escherichia coli*, *Salmonella*, and *Mycobacterium avium* subsp. *paratuberculosis* in wild European starlings in a feedlot in Kansas. Findings were minimal for *Salmonella*, as only 3 samples tested positive. Higher levels of generic *E. coli* were found, but none were

pathogenic strains. This caused researchers to conclude that starlings are not a significant threat for the spread of the pathogenic microorganisms tested, but perhaps they are more of a concern for the spread of avian disease between birds *(35)*. Conversely, results from several studies by Carlson et al. *(16-18)* demonstrated that starlings might be responsible for mechanical movement of *Salmonella* within feedlots. The objective of the first study was to assess the role of starlings in the transmission of *Salmonella* to cattle, feed bunks, and water within feeding operations *(16)*. Researchers conducted an odds-risk analysis, and determined that it is likely that starlings, specifically the presence of higher numbers of starlings at a feeding operation, contribute to *Salmonella* contamination of cattle feed and water. Additionally, though the statistical differences were not able to clearly identify the reason, the number of starlings on the feeding operation was the best explanatory variable for cattle fecal shedding of *Salmonella (16)*. Researchers also evaluated serotypes of the *Salmonella* isolates, and this analysis did not link starling feces to feed bunk contamination. This led to the concept that starlings mechanically transmit contaminated cattle fecal material from cattle pens to other locations, which was supported by the direct observation of cattle feces on the feathers and feet of starlings *(16)*. Other studies conducted by this group elaborate on this hypothesis. One study consisted of testing several different locations within 5 different confinement feeding operation for *Salmonella*, including the starling gastrointestinal (GI) contents, external starling wash, cattle feces, feed, and water samples *(18)*. Researchers could compare antimicrobial susceptibility and serotypes of microorganisms obtained at the aforementioned sampling locations. These data do not prove transmission from the

starlings to cattle, but do suggest that the birds might act as mechanical carriers, especially due to the prevalence of microorganisms isolated from external starling washes (17%). In another publication by Carlson et al. *(17)*, the molecular profiles of *Salmonella* colonies were mapped, and profiles of starling GI, external starling wash, feed and water sources, and cattle fecal samples were compared. Starling GI and external wash samples taken as rinseates were compared with cattle feed and water sources in order to more firmly show an interaction *(17)*. Results showed clades of *Salmonella* indistinguishable between starling GI tracts and cattle fecal samples, which suggests interspecies transmission, although it does not indicate the direction of this transmission. Further, the study showed clades of similar salmonellae between starling GI tracts and feed and water samples, which further proves the concept of starlings acting as carriers to mechanically move *Salmonella* into the feed and water presented in previous studies *(16, 18)*. The rock pigeon is another bird species that has been implicated in carrying *Salmonella* and other pathogens in animal operations. Pedersen et al. *(60)* investigated pigeons at dairies and in urban settings. Several *Salmonella* serotypes associated with dairies and human illness were found in samples collected from pigeons trapped on dairies in Colorado, which indicated that pigeons are potential carriers of *Salmonella* and that pigeons may transmit *Salmonella* to cattle *(60)*.

Another source involved in moving foodborne pathogens is wildlife. Rodents are known to intrude into feeding operations, and a study by Kilonzo et al. *(45)* reported that rodents were viable carriers of foodborne pathogens on farms, on cow/calf operations, and in feedlots. Based on results from this study, there was an increase in *Salmonella*

fecal shedding as rodent density increased. Despite this increase, the numbers were low, implying that rodents may not be a significant reservoir for *Salmonella (45)*.

Lymph nodes. The lymphatic system is an essential component of the mammalian immune system, and consists of three types of tissues *(14)*. The primary tissue composes organs that generate the immune cells, such as bone marrow and thymus. Secondary tissues include the LNs and spleen. Tertiary tissues are transient; the tissues that develop during inflammation but are not always structured *(14)*. LNs are a critical aspect of this system, as they have numerous functions relating to the destruction of pathogenic invaders in the body.

The structure of the lymphatic system is well understood, and has been primarily studied in humans and mice. The structure begins with the branched capillary system that conducts lymph to the LN, and then circulates it back into the blood through the thoracic duct *(63, 74, 79)*. Lymph is a clear, colorless fluid that collects and transports pathogens and other particulate matter to sites such as LNs for destruction *(63, 74, 79)*.

The LN has been defined as a discrete mass of fibro-vascular tissue enclosed within a dilated lymphatic vessel *(79)*. These organs are variable in shape due to the change in size of internal cells and accumulation of lymphocytes during the immune response *(79)*. There are three major structures in the LN, which make up a lobule *(76, 79)*. Each of these structures is associated with specific cells and function. The cortex, or superficial cortex, is the outermost portion associated with B-cells, and is the primary location for immune response. The paracortex is the intermediate structure that is associated with T-cells and is the location for interaction between T-cells and the

dendritic cells that carry the antigens. Finally, the medulla is the internal structure that is most closely related to the lymph vessels where they enter at the hilus. The function of this portion is not well understood, but it is primarily comprised of the sinuses and capillaries that drain the lymph *(76)*. The lobules created by these structures are the functional units of the LN, and are variable in number based on the size of the LN *(79)*.

The lobular structure of the LN is surrounded by a labyrinthine structure of sinuses, vessels, and tissue that conduct the lymph and mediate the transportation of lymphocytes, also known as white blood cells, through the organ *(63, 74, 79)*. Afferent lymph vessels deliver lymph to the LN at the hilus, and the lymph then is moved through the node via sinuses that surround the lobes *(74, 76, 79)*. The movement of lymph in this system has been described as "tree-like," as it moves toward the cortex in a "trunk" and then branches out as it flows through the LN in sinuses. Then, the lymph is filtered back out of the LN and carried away via efferent ducts *(74, 76, 79)*. These sinuses are crossed by a complex system of fibroblastic reticular cells termed the reticular meshwork *(79)*. The meshwork has several functions, but primarily serves as a structure for lymphocyte movement through the LN *(79)*. This meshwork is protected by high endothelial venules, which are endothelial cells displaying receptors for the lymphocytes *(79)*. These venules allow lymphocytes to migrate into the paracortex, and then lose their specialized structure *(79)*. The entire LN is surrounded by a capsule of smooth muscle cells and elastic fibers *(74)*.

