THESIS

EFFECTS OF POTENTIAL TYLOSIN SUBSTITUTES ON *SALMONELLA* PREVALENCE AND THE MICROBIOME OF SUBILIAC LYMPH NODES OF BEEF FEEDLOT CATTLE

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ABSTRACT

EFFECTS OF POTENTIAL TYLOSIN SUBSTITUTES ON *SALMONELLA* PREVALENCE AND THE MICROBIOME OF SUBILIAC LYMPH NODES OF BEEF FEEDLOT CATTLE

Tylosin, a macrolide antibiotic, is fed to feedlot cattle for liver abscess prevention. Tylosin alternatives are currently being investigated as pressures to reduce the amount of antibiotics used in livestock increase. Understanding effects of various feeding strategies on the safety of beef products is a priority as alternatives are investigated. This study investigated the effect of Tylosin, and two Tylosin alternatives on the prevalence of *Salmonella* in subiliac lymph nodes (SLN) and the microbiome of SLN from feedlot cattle. *Salmonella* harborage in the lymph node is a challenge for the beef industry as ground beef is made from beef trimmings that commonly contain lymph nodes. Consumption of contaminated ground beef is one of the possible foodborne routes of *Salmonella* infection. SLN (n=600) were collected from feedlot cattle (n = 5,481) at the time of slaughter. Overall 84.6% of the SLN were positive for *Salmonella* and the treatment did not influence prevalence (P > 0.8402). Samples from each pen of feedlot cattle (15 SLN/pen) were composited for microbiome analysis using 16s rRNA amplicon sequencing. Samples were analyzed using the open-source software Quantitative Insights Into Microbial Ecology (QIIME). The treatment did not influence the microbiome of the SLN (P = 0.223; P= 0.267). The top three phyla present were Proteobacteria (67.3%), Actinobacteria (10.2%), and Acidobacteria (9.5%). Although *Salmonella* was culturally isolated, it was not identified in the microbiome analysis because the genus could not be resolved for 18.9% of the Enterobacteriaceae family. Understanding why *Salmonella* is detectable in the

lymph nodes may hold the key for prevention, and characterizing the microbiome is crucial for this process.

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Thank you to Maggie Weinroth for eating with me, and for keeping me from throwing my computer across the room when I was analyzing my microbiome data.

DEDICATION

An Ode to the Node

This is an Ode to the Node

But I'm not going to write it in code

I have autoclaved many a load

And now I have knowed

That *Salmonella* can gload

For being found in the commode

And quantities in the node by the boat load

And causing bowel movements to be ever flowed

To where you feel you might explode

But not by the nematode

And now my intestines will erode

You may change the health code

You greatly increased my workload

But now I will have a new zip code

So now I thank my node

To the node I am owed

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CHAPTER 1

Literature Review

1.1 *Salmonella enterica* in Beef

Nontyphoidal *Salmonella enterica* is the leading cause of foodborne illness globally resulting in hospitalization, as well as the leading cause of foodborne illness resulting in death in the United States (CDC, 2011). In the United States, it is estimated that there are 1.3 million cases of gastroenteritis a year caused by *Salmonella enterica* (Scallan et al., 2011). One in seven *Salmonella* outbreaks (where the contamination was found) were attributed to beef (CDC, 2008) and beef is the third most common source of bacterial foodborne illness and the fourth most common source of *Salmonella* (Gould et al., 2013). Over 24 billion pounds of beef are consumed in the United States each year, and beef contaminated with *Salmonella* can present a public health risk (United States Department of Agriculture, 2017).

Using data from the Centers for Disease Control (CDC), Laufer et al. (2015) assessed outbreaks of *Salmonella* in the United States attributed to beef in the period between 1973 and 2011. Of the 28,599 foodborne outbreaks, 1,965 were attributed to *Salmonella,* and beef was responsible for 90 of those outbreaks. Among *Salmonella* outbreaks associated with ground beef, *Salmonella* serotypes Typhimurium and Newport accounted for over half of the outbreaks identified in ground beef. Before 2002, roast beef was the leading cause of *Salmonella* foodborne illness outbreaks. However, from 2002 to 2011, ground beef became the predominant vehicle of foodborne illness associated with *Salmonella,* responsible for 17 of 38 outbreaks reported (Laufer et al., 2015). From 1973 to 2011, nine multistate outbreaks of *Salmonella* have been

attributed to ground beef—and generally, has been attributed to ground beef prepared in the home (Laufer et al., 2015).

Previous researchers have investigated the prevalence and level of *Salmonella* in ground beef in the United States (Samadpour et al., 2006; Bosilevac et al, 2009). Samadpour and others (2006) assessed the prevalence of foodborne pathogens in retail foods collected from retail stores over a 12-month period in Seattle, Washington. (Samadpour et al., 2006). Interestingly, *Salmonella* was the most common pathogen to be isolated from the food samples taken (ground beef, sprouts, and mushrooms). Among beef products, ff the 1,750 ground beef samples, 4.2% were positive for *Salmonella.*

Additionally, Bosilevac and colleagues (2009) evaluated the presence of *Salmonella* in ground beef by collecting samples from 18 commercial ground beef plants during the period betwen July 2005 and June 2007 (Bosilevac et al., 2009). Overall prevalence of *Salmonella* was 4.2% (172 samples). The authors found a greater prevalence of *Salmonella,* which they believe is due to using immunomagnetic separation (IMS). There were no monthly differences in prevalence between difference regions where the samples were collected. However, the Southern High Plains had the greatest prevalence, while Northern California had the lowest prevalence of *Salmonella* (broadly Northern California). Overall, the summer months tended to have a higher prevalence. Among the 28 *Salmonella* serotypes isolated, Anatum, Mbandka, Montevideo, and Muenster represented 50% of the isolates. The season and region did not affect the distribution of serotypes. Interesting, serotypes Agona, Cerro, and Typhimurium were found in four to five regions, but were found infrequently. Multidrug resistant (MDR) *Salmonella* comprised 0.6% of the samples. Serotypes Dublin, Reading, and Typhimurium comprised most the MDR isolates.