The primary function of the LN is to sequester and destroy potentially pathogenic invaders and other debris collected by the lymph. The early immune response to an

invader is conducted by dendritic cells, which collect and transport the antigens to the LN and present the foreign substance to the T-cells to initiate an immune response by actively producing cytokines *(79)*. Lymphocytes within the LN are able to recognize only very specific antigens. B-lymphocytes, which are generated in the bone marrow and associated with the blood, acquire the ability to recognize specific foreign antigens by displaying immunoglobulins on the cell surface *(48)*. If a B-lymphocyte does not contact the specific antigen within a LN, it moves through the efferent vessel to another LN and continues to search *(79)*. Upon contact with the recognizable antigen, the B-cell produces antibodies and marks the foreign substance for destruction by the macrophages within the LN *(48)*. Unfortunately, upon study, some microorganisms also are able to colonize and proliferate in the phagocytic cells to escape destruction *(64)*. Moreover, St. John et al. *(70)* were able to demonstrate that *Salmonella* Typhimurium can alter LN structure, specifically the lymphocytes within the cortex and paracortex regions, in murine models. Further analysis led researchers to conclude that the *Salmonella* gene *msbB*, which is necessary for modification of lipopolysaccharides and enables the bacterium to be recognized by host cell toll-like receptor -4, is responsible for the structural modifications, as mutant *Salmonella* without this gene was unable to cause the same lymphocyte disruption. Therefore, the ability of *Salmonella* Typhimurium to utilize host-cell toll-like receptor signaling allows it to escape the host immune system, target and debilitate the active immune system, and survive within the LN *(70)*.

Researchers have attempted to develop methods of studying LN structure and function through novel technologies. Rasmussen et al. *(62)* utilized near infrared (NIR)

fluorescence in human subjects to visualize lymphatic channels and LNs throughout the body, and found differences in lymphatics between diseased and normal limbs. Other researchers have utilized LN dissection, surgically removing a LN from a living organism and monitoring immune function in the area *(14, 59)*. Novel techniques for research in this area continue to improve the body of knowledge surrounding LN function.

***Salmonella* presence in the bovine lymph node.** The ability of microorganisms to colonize in bovine LNs was first described by Lepovetsky et al. *(50)* in a study comparing the microbiological profiles of LNs, bone marrow, and muscle tissue. Gram-negative microorganisms were isolated from the LNs, but not from bone marrow or muscle, leading to a conclusion that LNs are the primary deep tissue source of spoilage. Researchers used the prescapular (now termed superficial cervical) LN, but other early studies focused on the mesenteric LNs *(50, 55, 66, 67)*. Moo et al. *(55)* provided a semi-quantitative observation of the bacterial flora in cattle jejunal and cecal LNs. Microorganisms were found in 58% of LNs cultured *(55)*. *Salmonella*, the main pathogen isolated, was found in 5% of the samples *(55)*. The LNs contained a wide range of *Salmonella* populations, with most samples harboring 10^2 to 10^5 bacteria per gram of LN and some with greater than 10^5 bacteria per gram *(55)*. Samuel et al. *(66)* investigated the prevalence of *Salmonella* at different sites along the GI tract and the associated LNs in both cattle and sheep. Of the 100 cattle surveyed, salmonellae were isolated from 77; salmonellae were found in the GI tracts of 72 cattle and LNs of 61. Researchers also compared sites within the GI tract and found that the cecum and rectum

were the most common points of contamination within the tract *(66)*. In a comparison of LNs, the jejunal and cecal LNs were the most frequent sources of *Salmonella (66)*. Arthur et al. *(4)* described the presence of *Salmonella* in peripheral LNs, which are of concern to the meat industry due to location within the fatty tissues that may be incorporated into beef trim and ground beef products. The objective of the study was to determine the prevalence and drug resistance status of *Salmonella* in LNs potentially destined for ground beef, and the group also compared the LN *Salmonella* prevalence between cull and feedlot cattle *(4)*. *Salmonella* was found in peripheral LNs at a prevalence of 1.6% of nodes overall, with a greater prevalence in cull cattle (2.46%) than feedlot cattle (0.7%). The prevalence of *Salmonella* was higher in flank (subiliac) than chuck (superficial cervical) LNs, with the highest prevalence in cull cattle flank LNs (3.86%) and the lowest in feedlot cattle chuck LNs (0.61%). Only a single LN in this study returned countable plates, and so only one enumeration was reported (5.8 CFU/g) *(4)*. Investigations of *Salmonella* in peripheral LNs, specifically, continue, as this presents a concern to the meat industry due to the possibility that the ability of the LN to protect *Salmonella* from antimicrobial treatments provides a source for contamination of beef trim and ground beef. Koohmaraie et al. *(46)* presented this hypothesis after a study comparing the pulsed-field gel electrophoresis profiles of *Salmonella* from several sources following beef harvest, including the plant, hides, carcass, trimmings, LNs, and ground beef. Results allowed researchers to conclude that the primary sources of *Salmonella* in beef trim are the hide and LNs. The *Salmonella* prevalence on hides was 96%, and prevalence dropped to 47% after hide removal. After intervention, though, the

Salmonella prevalence was 0%, yet the *Salmonella* prevalence in trim was 7.14%. Based on pattern mapping, researchers showed that, though not all of the *Salmonella* in the trim was similar to LN *Salmonella*, LNs were still a likely source of beef trim contamination *(46)*.

Following the publication of these studies, there was sufficient evidence to justify further investigation into the prevalence and microbial loads associated with *Salmonella* in LNs *(4, 50, 55, 66)*. In the past decade, the knowledge base has grown drastically. The prevalence of *Salmonella* in LNs of cattle in commercial feedlots and dairies has varied *(11, 13, 40)*. In some studies, there were no salmonellae found in peripheral LNs at a given feedlot, and, for *Salmonella* isolated from LNs, the prevalence ranged from 0.8% to 88.2% *(11, 40)*. A study by Brown et al. *(13)* reported prevalence of up to 100%, when a single breed type, *Bos indicus*, was analyzed. The enumerations of *Salmonella* reported in literature vary substantially as well. Moo et al. *(55)* reported bacterial loads above 10^5 bacteria/g LN, but *Salmonella* was not specifically isolated. Studies of *Salmonella* have reported counts of greater than 100 bacteria/g, 5.8 CFU/g, 1.4 $\log_{10}$ CFU/g, 3.0 CFU/g, and one study showed a range of 1.0 to >3.8 log CFU/g *(4, 13, 38, 66)*. These differences have led to new hypotheses evaluating breed type, seasonal, and environmental effects as possible contributors to differences in *Salmonella* prevalence.