An MDR *Salmonella* Typhimurium DT104 outbreak in the Northeastern United States was investigated (Dechet et al., 2006). There were 58 case patients identified in 9 states from 2003-2004. MDR *Salmonella* infections are more severe than *Salmonella* infections that are pan susceptible (Helms et al., 2002). This outbreak was associated with ground beef that had been bought from grocery stores, from one processor that was sold as tube stock. Illness was found to be associated with ground beef consumed as hamburgers at home, or eaten raw. From October 2003 through January 2004 PulseNet identified cases of Salmonellosis in Maine, Massachusetts, New Hampshire, Connecticut, and Vermont. The illnesses associated with this outbreak were severe, with many requiring hospitalization. The meat processor that is associated with the outbreak sources the ground beef rom culled dairy cows.. The same processor was also implicated in an outbreak of ground beef tainted with MDR *Salmonella* Newport in five states with 47 cases (January to April 2002) (CDC 2002).

Following the 1992-1993 *Escherichia coli* O157:H7 outbreak, substantial efforts have been made in the U.S. meat industry to reduce the risk of foodborne pathogens (Centers for Disease Control and Prevention, 1993). Though most efforts targeted *E. coli* O157:H7, including the declaration of this pathogen as an adulterant and the establishment of zero-tolerance policy for it by the Food Safety Inspection Service (FSIS), additional efforts also reduced the presence of other foodborne pathogens. The implementation of requirements for Hazard Analysis and Critical Control Point (HACCP) systems, and other programs, following the 1993 outbreaks have led to reduction in the presence of *E. coli* O157: H7 and the six non-O157 Shiga toxin-producing *E. coli* (STEC) serogroups found in beef. However, despite the decline in E. *coli* O157: related illnesses, *Salmonella enterica* related illnesses have gone relatively unchanged (Wheeler et al., 2014, CDC 2005, CDC 2016, CDC 2011).

1.11. Food Safety Inspection Services *Salmonella enterica* Regulations

Salmonella verification is part of the Pathogen Reduction/ Hazard Analysis Critical Control Point (PR/HACCP) Systems Final Rule and is a performance standard to insure process control in meat and slaughter facilities (9 CFR 310.25(b)(1)). *Salmonella* is a target organism due to its presence in all major species and widespread prevalence as a foodborne illness. *Salmonella* samples are taken to "measure the effectiveness of the slaughter and grinding process in limiting contamination" (FSIS 2016). As *Salmonella* is not an adulterant, product does not have to be held or recalled if it is deemed positive for *Salmonella*. The performance standard for ground beef is 7.5% positive for *Salmonella*, or no more than 5 positive samples out of 53 samples (FSIS Directive 10,250.1). Performance standards are in place to determine overall process control. In 2013, USDA-FSIS tested 17,161 ground beef samples, and 1.6% tested positive for *Salmonella* (FSIS, 2013). When compared to previous years, *Salmonella* prevalence seems to be slightly lower, as prevalence from 2010 to 2012 ranged from 1.9% to 2.4% (FSIS, 2013). However, it is of interest to note that the sampling size increased each sampling period.

1.12. Geographical and Seasonal Distribution of *Salmonella enterica* in the United States in Relation to Beef

Rivera-Betancourt et al. (2004) investigated the prevalence of *Salmonella, E. coli* O157:H7*,* and *Listeria* in two commercial beef processing plants in geographically different regions (south and north). Their results indicate a higher prevalence of *Salmonella* and *E. coli* O157:H7 in the plant located within the southern United States in comparison to the other plant in the north. An additional study performed by Kunze and others (2008) examined the presence of *Salmonella* in four abattoirs and six feedlots in the southern United States. Of the 1,681 total samples collected, *Salmonella was* recovered in 934 (55.6%). *Salmonella* was recovered from

30.3% of the fecal samples and 69.6% of the hide swab samples (taken post stunning and prior to antimicrobial treatment). Neither animal type nor season affected the prevalence of *Salmonella* on the hides of animals at slaughter. The concentration of *Salmonella* on the hides was greatest for feedlot origin cattle compared to beef-type cows and dairy-type cows. There were no differences in the feces of the animals based on animal type or season. *Salmonella* serotypes Anatum, Montevideo, Cerro, Mbandaka, and Kentucky were most commonly isolated from the hides, while serotypes Anatum, Montevideo, Mbandaka, Kentucky, Reading, and Cerro were most commonly isolated from the feces of the feedlot cattle. Additionally, 33.1% of the isolates were resistant to one drug, 8.35% were resistant to two drugs, 3.75% were resistant to three drugs, and 3.75% were resistant to four or more drugs. A similar study performed by Callaway et al. (2006) among four feedlots in the Southern Great Plains states found a fecal prevalence of 3.75%, with serotypes Orion, Give, Kentucky, Cerro, Anatum, and Oranienburg being the most common.

Seasonal prevalence of *Salmonella* was studied in three Midwest fed-beef processing plants by Barkocy-Gallagher et al., (2003). They observed the highest fecal *Salmonella* prevalence in the summer, while the summer and fall months exhibited the highest prevalence on the hide and pre-evisceration carcasses. Pre-evisceration carcass samples also had a higher prevalence of *Salmonella* than the fecal samples, following the pattern of higher numbers of *Salmonella* on the hides. This study showed that pathogen levels decrease after antimicrobial interventions in the plant, as only one post-intervention carcass was positive for *Salmonella.*

1.13. The Lymph Node

The immune system is the defensive center of the host. This system is responsible for protecting the host body from bacteria, viruses, fungi, parasites, and tumors. There are two

branches of the immune system, acquired and innate. Physical barriers, such as skin and stomach acid, help to keep pathogens (also known as antigens) out of the body. Phagocytic cells (such as macrophages) patrol the body for pathogens and can phagocytose pathogens. Macrophages can discriminate from “self” and “foreign” molecules, such as mannose that is not expressed on cell walls of vertebrates (Fraser et al., 1998). Acquired immunity comes from B and T cells that can specifically identify pathogens of interest during infection. The immune system can remember pathogens it has encountered in the past, so that a timely immune response can be elicited. Lymph nodes are an incredibly important part of the immune system. Responses to pathogenic antigens are initiated and the immune response is controlled within the lymph node. The lymph node acts as a filter by grabbing antigens from the circulating lymph fluid that passes through the node (Buettner and Bode, 2012). Thus, the lymph node acts as a surveillance of the body’s tissues to identify any antigens through the flow of the lymph.

Lymph nodes have three compartments and vessels that enter and exit the node. The three parts of the lymph node are the cortex, paracortex, and the medulla, which are commonly looked at during examination (Haley et al., 2005). The functional unit of the lymph node is the lymphoid lobule (Kelly 1975). A single afferent lymphatic vessel transports lymph entering the node to the subcapsular sinus and then drains to a single efferent lymphatic vessel that exits the node (Sainte-Marie et al.,1982). The paracortex houses the T cells and the cortex houses the B cells (Willard-Mack, 2006). Dendritic cells enter the node from the afferent lymphatic and present antigens to the T cells in the paracortex. These T cells then differentiate and proliferate and migrate to the cortex to assist B cells so that they can make antibodies to the antigen. These now activated T and B cells leave the node via the efferent lymphatic travel to the area of infection (Buettner and Bode, 2012).

The medulla of the lymph node houses the macrophages, which are the immune cell of interest in a *Salmonella* infection. Gray and Cyster (2012) stated that the three most important functions of the medulla are phagocytosis of pathogens from the lymph, supporting plasma cell survival, and creating a path for antibodies to reach the rest of the lymph. The lymph exiting the node must pass through the medulla before exiting into the body. Roozendaal et al., (2008) describes the size exclusion of the conduit system of the lymph to be about 70 kDa, this prevents pathogens from entering the blood stream because larger particles are likely to be caught by the macrophages within the subcapsular sinus and the medullary region. Antigens smaller than 70 kDa are transported to the B and T cell regions.

Within the medulla there are macrophages (phagocytic cells) that filter and destroy particulate antigens (Willard-Mack, 2006). Macrophages internalize and degrade antigens by phagocytosis and release cytokines that alert the adaptive immune system (Gray and Cyster, 2012). This is useful if there is an active infection and bacteria are moving through the host. The macrophages in the lymph node will "catch" the bacterium, thus preventing it from causing further infection. Parts of a bacterium can also be transported in the blood indicating an infection somewhere else in the body, and these smaller parts can elicit a specific immune response within the lymph node. The whole live *Salmonella* bacteria are of concern in the lymph node, because they can go on to cause further infection so the macrophage response in the medulla is of interest. Szakal et al., (1983) and Fossum and Vaaland (1983) found that macrophages in the subcapsular sinus of the lymph node have little opsonization and degradation of antigen activity.

Macrophages have multiple receptors to help recognize a wide variety of antigens. For example, the LPS receptor recognizes the lipopolysaccharide from gram negative bacteria, which can be an indication of infection (Wright et al., 1989). Phagocytosis by macrophages is an

important defense against pathogen invasion. Pathogens, or other material engulfed by phagocytosis by macrophages, is delivered to the phagosome, which combine with lysosomes and endosomes to enable destruction of the pathogen (Stuart and Ezekowitz, 2005; Desjardins et al., 1994). The phagolysosome of macrophages is hydrolytic and bactericidal (Garin et al., 2001; Stuart et al., 2007). The bacterium is essentially killed and broken up within the macrophage and then the macrophage presents specific parts of the antigen to other cells in the immune system, making it a professional antigen presenting cell. The macrophage can present the antigen to a helper T cell to alert the rest of the immune system that there was an invader. Most the antigen capture in the lymph node occurs in medullary sinus macrophages, indicated by the large size of lysosomes and a variety of vesicles (Steer, 1987).

1.14. *Salmonella enterica*

Salmonella enterica is a facultative, gram negative, intracellular pathogen. *Salmonella* can invade non-phagocytic cells, such as the intestinal epithelium, thus ingestion is the normal route of infection in animals. *Salmonella* Pathogenicity Island I (SPI-I) encodes a Type 3 Secretion System (T3SS) that is essential for gastrointestinal infection which are, collectively termed the 'invasion genes' (Mills et al., 1995). The SPI-I causes cytoskeletal rearrangement resulting in membrane ruffling which facilitates bacterial mediated endocytosis into the intestinal epithelial cell (Finlay et al., 1992a; Francis et al., 1992b). Invasion of the intestinal cells results in the symptoms associated with *Salmonella* inflammatory gastroenteritis (in cattle fluid accumulation within the gut [Wallis et al., 2000]). *Salmonella* can access the Peyer's patches near the M cells through invasion of the non-phagocytic cells in the intestine, which then allows access to the lymphatic system (Penheiter et al., 1997). Dendritic cells in the Peyer's Patch may take up the *Salmonella* after M cell invasion (Hopkins 2000).

Once *Salmonella* is within host cells it becomes easier for the bacterium to travel throughout the body through the lymph and circulatory system. The bacterium can eventually go to a lymph node either from circulation or through an immune cell. Macrophages within the lymph node will either phagocytose the bacterium or the bacterium will enter the macrophage through membrane ruffling. *Salmonella's* ability to survive in the host cell is a result of has multiple genes that are turned on upon entry into the macrophage for intracellular survival. The Pathogenicity Island II (SPI2) is required for intracellular survival. The SPI2 encodes a T3SS that is activated during intracellular conditions and is required for proliferation (Shea et al., 1996, Hensel et al.,1998). Almost immediately upon entry into the cell a phagolysosome is formed, called the *Salmonella* containing vacuole (SCV) that enables intracellular growth (Mills and Finlay, 1998; Garcia-del Portillo, 2001). The SCV is advantageous because it has less antibacterial activity and is less acidic. The SCV induces the formation of *Salmonella* induced filaments (SIFs) which allows an interchange between the tubular network and the luminal content to facilitate intracellular growth and receiving of host nutrients by endosomal remodeling (Liss et al., 2017). *Salmonella* Typhimurium 14028 inhibits phagosome-lysosome fusion within the macrophage (Buchmeier and Heffron 1991). The SPI2 is essential for intracellular survival and dissemination throughout the lymphatic system through oral inoculation (Cirillo et al., 1998).