In a study by Arthur et al. *(4)*, researchers compared cull cows to feedlot steers and determined the prevalence of *Salmonella* in LNs was significantly higher in cull cattle. The prevalence of *Salmonella* overall was only 1.60%, but in cull cattle, the

prevalence was 2.46%, compared to 0.70% in feedlot cattle. Conversely, Gragg et al. *(38)* identified a higher prevalence in feedlot cattle than cull cattle, 14.7% compared to 1.8% respectively, in a study with an overall prevalence of 7.5%. Brown et al. *(13, 38)* hypothesized that breed difference was the cause of the significant difference in *Salmonella* presence between fed and cull cattle. Researchers compared beef and dairy cattle, with *Salmonella* prevalences in LNs of 59.7% and 62.1%, respectively, which were not significantly different *(13, 38)*. Further, the numerical difference in prevalence conflicted with findings by Gragg et al. *(38)*. The group suggested that perhaps the early exposure of dairy animals to *Salmonella* allowed for lower prevalence later in life. This idea is supported by findings reported by Rodriguez-Rivera et al. *(65)*, in which researchers determined that environmental *Salmonella* is ubiquitous on dairy farms, with the highest likelihood of *Salmonella* occurrence in maternity pens. Brown et al. *(13)* also compared *Bos indicus* and *Bos taurus* breed types. No significant difference was seen between the two types, with a prevalence within *Bos taurus* of 97% and within *Bos indicus* of 100% *(13)*.

The season in which cattle are harvested also has been identified as a factor affecting *Salmonella* within bovine LNs. Li et al. *(51)* developed a stochastic simulation model to assess the contribution of LNs to *Salmonella* contamination of ground beef, and, as a result, determined seasonal effects that alter *Salmonella* presence. In an experiment by Gragg et al. *(37)*, seasonal differences were seen after evaluating the *Salmonella* burden in LNs at harvest over the year and a half long experimental period. *Salmonella* prevalence in LNs of cattle slaughtered in the first fall was 8.8%, in the

winter/spring was 1.3%, and in the following summer/fall was 12% *(37)*. In a study by Brown et al. *(13)*, the highest prevalence was seen in June and the lowest was in May. In a study measuring the seasonal prevalence levels of several microorganisms, including *Salmonella*, Barckocy-Gallagher et al. *(6)* measured the prevalence and levels of the microorganisms both pre- and post-harvest. A seasonal difference was apparent in *Salmonella* prevalence, though LNs were not specifically included in the study. The highest prevalence occurred during the summer, especially on hides, which were 97.7% positive, and the lowest prevalence occurred during the winter/spring months *(6)*. Conversely, in a similar study, Kunze et al. *(47)* did not see a seasonal effect, but admitted that the year of the study was not representative, as the Spring was unusually dry and the summer was unusually wet. Therefore, it would still be reasonable to conclude that season has an impact on *Salmonella* prevalence within the LN, with the summer months yielding the highest prevalence.

Finally, different environments have been shown to affect the carriage and shedding of *Salmonella*. Nesemeier et al. *(56)* showed that fecal shedding was highest immediately post-weaning, with the highest prevalence occurring on pasture. Haneklaus et al. *(40)* investigated whether the prevalence varied between feedlots across the state of Texas. Prevalence of *Salmonella* within LNs at feedlots varied significantly: 0%, 4%, 24%, 40%, 40%, 42.9%, and 88%. Rodriguez-Rivera at al. *(65)* suggested that *Salmonella* is ubiquitous on dairy farms, with higher environmental prevalence in maternity pens and the lowest in cow and calf housing. Though it is still not clear how the environment impacts *Salmonella* presence, the difference in prevalence between

environments makes it clear that environments/housing types may play an important role in *Salmonella* prevalence in LNs.

Remarkably, the presence of *Salmonella* within LNs appears to be transient. In a recent study by Edrington et al. *(28)*, cattle were inoculated with specific strains of *Salmonella* and slaughtered at intervals beginning at 6 h up to 21 d post-inoculation to determine the point at which *Salmonella* is drained from the LN. Generally, *Salmonella* was culturable from LN by 24 h, and continued to be culturable until 14 d, with a peak between 7 and 9 d. Only 50% of LN were positive for *Salmonella* by 21 d. In a second experiment, the timeline was extended and cattle were slaughtered up to 28 d. In this experiment, *Salmonella* levels were low through 20 d, but increased again at the 24 and 28 d slaughter points. Moreover, new serotypes were found on these days. These data led researchers to hypothesize that this increase was due to the acquisition of new *Salmonella* strains as opposed to the continued harboring of the inoculum *(28)*. Overall, researchers concluded that the *Salmonella* is drained from LNs approximately 24 to 28 d post-inoculation. However, this study reported areas of uncertainty, and so the concept requires further investigation.

Proposed mechanisms for *Salmonella* to be sequestered in lymph nodes. There are three major hypotheses proposed to explain the mechanism by which *Salmonella* becomes sequestered in the LNs: (1) oral route in which *Salmonella* is consumed, either in feed or drinking water, (2) inhalation route, and (3) transdermal route in which *Salmonella* is introduced through fly bites or skin abrasions.