Virulent *S.* Typhimurium strains and noninvasive *Salmonella* mutants were studied in RAW264.7 macrophages for the ability to induce apoptosis (Monack et al., 1996). Membrane ruffling is necessary for the signaling to induce apoptosis in the macrophage. It was found that *S.* Typhimurium taken up by the macrophage through membrane ruffling or by phagocytosis could replicate intracellularly. The virulent strain caused macrophages to detach from the monolayer

and die, while the noninvasive strain did not incur cytotoxicity. The virulent strains showed characteristics of macrophages about to undergo apoptosis, while macrophages infected with the noninvasive strains only showed bacteria within the vacuoles. Virulent strains and noninvasive strains that were opsonized by the macrophage did not induce apoptosis in the macrophage.

In a study by Eriksson et al., (2003) *Salmonella* Typhimurium infected J774-A1 macrophage cells by complement-opsonization to study genes expressed during intracellular growth in the SCV. To accomplish this, RNA was extracted at times when the host oxidative burst was expected, after bacterial replication begins, and when the macrophage nitric oxide burst is produced. The study showed that 919 of the 4451 *S.* Typhimurium coding sequences changed expression while the macrophage. There was an increase in the coding for RpoS (the virulence associated sigma factor) that could be indicative of post-transcriptional regulation. The most important regulator of intracellular gene expression, PhoPQ, is a two-compartment system and expresses several known virulence functions that responds to environmental concentration of Calcium and Magnesium (Kier et al 1979; Vescovi et al., 2001). It is known that macrophages express two enzymes: NADPH phagocyte oxidase which produced superoxide and inducible nitric oxide synthase, which produces NO (Mastroeni et al., 2000; Vasques-Torres et al., 2000). These compounds are harmful to bacterial cells by oxidizing DNA. *S.* Typhimurium can avoid these reactive compounds by utilizing a two-step detoxification of the superoxide. The superoxide is converted to hydrogen peroxide by superoxide dismutase and the second step involves a catalase mediated destruction of hydrogen peroxide (Carmel-Harel, 2000). The thioredoxin and glutaredoxin systems are used to restore protein function by reducing the oxidized residues in the macrophage (Aslung and Beckwith, 1999). Stevanin et al., 2002 found

that flavohemoglobin Hmp helps to protect *S.* Tphimurium from nitric oxide produced by macrophages by detoxifying NO aerobically and reducing NO anaerobically.

S. Typhimurium can use the Emden-Meyerhof, the pentose phosphate and the Entner-Doudoroff pathways for intermediate sugar catabolism (Fraenkel, 1996). The Entner-Duodoroff pathway is the pathway of choice for intermediate sugar metabolism in *S.* Typhimurium in the intracellular environment. The genes involved for the Emden-Meyerhof and the pentose phosphate pathways were repressed. Gluconate and related carbohydrates are the carbon of choice for the intracellular bacteria. Gluconate is converted to pyruvate and glyceraldehyde 3-phosphate. The Entner-Duodoroff pathway is a source of NADPH that can be used in redox cycling (Fraenkel, 1996). The iron acquisition genes (Fur) are not induced during intracellular macrophage infection, indicating that the SCV is devoid of Fe^{3+} and rich in Fe^{2+} (Eriksson et al., 2003).

Macrophages produce antibacterial peptides against *Salmonella* (Hiemstra et al., 1993) and the PhoP/Q system in *Salmonella* functions to enable resistance to these antimicrobials in the SCV. To try and reduce immune response, it was found that *Salmonella* downregulated synthesis of flagella and type 1 fimbriae in the SCV (Hiemstra et al., 1993). The SCV is an aerobic environment because the succinate dehydrogenase operon is induced. ATP synthase genes were repressed in the SCV and Adams et al., 2001 found that the decrease in flagella expression would block the proton influx through the H+/ATPase and the flagellar base structure. Heimstra et al., (1993) concluded that *Salmonella* are not starved for amino acids or iron, the SCV is low in phosphate and high in magnesium. *Salmonella* requires an acidic environment in the SCV for replication and uptake of nutrients and the proton gradient aids in importing the primary carbon source and other proton channels are downregulated to maintain the pH required for growth

within the SCV. An important virulence mechanism *S.* Typhimurium is killing of the infected macrophage after replication and formation of the SCV, so that the bacterium can go infect other cells (Lindgren et al., 1996).

Waterman and Holden (2003) concluded that the SPI2 inhibits some endocytic traffic by blocking fusion between lysosomes and SCVs, avoiding macrophage NADPH oxidase-dependent killing, and interference with the nitric oxide synthase produced by the macrophage. The SPI2 helps to control the environment of the SCV and surrounds itself with F-actin and cholesterol. Waterman and Holden (2003) hypothesized that this could help stabilize the SCV or facilitate transport vesicles. *S.* Typhimurium has been shown to induce a delayed apoptosis within the host cell which can promote bacterial spread to the rest of the body, specifically the liver and spleen. Another study found that *Salmonella* Typhimurium kills phagocytes not by apoptosis, but by a caspase-1-dependent necrosis (Brennan and Cookson 2000). Type I interferon is produced in response to *S.* Typimurium to drive necroptosis and avoid detection by the immune system (Robinson et al., 2012). The macrophage dies and all the *Salmonella* that have replicated in the vesicle are released to go infect other cells.

1.15. *Salmonella* in the Lymph Nodes of Cattle

Multiple studies have highlighted the isolation of *Salmonella* from the lymph nodes of cattle (Gragg et al., 2013b, Haneklaus, et al., 2012, Brichta-Harhay et al., 2012, Gragg et al., 2013a, Vipham et al., 2015, Moo et al., 1980). In one of the initial studies of *Salmonella* in the lymph nodes of food-producing animals, Moo et al. (1980) isolated *Salmonella* from jejunal and caecal lymph nodes in slaughtered animals (Moo et al., 1980). Using enrichment methods *Salmonella* was isolated from 15 cows (30%) and 2 yearling cattle (5%). It is of interest to note that sheep and pigs also had *Salmonella* positive lymph nodes. *Clostridium perfringens* was also

isolated from the yearling cattle. *Salmonella* Typhimurium and Anatum were some of the serotypes isolated from the cattle lymph nodes. There were counts of up to 1,500 per gram of *Salmonella* in the lymph nodes.

Bichta-Harhay et al. (2012) evalauted 906 subiliac lymph nodes and 180 resulting pooled samples of adipose trim from the lymph nodes. The subiliac lymph node *Salmonella* prevalence was found to be 0.8% and the prevalence for the pooled adipose samples was 5.0%. Serotpyes Anatum, Dublin, Cubana, Typhimurium, and Montevideo were found in the lymph nodes, while serotypes Newport, O3,10:R1H, Montevideo, Typhimurium, and Mbandaka were found in the pooled adipose trim samples.

In a study by Gragg et al., (2013a), *Salmonella* was isolated from mesenteric, subiliac, mandibular, and mediastinal lymph nodes of 68 beef carcasses in Mexico. Prevalence of *Salmonella* among the various types of lymph nodes was 55.9% for mandibular, 91.2% for mesenteric, 7.4% for mediastinal, and 76.5% for subiliac. Serotypes Kentucky, Anatum, Reading, Meleagridis, and Cerro were found in the subiliac lymph nodes. Reading was the only serotype found in mediastinal lymph nodes. Serotypes Kentucky, Anatum, Meleagridis, Cerro, Give, and Mbandaka were found in mesenteric lymph nodes. Within the mandibular lymph nodes, serotypes Kentucky, Anatum, Reading, Meleagridis, and Muenster were found. The isolated *Salmonella* expressed resistance to tetracycline, nalidixic acid, amoxicillin-clavulanic acid, ampicillin, cefoxitin, sulfisoxazole, trimethoprim-sulfamethoxazole, and streptomycin.

The effect of a direct-fed microbial (DFM) on the prevalence of *Salmonella* in subiliac lymph nodes of cattle from a commercial feedlot and research feedlot was evaluated by Vipham et at. (2014). In the feedlot study, the use of a DFM reduced *Salmonella* prevalence in the DFM group (57.5%) versus the control group (76.3%). Similarly, in the research feedlot, 25.9% of the

subiliac lymph nodes from the control group were positive for *Salmonella* and only 4.7% of the DFM samples were positive.

Haneklaus et al. (2012) evaluated the prevalence of *Salmonella* in cervical and iliofemoral lymph nodes of cattle finished at different feedyards. Prevalence differed among feedyards and ranged from 0% to 88.2%. Similarly, Gragg et al. (2013b) examined the prevalence of lymph node *Salmonella* in cull and feedlot cattle from different regions of the United States over a 12-month period (Gragg et al, 2013b). *Salmonella* point prevalence was greater in feedlot cattle than in cull cattle, and was greater in the southwest region. Additionally, prevalence was greater in the summer/fall when compared to winter/spring. Cull cattle *Salmonella* prevalence is not affected be region or season.

Arthur et al. (2008) evaluated the prevalence of *Salmonella* in lymph nodes that would be incorporated into ground beef trimmings, specifically from the chuck and flank (superficial cervical and subiliac). Overall, 1.6% of lymph nodes were positive for *Salmonella,* and lymph nodes from carcasses of culled cattle had a higher prevalence of *Salmonella* than those from fed cattle. Subiliac lymph nodes from culled cattle had the greatest prevalence of *Salmonella* (3.86%), while the lowest prevalence was observed in the superficial cervical lymph node of fed cattle (0.35%). The serotypes found in culled cattle were Anatum, Blockley, Cerro, Montevideo, Muenster, Newport, Thompson, and Typhimurium. The serotypes found in fed cattle were Cerro, Montevideo, Seftenberg, and Typhimurium. Three isolates from cull cattle were resistant to six to eight antibiotics.

The source(s) of *Salmonella* contamination in ground beef in dairy cows was studied in a U.S commercial beef processing plant (Koohmaraie et al., 2012). Samples were collected throughout the harvest process; 96% of the hides positive for *Salmonella* and 47% positive for

pre-intervention treatment carcasses were positive for *Salmonella*. Post-intervention, 0 carcasses were positive for *Salmonella.* Superficial cervical lymph nodes were 18% positive for *Salmonella.* Trim was 7.14% positive and ground beef was 1.67% positive for *Salmonella.* This study suggested that the hide and lymph nodes were important sources of *Salmonella* in ground beef. Lymph nodes were a larger contributor of contamination in ground beef since they are shielded from antimicrobial carcass sprays within the carcass. Nonetheless, this study showed that interventions in the plant are effective in eliminating contamination from hides during processing.

Recently, Brown et al. (2015) assessed the influence of breed on the prevalence of *Salmonella* in lymph nodes. Breed had no measurable influence on *Salmonella* prevalence. However, the prevalence of *Salmonella* in Holstein and beef steers decreased from May to October.

1.16 Transmission of *Salmonella* to Peripheral Lymph Nodes of Cattle

Based on current research, there appears to be multiple routes of inoculation for *Salmonella* to reach peripheral lymph nodes in cattle. Like for infection in humans, Brown et al. (2015) assessed oral inoculation of *Salmonella* in the subsequent uptake into the peripheral lymph nodes of cattle. They reported *Salmonella* could be found in peripheral lymph nodes after an oral experimental inoculation of 10^6 to 10^7 CFU of *Salmonella* per day for 14 days. Cattle deprived of feed and water were not more likely to harbor *Salmonella* within the peripheral lymph nodes following experimental inoculation compared to a control group. This paper suggested that a lower dose inoculation of *Salmonella* (10^2 to 10^3), like that found in drinking water (unpublished data from the authors), over a longer period (15-150 days) could result in *Salmonella* positive peripheral lymph nodes. It is important to note that this study recovered

Salmonella positive lymph nodes from control calves that were not provided a *Salmonella* inoculation.