Early investigations into LN contamination were based on the oral transmission hypothesis. Mesenteric LNs were sampled based on the suggestion that *Salmonella* and other microorganisms would drain from the intestinal tract and colonize the associated LNs *(55, 66, 67)*. Pullinger et al. *(61)* demonstrated that *Salmonella* translocates from the distal ileum through lymphatics, using the type III secretion system to force phagocytosis and induce enteritis, in addition to passive uptake. Brown et al. *(12)* extended this concept to peripheral LNs in an experiment by inoculating cattle with *Salmonella* via drinking water with for ten days, and compared this to a positive control that consisted of a very high single dose (10^7) of *Salmonella* in drinking water and a negative control of no fed *Salmonella*. The negative control group resulted in a 12.5% prevalence, which was the same as the water-inoculated treatment group, suggesting that the dose was not sufficient to cause peripheral LN uptake. The positive control group resulted in a prevalence of 62.5% *(12)*. This implies that it is possible to induce peripheral LN carriage with an oral dose, but it appears to require a higher dose than has been found to occur normally in feedlot environments *(12)*. Even in an early study of *Salmonella* in LNs, Samuel et al. *(67)* suggested that microorganisms were unable to spread beyond the infected nodes. Researchers were unable to isolate *Salmonella* from tissues where isolation would be expected (spleen) if the mesenteric nodes did drain the *Salmonella (67)*. Further evidence against the oral hypothesis was published by Gragg et al. *(38)* in a study evaluating the diversity of *Salmonella* isolates recovered from LN, feces, and hide swabs at slaughter. Different serotypes and genetic origins of *Salmonella* were isolated from different parts of beef carcasses; strains also varied between different

LNs *(12)*. If *Salmonella* entered through the GI tract and migrated to the peripheral LNs then one would expect more homogeneity between *Salmonella* spp in each LN. Researchers suggested the introduction of *Salmonella* is more regional within the animal, and proposed a transdermal route. It also has recently been suggested that oral transmission might be possible as *Salmonella* move through the liver. A study by Amachewadi and Nagaraja *(3)* indicated serotypes of *Salmonella* found in liver abscesses matched those described as being found in LNs. The proposed mechanism is that the *Salmonella* in the gut crosses the epithelial barrier, enters portal circulation, and is trapped in the portal capillary system to initiate infection. Then efferent drainage may spread these microorganisms to peripheral LNs *(3)*. However, more investigation must be done on this topic to conclude that this mechanism is possible.

An inhalation route also has been suggested for *Salmonella* transmission in cattle and other species. Unfortunately, there are little data relating this route of transmission to peripheral LNs. It has been demonstrated that *Salmonella* can survive in an aerosol in animal production facilities for long periods of time *(29, 41, 78)*. Wathes et al. *(78)* described viable *Salmonella* Typhimurium in air samples 90 minutes after inoculation, though viability had been reduced to 1%. McDermid and Lever *(52)* saw greater survival, and in 2 serotypes of *Salmonella* (*S.* Typhimurium and *S.* Enteritidis) both were able to survive for over 120 m, *S.* Typhimurium without any loss of viability and *S.* Enteritidis with a 30% loss of viability. Researchers in this case were able to conclude that the survival ability of *Salmonella* was sufficient to cause enteric disease in chickens, though they did not compare conditions to larger animals. Conversely, Okraszewska-

Lasica et al. *(57)* found very low pathogen counts when evaluating *Salmonella* and *Listeria* populations in commercial beef and pork slaughter facilities, leading to a conclusion that an airborne route is unlikely to be important in carcass contamination. However, it could be that the aerosol *Salmonella* is of more importance in the live animal than carcass, which could account for the varied results. There has been some description of aerosol *Salmonella* entering LN. Wathes et al. *(78)* describe finding *Salmonella* in pulmonary LNs of calves, and Fedorka-Cray et al. *(30)* describe *Salmonella* in the ileocolic lymph nodes. However, there are no data establishing the ability of *Salmonella* in thoracic and mesenteric LN to migrate to peripheral LN, so the airborne hypothesis may not be the most significant route for *Salmonella* presence in peripheral LN.

Gragg et al. *(38)* suggested a transdermal route of infection based on the substantial within-animal diversity of *Salmonella*. The diversity implies a within-animal regional distribution of *Salmonella* infection that could be achieved through a transdermal route. Edrington et al. *(27)* developed this hypothesis by creating a *Salmonella* challenge model using 3 studies. In the first, researchers inoculated 10^8 CFU/ml of *Salmonella* with syringes into the legs of a steer, using different serotypes for each leg. Then, the steer was slaughtered and peripheral LNs were evaluated for *Salmonella*. Most peripheral LNs contained the expected strain (the serotype inoculated into the leg associated LN), except the subiliac LNs were negative *(27)*. A second study used similar methods, but using allergy testing lancets for intradermal instead of transdermal inoculation. *Salmonella* of expected serotypes were isolated from LN

associated with the left side, but not the right, and all concentrations were below the limit of detection *(27)*. In the third study, 2 steers were inoculated using the allergy testing lancets with only 1 serotype, but at different times relative to slaughter. Results were similar to the second study *(27)*. Researchers concluded that a transdermal route of inoculation is predictable and can be used as a challenge model. Moreover, they concluded that a portion of *Salmonella* observed in peripheral LNs crosses the integument transdermally *(27)*. It is possible, then, that *Salmonella* is introduced transdermally, potentially through biting flies or other insects *(53, 58)*. Olafson et al. *(58)* evaluated the ability of biting flies to transmit *Salmonella*. Results showed flies could collect *Salmonella* from the hide of cattle and carry the pathogen for at least 5 d. Moreover, when *Salmonella*-contaminated flies fed on cattle there was a greater prevalence of *Salmonella*-positive peripheral LNs than control cattle inoculated with lancets as described above. The prevalence for *Salmonella* in the peripheral LNs was 8%, 50%, and 42% for cattle exposed to flies for 5 d, 11 d, and 19 d, respectively *(58)*. These results led to the conclusion that fly bites are a valid transmission mechanism for *Salmonella* entry into the peripheral LN.

***Salmonella* mitigation and prevention in live cattle.** There are many tools used at feedlots in an effort to reduce the spread and carriage of diseases amongst the live animal population. Many of these have been investigated as potential methods for reducing *Salmonella* in the GI tract and on hides of cattle. First, it is important to understand environmental factors that impact the shedding of *Salmonella*, as this is a potential route of transmission between animals, as well as a source of hide

contamination, which then can contaminate the carcass during dressing. Green et al. *(39)* evaluated 55 variables that might impact fecal shedding. Of these, they were able to identify several that increased the likelihood of shedding, including pens with cattle from more than one point of origin, season, the inclusion of cottonseed hulls in rations, and feeding corn gluten and brewer's grains *(39)*. Cottonseed hulls have been implicated as a source of *Salmonella*, and have tested positive for the microorganism in other studies. Corn gluten and brewer's grains, on the other hand, affect shedding by creating a ruminal environment more suitable for the survival of *Salmonella (39)*. Managing these variables may reduce the bacterial loads and fecal shedding of these organisms. In one study, Edrington et al. *(26)* evaluated the usage of sprinklers on a dairy farm to alleviate heat stress and therefore reduce the fecal shedding of *Salmonella* and *E. coli* O157:H7. Researchers compared both sprinklers at feeding bunks and holding pens to controls. While there were no differences between feeding bunk sprinkler and control groups, there was a difference in *Salmonella* enumeration, assessed by direct plating, between holding pen sprinkler (1.4%) and control (7.7%) groups *(26)*. Therefore, researchers suggested that implementing sprinklers in holding pens may reduce *Salmonella* prevalence. Two potential reasons for the decreased prevalence were discussed. Perhaps the sprinkler use decreased heat stress in the cattle, thus reducing the amount of energy used in temperature regulation and allowing more energy to be used to resist *Salmonella* colonization. Alternatively, the sprinklers may remove fecal contamination from cattle hides and therefore reduce the cross-contamination with other animals *(26)*.

In addition to general management practices, there have been several proposed treatments to reduce, if not eliminate, *Salmonella* in feedlot cattle. One current area of research is in direct fed microbials (DFMs), a form of probiotic feeding that has traditionally been used for performance enhancement, but has more recently been adopted to reduce pathogenic bacteria *(15)*. The method has been utilized for *E. coli* O157:H7 reduction, so more recent studies have applied the technique to reduce *Salmonella*, both in fecal shedding and LNs *(15, 71, 75)*. Stephens et al. *(71)* evaluated levels of *Salmonella* in feces and on hides when cattle were treated with three different doses of DFMs, and determined that *Salmonella* was less likely to be found in feces with DFM feeding, but there was no significant difference between treatments and control in hides. Additionally, Stephens et al. *(71)* showed that, although the required dose was higher than that used to control *E. coli*, DFMs are potentially useful for the reduction of fecal *Salmonella.* This was investigated further by Vipham et al. *(75)* in a two-part study that investigated the effects of DFM on *Salmonella* prevalence in LNs. In a commercial feedlot, researchers found a prevalence of 57.5% in cattle treated with a dose of 10^9 CFU/hd/day, which is significantly lower than the control group, at 76.3%. In a second study, in a controlled research facility, results showed 25.9% prevalence in the control group and 4.7% in the treatment group, which indicated that *Salmonella* is 82% less likely to be isolated from a treated LN *(75)*.

Salmonella vaccines also have been developed, and researchers have evaluated their ability to reduce fecal shedding of *Salmonella* among the tested cattle populations. However, many suggest that vaccination is not an effective measure to reduce

Salmonella in cattle operations. Heider et al. *(42)* utilized a commercial subunit vaccine that targets siderophores in dairy cows. Researchers chose two dairy farms with a history of subclinical *Salmonella* fecal shedding and administered a vaccination to a proportion of the population. At the beginning of the experiment, the prevalence of fecal *Salmonella* was 30%, and researchers expected this to be reduced to 0% amongst the vaccinated populations. However, 8.6% of the vaccinated cattle tested positive for *Salmonella* throughout the experiment, compared to 6.5% of the control cattle. Therefore, researchers concluded that this was not an effective method for reducing subclinical infection of dairy cows *(42)*. Dodd et al. *(24)* conducted a similar experiment in feedlot cattle using a *Salmonella* Newport siderophores receptor and porin protein vaccine. Again, there were no differences between vaccinated and control cattle. Researchers suggested three possible explanations for this: (1) a lack of efficacy of the vaccine, (2) not enough environmental *Salmonella* to cause infection in the control group to create a difference, or (3) the vaccine caused herd immunity, protecting control cattle from *Salmonella* exposure *(24)*.

Antimicrobial interventions in beef carcasses. Despite efforts to prevent *Salmonella* infection in live cattle, there are still carrier animals brought to harvest plants that have the potential to contaminate meat products. Numerous interventions are currently being used throughout the beef industry to reduce the presence of pathogens on carcass surfaces. Water washes are used antemortem and in carcass spot cleaning, in addition to spray rinsing and steam vacuuming of carcasses and shanks *(69)*. The goal of water rinses is to remove contaminants from the surface, though there are concerns that

if the water is not sufficiently hot (90°C), or the pressure is too high, allowing pathogens to be spread onto a greater area of the carcass instead of being removed *(23)*. As a result, these carcass washes are generally followed by chemical sprays, either acid or non-acid solutions. Acid sprays are typically organic acid solutions, most frequently lactic acid, but can also consist of acetic acid or citric acid at 1.5 to 2.5% *(69)*. The approved non-acid sprays can contain chlorine, chlorine dioxide, sodium tripolyphosphate solution, or cetylpyridinium chloride *(69)*.

In 2008, Arthur et al. *(5)* evaluated sufficiency of antimicrobial interventions used in beef processing plants for pathogen (*Salmonella* and *E. coli*) reduction, specifically antimicrobial resistant microorganisms. Findings showed both antimicrobial resistant and non-resistant *Salmonella* strains were equally susceptible to all acid treatments (acetic acid, lactic acid, and FreshFX commercial solution), but some strains were more susceptible than *E. coli* O157:H7. Interestingly, when non-acid treatments were applied, the antimicrobial resistant *Salmonella* were more susceptible than non-resistant *Salmonella* and *E. coli*, although reduction was still seen in all strains *(5)*. This led researchers to conclude that interventions currently in place at beef processing plants are equally effective at reducing the foodborne pathogen loads of beef carcasses between antimicrobial resistant and susceptible bacteria. Schmidt et al. *(68)* reached similar conclusions in a study designed to determine the prevalence and concentrations of several bacterial strains including *Salmonella*. In this study, hides showed the highest prevalence of *Salmonella* when measured both in the feedlot (26.1%) and in the processing facility (99.5%). After hide removal, there was a 2.2% *Salmonella* prevalence

in pre-evisceration carcasses, but a 0% *Salmonella* prevalence on the final carcass *(68)*. Again, researchers concluded that the post-harvest interventions employed in beef processing facilities are effective. Overall, it is reasonable to expect that the antimicrobial interventions currently in place at beef packing and dressing facilities are sufficient to reduce microorganisms on carcass surfaces.