Insects, like flies, are normal inhabitants surrounding livestock and are commonly found at feedlots. *Haematobia irritans,* the horn fly, is a blood feeding and biting fly that parasitizes cattle, it has been shown to be a mechanical vector of *Staphylococcus aureus* in dairy cattle (Owens et al., 1998). Horn flies feed almost hourly (Harris et al., 1974), and therefore present potential for bacterial inoculation at each feeding. Survival of *Salmonella enterica* Montevideo in horn flies was studied to try and better understand *Salmonella* transmission and uptake into peripheral lymph nodes (Olafson et al., 2014). A fluorescently labeled *Salmonella enterica* Montevideo was used to look at the survival of the bacterium within the fly. Tactile exposure of *Salmonella* resulted in *Salmonella* being found on the mouthparts and subsequent digestion in the fly. A blood meal of the fluorescently labeled *Salmonella* (10^2 to 10^6) resulted in colonization for up to three days after ingestion (Olafson et al., 2014). *Salmonella* were found in feces of 100% of flies fed the inoculated blood meal 6-7 hours after feeding. Mean quantity of *Salmonella* excreted was 5.65- 67.5 x 10^2 CFU per fly, which can could account for microbial contamination on the hide of cattle.

Olafson et al., (2016) assessed transmission of *Salmonella* to peripheral lymph nodes using horn flies as a vector. Horn flies could feed on *Salmonella* inoculated blood meal for 12 hours before inoculation of cattle. A prolonged exposure time of the horn flies to cattle resulted in lymph nodes that cultured positive for *Salmonella.* After 5, 11, or 19 days of exposure, 8%, 50%, and 42% of lymph nodes were positive for *Salmonella.*A microlancet inoculation was used as a control, and the repeated inoculations by the horn flies resulted in a higher concentration than the microlancet inoculation. *Salmonella* was recovered from popliteal, superficial cervical,

and subiliac lymph nodes at different time points in the study. However, a negative control animal was also found to be positive for *Salmonella*.

Edrington et al. (2016) performed experimental transdermal inoculation in the lab with *Salmonella* inoculation with an allergy skin test device on cattle. Cattle were inoculated on the lower legs, abdomen, and back, and were euthanized up to 21 days post inoculation to look for *Salmonella* in superficial cervical, popliteal, and subiliac lymph nodes. The challenge strain used was identified 6 hours after inoculation and until 21 days post inoculation. The *Salmonella* levels within the lymph nodes was found to be from 0.8 to 1.8 log CFU/g. This experiment found that *Salmonella* should be eliminated from the lymph nodes in about 28 days after a single inoculation event of an allergy skin test device with *Salmonella*. Two experiments were conducted since elimination of *Salmonella* from the lymph nodes was not complete by day 21 post inoculation, so a second experiment was completed until 28 days post inoculation. Elimination of *Salmonella* from the lymph nodes starts to occur on day 14, but can still be found in the lymph nodes in smaller numbers.

1.17. Bacteria in the Lymph Nodes of Other Animals

Salmonella also have been found in lymph nodes of sheep and goats (Hanlon et al., 2016). Lymph nodes were collected from 311 goats and 357 lambs in California, New Mexico and Texas. *Salmonella* were detected in mandibular, mesenteric, and subiliac lymph nodes of goats. The subiliac lymph node of goats had the highest prevalence of *Salmonella* at 9.62%. The type of lymph node was significant in goats when comparing prevalence. In lambs, mesenteric lymph nodes had the highest prevalence of *Salmonella*, and there was not a significance in lymph node type in lambs for *Salmonella* prevalence. *Salmonella* prevalence were greatest in the month of March over the five months of sample collection.

Pork carcasses were sampled for *Salmonella* in Portugal (Vieira-Pinto et al., 2005). The study looked at carcass, lymph nodes (including tonsils) and ileum *Salmonella* contamination. A total of 101 pigs were sampled. Ileocolic lymph nodes most frequently were positive for *Salmonella* (18.8%). The ileum was sampled alongside the ileocolic lymph nodes and had a prevalence of 13.9%. Mandibular lymph nodes had a prevalence of 12.9% and tonsils had a prevalence of 9.9%. The most prevalent serotype identified was Typhimurium and it was found in 47.8% of the isolates in the study.

Salmonella can be found in the lymph nodes of orally inoculated swine (Broadway et al., 2015). Thirty-eight pigs were inoculated with *Salmonella* Typhimurium in two different phases, one phase with either phosphate buffered saline or phosphate buffered saline with *Enterobacter cloacae* and the other phase with or without the inclusion of a yeast cell wall product. The ileocecal, subiliac, popliteal, and mandibular lymph nodes were sampled. *Salmonella* was not present in the popliteal lymph node. Ileocecal lymph node had the highest prevalence, 41.6% and 37% for the two phases respectively. Mandibular and subiliac lymph nodes had a prevalence of 2.7% for phase one. There were no statistical differences between phases for *Salmonella* prevalence.

Gnotobiotic mice (germ-free) were used to study the translocation of bacteria from the gastrointestinal tract to the mesenteric lymph nodes (Steffen and Berg 1983). The mice were given an inoculum of indigenous bacteria of the cecum. A relationship between the population of the bacteria within the cecum and the number of bacteria found in the mesenteric lymph node was found. The higher the bacterial population in the cecum, the larger the number of viable bacteria found in the mesenteric lymph node.

1.2. The Microbiome of Lymph Nodes

Culture independent methods are a relatively new development in the scientific field. Charles Darwin and Gregor Mendel were the pioneers for genetics in the 1800s. Darwin wrote "On the Origin of the Species by Means of Natural Selection" and Mendel came up with the Laws of Inheritance. Many scientists have made major accomplishments in the field of genetics to get science to where it is today. Watson and Crick won the Nobel Prize for discovering that DNA is in the shape of a double helix (Watson and Crick 1953). Marshall Nirenberg cracked the genetic code (Nirenberg and Leder 1964). In 1975 DNA could be sequenced, a method refered to as Sanger Sequencing after Frederick Sanger (Sanger and Coulson 1975). It is now possible to sequence the genome of eukaryotes, prokaryotes, and viruses. There is an interest in looking at an entire microbiome (all the microorganisms that inhabit an environment). The human microbiome has been characterized (Human Microbiome Project Consortium, 2012) and continues to be characterized to try and understand how the microbial environment impacts health of an individual. A method of utilizing the 16S rRNA subunit for phylogenies was first done by Carl Woese and George E. Fox (Woese et al., 1990). The 16S rRNA gene can be used as a molecular marker for microbial ecology studies andnis unique to bacteria and is highly conserved among species (Coenye et al., 2003, Case et al.,2007)). There are nine hypervariable regions of the 16S rRNA gene that aid in the identification of bacteria (Gray et al., 1984). The Illumina platform is generally utilized for 16S rRNA sequencing because of the cost and deeper community coverage and low error rate (Burke et al., 2016). The V4 hypervariable region can be used to predict taxonomic levels and is used in many studies (Yang et al., 2016). A shortcoming of 16S rRNA sequencing is the inability to differentiate between closely species (Yang et al.,

2016). Microbiome analysis represents the entire bacterial population, which is one of the benefits of using a culture free method.