CHAPTER III

MATERIALS AND METHODS

Treatment design. Eighty Angus-sired beef steers that were either 0%, 12.5%, or 25% *Bos indicus* influenced and of similar age were selected from the existing cattle herd maintained at the Texas A&M University McGregor Research Center (McGregor, TX). Calves were raised at this facility until the time of weaning. Twenty steers were then transported to the Texas A&M University Rosenthal Meat Science and Technology Center (RMSTC; College Station, TX) for harvest using methods pre-approved by the Texas A&M University Institutional Animal Care and Use Committee (Animal Use Protocol #2015-0241). This initial harvest was completed in early October. The remaining 60 steers were divided equally between two South Texas feedlots with historically different levels of *Salmonella* prevalence within LNs *(40)*. Upon arrival, these steers were processed using typical practices unique to each feedlot and placed into their respective backgrounding/stocker programs (approximately 30 d in a preconditioning pen followed by 120 d on pasture). At the conclusion of the backgrounding phase, in early March, 10 steers from each feedlot were transported to the RMSTC for harvest, whereas the remaining steers entered feedlot pens. Following 60 d of feeding diets typical to each feedlot, in early May, 10 steers from each feedlot were selected and transported to a commercial beef harvest and processing facility in South Texas for harvest. The remaining 10 steers from each feedlot completed the designated 120 d on feed and were transported to the commercial processing facility for harvest in

early July. Live animal and carcass weights were recorded for each steer at the time of harvest. Four steers did not complete the study.

Lymph node collection and processing. At the completion of each feeding stage [(1) weaning, (2) background/stocker, (3) 60 d on feed, (4) 120 d on feed], left and right superficial cervical and subiliac lymph nodes (n = 304 LN) were removed from warm carcasses immediately after harvest. Within animal, left and right LNs for each type were pooled (n = 152 total samples). A single LN was procured from the head of one steer carcass to be inoculated in the laboratory for use as a positive control sample.

Pooled LNs were placed into sterile sample bags (VWR, Radnor, PA), and transported, in insulated containers with refrigerant material, to the Meat Science Laboratory at Texas A&M University (College Station, TX) for processing. All LNs were trimmed aseptically of fat cover using flame-sterilized forceps and a flame-sterilized scalpel. Denuded LNs were drenched with ethanol and flame-sterilized to remove any surface contamination, weighed, placed into a sterile filter bags (Whirl-pack, Nasco, Sandy Springs, GA), and pulverized using a rubber mallet. Pulverized LNs were stored in refrigerated conditions (approximately 4°C) overnight until microbiological analyses could be performed.

***Salmonella* isolation and confirmation.** Pulverized LNs were transported to the Texas A&M University Food Microbiology Laboratory (College Station, TX). *Salmonella* were enriched and isolated using the protocol described in the USDA-FSIS Microbiology Laboratory Guidebook (MLG) #4.08 for Raw Meat and Raw Beef Mixed products *(73)*. LNs underwent pre-enrichment in modified Tryptone Soya Broth (mTSB;

Fisher Scientific, Waltham, MA) added at a 1:4 ratio by mass under a sterile biological safety hood. The pre-enriched samples were hand-massaged for 60 s before incubation. After the addition of mTSB, 1 mL *Salmonella* LT2 was added to the positive control LN. The pre-enrichments were incubated at 42 ± 1°C for 15 to 24 h. Following pre-enrichment incubation, all samples underwent enrichment in two selective broth media. 0.5 ± 0.05 mL of each pre-enrichment was added to 10 mL Tetrathionate Broth Base, Hajna (Fisher Scientific) and 0.1 ± 0.02 mL of each pre-enrichment was added to modified Rappaport Vassiliadis Broth (Sigma-Aldrich, Saint Louis, MO) under a sterile biological safety hood. The enrichments were incubated at 45°C for 22 to 24 h. Following incubation, each enrichment then was streaked for selective plating onto Xylose-Lysine-Tergitol 4 (XLT-4; Sigma-Aldrich) and Brilliant Green Sulfa agars (BGS; Fisher Scientific). Streaking was done under a sterile biological safety hood using disposable 10 μL loops (VWR). Plates were inverted and incubated at 35 ± 2°C for 18 to 24 h.

If present, 3 to 4 colonies per LN of typical *Salmonella* morphology were picked from the selective agar plates for further analysis. A colony was considered positive based on the description of typical appearance described in MLG #4.08 *(73)*. Typical colonies on XLT-4 agar appeared black or red, with or without black centers. Typical colonies on BGS agar appeared pink and opaque surrounded by a red color in the medium. Each selected colony was inoculated into two differential slants, one containing Triple Sugar Iron (TSI; Sigma-Aldrich) and another containing Lysine Iron Agar (LIA; Sigma-Aldrich) with a single pick from the positive colony by stabbing the butts and

streaking the slants. Slants were incubated at 35 ± 2°C for 22 to 26 h. After incubation, slants were observed for evidence of *Salmonella* growth. Slants were considered positive based on descriptions from MLG #4.08 *(73)*. A positive TSI slant would contain a yellow butt with a red slant, with or without blackening. A positive LIA slant would contain a purple butt, with or without blackening. If a colony yielded positive results on both slants, the colony then was considered a presumptive positive. Presumptive positive colonies were picked from the original XLT-4 or BGS plate and streaked onto a nutrient agar slant (Fisher Scientific) in preparation for confirmation testing. Nutrient slants were incubated at 35 ± 2°C for 22 to 26 h and, following incubation, were stored (approximately 4°C) for no longer than 2 weeks before confirmation testing.