Eight healthy slaughter pigs in Austria were investigated for metabolically active bacteria in the tonsils and mandibular lymph nodes (Mann et al., 2015). The hypervariable V1-V2 region of the bacterial 16S rRNA genes were amplified, sequenced, and analyzed using *mothur*. Sixteen phyla and 230 genera were represented in the lymph node and tonsil samples with a total of 576 operational taxonomic units (OTUs). There was variability in the number of OTUs detected in each sample. The lymph node samples had between 23 and 171 OTUs per sample, with the median at 66 OTUs. In the lymph nodes, *Serratia proteamaculans* dominated 41.8% of the sequences. *Pseudomonas marginalis* and *Herbaspirillum huttiense* were at 5.6% and 4.1% relative abundance respectively. Spearman correlations were calculated for genera, and *Treponema, Anaerovirgula,* and *Proteocatella* were highly positively correlated, as well as *Gemella, Porphyromonas,* and *Fusobacterium* were postivitely correlated in lymph nodes.

Using meta-transcriptomics and 16S rRNA amplicon sequencing the retropharyngeal lymph nodes of five mule deer were analyzed for bacterial and viral microbial communities (Wittekindt et al., 2010). Bacterial diversity of lymph nodes was greater using the meta-transcriptomic approach compared to amplicon sequencing. The V6 hypervariable region of the 16S rRNA was sequenced to evaluate the bacterial diversity of the lymph node samples. The transcript-tags could be assigned to 99.3% eukaryotic origin (mainly *Bos taurus*) and 0.3% assigned to bacteria. Of the bacterial hits, Proteobacteria dominated at 60%, and Enterobacteriaceae was the most prevalent family within this group. Firmicutes represented 22% of the identifiable taxa, followed by Actinobacteria at 5%. Interestingly, 37 transcripts were assigned to viruses, with the greatest matches to Retroviridae and Poxviridaes, and the remainder

of the transcripts belonging to phages and a single assignment to herpesvirus. This study used the metagenomic approach as well, which only identified four bacterial genera. The genera identified were *Xylella, Burkholderia, Acidovorax,* and *Bartonella.* These authors suggested that these might not have been actively replicating organisms. Using the transcriptome libraries, intestinal, skin-dwelling, soil, and freshwater bacteria were identified in the lymph nodes. *Ruminococcus* was found in all the samples and is normally found in the intestines of deer. Pathogenic bacteria, like *Escherichia* and *Streptococcus,* also were found in the lymph nodes. Diversity among samples was substantial; two of the samples had a higher diversity than the other samples, and contained genera such as *Legionalla, Enterobacter, Salmonella, Yersinia, Vibrio, Listeria,* and *Acinetobacter,* which are known pathogens. These two samples also had the most reads assigned to the Enterobacteriaceae family. This study by Wittenkindt et al (2010) confirmed that there are viable organisms in the lymph node. The microbiome is made up of organisms found in the environment associated and commensal organisms normally found in the mule deer.

Ileocecal lymph nodes of slaughter pigs were characterized to understand microbial diversity and community shifts of different pathologies of lymph nodes (Mann et al., 2014). The various lymph nodes utilized were unreactive, enlarged, purulent, and granulomatous formations. There were 32 lymph nodes that were pyrosequenced by the 16S rRNA amplicon. Proteobacteria (50%) was the most common phylum followed by Firmicutes (15-33%) and then Bacteroidetes (4-11%). The lymph nodes were highly diverse. Each pathology group of lymph nodes had similar microbiomes (distinct OTUs and abundance). An unreactive lymph node had a balanced microbial community, and the most common genera were *Faecalibacterium, Cloacibacterium rupense,* and *Novosphingobium panipatense.* Enlarged lymph nodes had an increase in *Lactobacillus amylovorus* and *Clostridium glycolicum. Mycobacterium hysosynoviae* was the

causative genera of granulatomous lymph nodes. Within the purulent lymph nodes, *Escherichia, Pseudomonas,* and *Acinetobacter* were dominant.

Sixteen ileocecal lymph nodes were taken from 16 slaughter pigs (Mann et al., 2015). Half of the lymph node was sequenced using 16S rRNA amplification and the other half was subjected to cultivation techniques. Pigs were either asymptomatic (normal appearance of lymph nodes) or had hyperplasia, purulence, granulomas formations of the lymph nodes. Assignment to taxa identified that Proteobacteria was the most prevalent (68%), followed by Firmicutes (27%) and then Actinobacteria (5%). The pathogens *Salmonella enterica* were identified in the isolates, as well as *Streptococcus suis.* The most abundant isolate was *Escherichia coli.* The *E. coli* isolates had a high diversity and at least 24% of the isolates were positive for at least one gene associated with enterohemorrhagic disease.

1.3. Tylosin

Tylosin (or Tylan), manufactured by Elanco Animal Health, is a macrolide antibiotic fed to feedlot cattle for the prevention of liver abscesses (Nagaraja and Chengappa 1998). Tylosin is fed in 71.2% of U.S. feedlots. Tylosin is fed in 77.2% of the feedlots that can feed more than 8000 head (USDA-APHIS, 2013). Livers are important exports for the United States, and liver abscesses cost the U.S. beef industry $15.8 million a year (USDA, 2014). It has been demonstrated that Tylosin can reduce liver abscesses by 40 to 70% (Nagaraja and Chengappa 1998). Liver abscesses can negatively affect live animal and carcass performance in cattle (Hicks, 2011).