The nutrient slants were transported to the Quantitative and Functional Genomics Laboratory at Texas A&M University (College Station, TX) for genetic testing to confirm presumptive positive isolates as *Salmonella*. DNA was isolated from each colony using an UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) per manufacturer instructions. Isolated DNA was frozen at -40°C and stored until PCR detection could be conducted. PCR was conducted using methods and primer sequences described by Brandt et al. *(9)* for the *InvA* gene. Primer sequences were: 5' – GAATCCTCAGTTTTTCAACGTTTC – 3'(forward) and 5'– AGCCGTAACAACCAATACAAATG – 3' (reverse). The PCR conditions used were Initial: 94°C for 120 s, 35 Cycles: 94°C for 30 s; 60°C for 30 s; 72°C for 30 s, Final: 72°C for 420 s, Hold: 4°C. PCR products were evaluated on a 1% agarose gel stained with BioRed (Phenix Research, Candler, NC) and visualized under UV light. A LN

sample was considered positive if at least one colony was confirmed to be *Salmonella* positive by PCR.

***Salmonella* serotyping.** A single representative colony was selected from each positive LN sample, streaked onto a nutrient agar slant, and incubated for 22 to 26 h at 35 ± 2°C. Following incubation. Slants were packaged for shipping following Texas A&M University Environmental Health and Safety Department personnel instruction. The slants containing the *Salmonella* isolates were transported to the USDA – Animal and Plant Health and Inspection Service National Veterinary Services Laboratory (NVSL; Ames, IA) for serotyping.

Statistical analysis. Data were analyzed using JMP Pro software v12.0 (SAS Institute, Inc., Cary, NC). For live and carcass weight data, least squares means were calculated, and, where appropriate, means were separated using an $\alpha = 0.05$. For *Salmonella* prevalence data, contingency tables were produced for each feeding stage and within-table differences were determined using Fisher's exact test.

CHAPTER IV

RESULTS AND DISCUSSION

Mean live steer and carcass weights (kg) are presented in Table 1. At the conclusion of feeding stages 2 and 3, live weights for Feedlot B steers and their associated carcass weights were heavier ($P < 0.05$) than those from Feedlot A. Weights did not differ ($P > 0.05$) between feedlots at the completion of feeding stage 4.

TABLE 1. *Least squares means ± SE for live and carcass weights (kg) by location for each feeding stage*[a]

	n	*Mean live weight (kg)*	*Mean carcass weight (kg)*
Stage 1 (Weaning)			
McGregor	20	216.5 ± 7.5	117.8 ± 5.0
Stage 2 (Stocker)			
Feedlot A	10	250.2 B ± 10.1	147.1 B ± 6.7
Feedlot B	9	385.7 A ± 10.6	235.3 A ± 7.0
Stage 3 (60 d on feed)			
Feedlot A	9	386.9 B ± 20.8	213.7 B ± 12.5
Feedlot B	9	505.8 A ± 20.8	292.9 A ± 12.5
Stage 4 (120 d on feed)			
Feedlot A	10	526.8 ± 16.0	299.0 ± 9.2
Feedlot B	9	529.5 ± 16.8	301.6 ± 9.7

A, B: Values within a column and feeding stage lacking a common letter differ ($P < 0.05$).
[a] At the conclusion of each feeding stage, steers from each location were harvested and left and right superficial cervical and subiliac LNs ($n = 304$ LNs) were removed. Within animal, left and right LNs of each type were pooled ($n = 152$ total samples).

TABLE 2. *Prevalence of* Salmonella*-positive peripheral lymph nodes (LNs)*[a] *by location for each feeding stage*[b]

Location	Stage 1	Stage 2	Stage 3	Stage 4
McGregor	00.0 (0/40) (n = 20 steers)	--	--	--
Feedlot A	--	00.0 (0/20) A (n = 10 steers)	00.0 (0/18) A (n = 9 steers)	00.0 (0/20) A (n = 10 steers)
Feedlot B	--	22.2 (4/18) B (n = 9 steers)	77.8 (14/18) C (n = 9 steers)	94.4 (17/18) C (n = 9 steers)

A, B, C: Values lacking a common letter differ ($P < 0.05$).

[a] At the conclusion of each feeding stage, steers from each location were harvested and left and right superficial cervical and subiliac LNs (n = 304 LNs) were removed. Within animal, left and right LNs of each type were pooled (n = 152 total samples).

[b] Feeding stages were identified as (1) weaning, (2) background/stocker, (3) 60 d on feed, (4) 120 d on feed.

Salmonella prevalence data across the four feeding stages and three feeding locations are presented in Table 2. There was no *Salmonella* detected in any LNs from stage 1 cattle (McGregor) or from Feedlot A at any stage of feeding. The feedlot data are similar to those reported by Haneklaus et al. *(40)*, in which a prevalence of 0% was found at the same location. Within Feedlot B, the *Salmonella* prevalence in LNs of cattle from feeding stage 2 (22.2%) was lower ($P < 0.05$) than later stages of feeding, however, no difference ($P > 0.05$) was found between feeding stages 3 (77.8%) and 4 (94.4%). These data further underscore the environmental differences in *Salmonella* prevalence between feeding locations. As the cattle moved into different feeding locations (i.e. from grass to feedlot pens) the *Salmonella* prevalence within LNs increased, but there was no increase between the two harvest periods when cattle were kept at the same location (feeding stages 3 and 4). There were no differences ($P < 0.05$) in prevalence between types of LN (subiliac vs superficial cervical) at any feeding stage or location (data not shown).

The *Salmonella* prevalence in LNs reported by Haneklaus et al. *(40)*, at the same facility that is labeled Feedlot B in the current study, was 42.9%. LNs were collected from cattle at finishing, which is equivalent to feeding stage 4 of the current study (94.4%). The substantially higher prevalence found in the current study provides opportunity for future research to evaluate changes in feedlot environmental conditions or management practices that led to these differences.

Although there was no significant difference within Feedlot B in *Salmonella* prevalence between feeding stages 3 and 4, there was a numerical difference. The main

influence, therefore, of this increased number was due to more LNs within a single steer becoming contaminated by *Salmonella*, rather than more cattle becoming infected. Edrington et al. *(27)* demonstrated that a transdermal inoculation of a specific serotype of *Salmonella* would result in an uptake of that serotype by the peripheral LN in the region of the body near the site of inoculation. This implies that there is specificity between region of *Salmonella* introduction and the colonized LN. When applied to the present study, this implies that a longer period of time in the feedlot environment simply provides greater opportunity for transdermal *Salmonella* introduction. Inoculation events on different portions of the body would explain an increase in the number of LN containing *Salmonella* while the number of steers containing at least one positive LN remained the same. This concept is further justified in a second study by Edrington et al. *(28)* in which cattle were transdermally inoculated with specific serotypes of *Salmonella* and harvested at intervals following inoculation to determine when the *Salmonella* serotype was no longer in the LN. Researchers found that a single inoculation event would likely be completely cleared after approximately 28 d, though researchers suggested that introduction of new *Salmonella* could cause an increase in levels of *Salmonella* within LN despite a decrease in the level of inoculated strain *(28)*. Therefore, it is not likely that *Salmonella* would continue to dwell in the LN from feeding stage 3 until feeding stage 4; it is more probable that there are multiple inoculation events throughout feeding that caused an increase in prevalence.

Thirty-five unique colonies representing all positive LN samples were submitted to NVSL for serotyping. Results are summarized in Figure 1. Of isolates with single,

identifiable serotypes, *S.* Montevideo was the most prevalent (20%), followed by *S.* Mbandaka (17%), *S.* Anatum (11%), *S.* Muenchen (9%), and *S.* Infantis (3%). The serotypes isolated during this experiment are generally in agreement with previous studies. *Salmonella* ser. Montevideo and Anatum have both been reported in LNs *(13, 38, 67)*. Infantis and Muenchen also have been described at a lower rate of prevalence as compared to the previously mentioned serotypes *(37)*. Serotypes that were not isolated in the present study have been reported, including *Salmonella* ser. Meleagridis, Reading, and Thompson *(37, 38)*. Based on results from available literature, *S.* Mbandaka has not been reported in feedlot cattle, though it has been recovered from cull cows *(38)*. It is important to note that serotype results from the present study are not a complete representation of the *Salmonella* load in a given LN, as only a single colony was selected to represent a pooled LN sample. In a study of *Salmonella* in feedlots of South Texas, *S.* Montevideo, *S.* Anatum, and *S.* Muenchen were isolated from soil samples, *S.* Montevideo and *S.* Anatum were isolated from feces, and *S.* Anatum were isolated from feed, which implies that the environment is a reasonable source for the *Salmonella* that were found in LNs *(80)*. Many (40%) of the isolates exhibited characteristics of multiple serotypes and could not be conclusively serotyped. This may have occurred from researchers picking isolated colonies that consisted of clumps of *Salmonella* bearing multiple serotypes with similar phenotypic appearances on media.

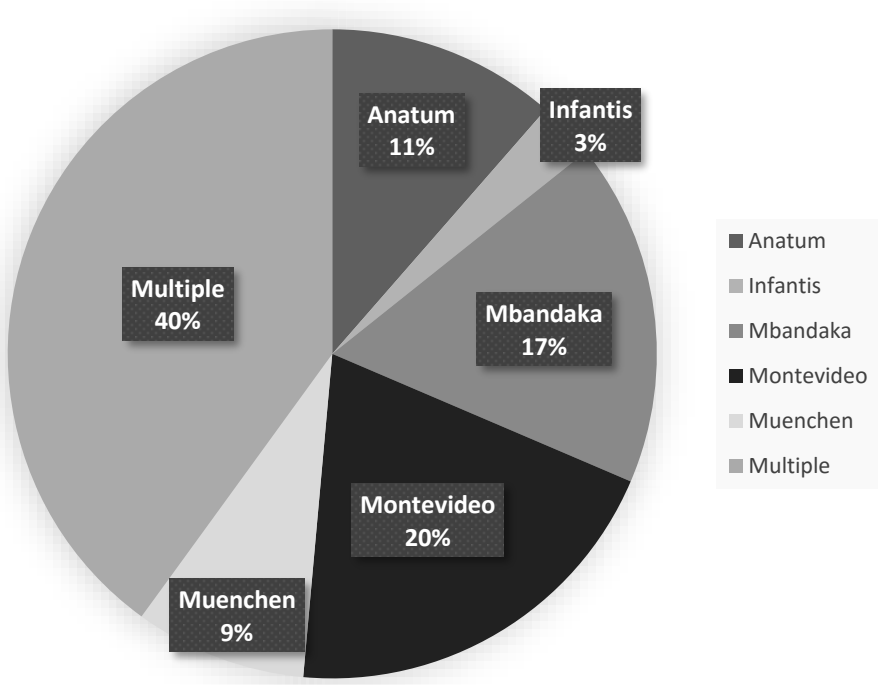

Fig. 1. Salmonella *serotypes isolated*[a] *from bovine peripheral lymph nodes*[b] *(LN)*

[a] *Salmonella* was isolated following protocols described by Microbiology Laboratory Guidebook 4.08. 3-4 presumptive positive colonies were selected for confirmation by PCR. One confirmed positive colony for each LN sample was selected for serotype testing (n = 35). Serotyping was conducted at the NVSL facility (Ames, IA).

[b] Left and right superficial cervical and subiliac LNs were collected from steers (n = 304 LNs). Within each animal, left and right LNs of each type were pooled (n = 152 samples).

CHAPTER V

CONCLUSION

Overall, the findings from this research support the notion that differences in feedlot environmental conditions and/or management practices play a key role in *Salmonella* exposure and uptake in fed beef cattle. There are numerous risk factors associated with fecal shedding of *Salmonella* that are present upon cattle entry to the feedlot, such as the mixing of groups of cattle, change in feedstuff, and onset of stress. Based on currently published literature, this is the first study that investigated prevalence in beef cattle through different stages of feeding, and not solely upon reaching market weight. Findings from this study indicate that beef cattle in feedlots may be at increased risk for *Salmonella* uptake as they enter finishing stages before harvest. Little is known about the causes of variations in *Salmonella* prevalence within bovine LNs at different feeding stages; perhaps there are isolated exposures unique to later stages of feeding, or there is a cumulative effect from persistent challenges for the duration of the feeding period. Regardless of feeding stage, one feedlot continues to produce cattle with significantly lower *Salmonella* prevalence in peripheral LNs than the other feedlot. Although the reasons for this difference are still unexplained, results indicate that management and environmental factors may have a greater influence on *Salmonella* prevalence than incoming cattle source. Future research to understand the factors contributing to greater *Salmonella* prevalence within LNs at later feeding stages

may provide opportunity for implementation of interventions or environmental controls to reduce *Salmonella* prevalence to create a safer product for consumers.

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