Macrolides are bacteriostatic and inhibit protein synthesis by binding to the 23S rRNA of the 50S subunit of the bacterial ribosome (Schlect 2015). Macrolides are generally effective against gram positive bacterial organisms.

CHAPTER 2

Effects of Potential Tylosin Substitutes on *Salmonella* Prevalence and the Microbiome of Subiliac Lymph Nodes of Beef Feedlot Cattle

Introduction

Non-typhoidal *Salmonella* is a major contributor of foodborne illness in the United States (CDC, 2010). Approximately 1.3 million cases of gastroenteritis are caused by *Salmonella enterica* annually (Scallan et al., 2011). In one out of every seven cases in which the food vehicle of contamination was identified, contamination was attributed to beef (CDC, 2008). Post-harvest food safety interventions are effective for surface control of contamination (Arthur et al., 2007 and Bosilevac et al., 2005), but if *Salmonella* contamination is within the carcass (i.e., not on the carcass surface), antimicrobial sprays and carcass washes will not be effective. Multiple studies have found *Salmonella* within the lymph nodes of beef cattle (Gragg et al., 2013, Haneklaus, et al., 2012, Brichta-Harhay et al., 2012, Gragg et al., 2013, Vipham et al., 2015, Arthur et al., 2008). As lymph nodes can be incorporated into beef trimmings for ground beef, *Salmonella* positive lymph nodes have the potential to cause foodborne illness.

Important to the challenge of mitigating *Salmonella* in the lymph node is understanding the lymph node's role and how it may become contaminated. The microbiome of the lymph node may yield important information in mitigating *Salmonella,* because lymph nodes act as a filter for the body to sequester and terminate pathogens, and to initiate the subsequent immune response (Sainte-Marie 2010). Thus, the lymph node microbial community is representative of whatever microorganisms are present within the host, and can differ from animal to animal. The microbiome of pig and mule deer lymph nodes have been previously characterized (Mann et al.,

2015 and Wittenkindt et al., 2010), but the microbiome of bovine lymph nodes has not. Further, no prior studies have evaluated the effect of in-feed antibiotic supplementationon the microbiome of lymph nodes in beef feedlot cattle. Subiliac lymph nodes were sampled after slaughter to assess the influence of pre-harvest management strategies on post-harvest beef safety.

Materials and Methods

Cattle Population

Commercial steers and heifers (n =5,481 hd) were sourced for enrollment in a feeding trial at a commercial feedyard in the panhandle of Texas. Upon arrival at the feedyard (Spring 2016), cattle were randomly assigned to one of four treatment groups within ten pen blocks (one pen of each treatment group per block; (n= 10 pens/treatment). The four treatment groups reflected the inclusion of differing supplements in finishing diets as possible substitutes for tylosin and were as follows: i) finishing ration with tylosin (90 mg/hd/d; Elanco Animal Health, Indianapolis, IN); ii) finishing ration without tylosin, iii) finishing ration without tylosin, but with an essential oil (1g/hd/d of CRINA-L; source of limonene); and iv) finishing ration without tylosin but with Diamond V-Prototype (18g/hd/d).

Lymph Node Collection

Cattle were harvested at a commercial beef processing facility in Texas within a three-week period in August and September 2016. Fifteen subiliac lymph nodes (SLNs) were collected from each pen (40 pens x 15 SLN = 600 SLN) at the time of slaughter. Lymph nodes were collected immediately following evisceration and carcass splitting. Lymph nodes encapsulated in the surrounding fat were placed into sterile sample bags (WhirlPak, Nasco, Modesto, CA) for refrigerated transportation to the Food Safety Microbiology lab at Colorado State University

(Fort Collins, CO). Upon arrival to CSU, the SLNs were kept at 4°C until processing (within 12 h).

Sample Processing

In order to appropriately assess the presence of *Salmonella* inside of the lymph node, eliminating external contamination was imperative. To do this, the individual SLN was immersed in ethanol and the external surface was flame sterilized. Afterwards, SLNs were placed on sterile cutting boards (wrapped in aluminum foil sprayed with 70% ethanol) and trimmed of all excess fat and fascia using sterile instruments. The exposed SLNs were then immersed in boiling water (about 100°C) for 3 to 5s. Next, the SLN was then placed in filtered sample bags (WhirlPak) and pulverized with a rubber mallet to disrupt the lymphatic tissue before the addition of 80 ml of Tryptic Soy Broth (TSB, Neogen, Lansing, MI). Samples were homogenized for 2 mins at 230 rpm using a commercial stomacher (Stomacher 400 Circulator, Seward, England).

Culture Detection of Salmonella

Following homogenization, the TSB lymph node homogenate was incubated at 42°C for 12 h. Following incubation, immunomagnetic bead separation was performed using anti-*Salmonella* Dynabeads (Invitrogen, Carlsbad, CA) following the manufacturer's guidelines. The resultant bead-bacteria complex in 100 µl of PBS-Tween (phosphate-buffered saline, Sigma Aldrich, St Louis, MO; Tween 20, ThermoFisher Scientific) was transferred into 3 ml of Rappaport-Vassiliadis Broth (RV; Difco, Becton Dickinson and Company, Sparks, MD). The RV tubes were incubated for 18 to 20 h at 42°C. After incubation, a disposable inoculating loop was used to remove 10 µl of the enriched RV for streaking onto split plates containing Brilliant Green Agar with Sulfadiazine and Xylose-Lysine-Deoxycholate Agar (BGS/XLT4; Hardy

Diagnostics, Santa Maria, CA). Following incubation (35°C for 24 h) of the BGS/XLT4 split plates, three isolated colonies were picked from each agar type and streaked onto separate Brilliant Green Sulfa Agar (Difco, Becton Dickinson and Company) or XLT4 (Difco, Becton Dickinson and Company) plates. Plates were incubated at 35°C for 24 h. Following sequential streaking to obtain pure isolates, an isolated colony from each agar type was inoculated into 10 ml of TSB and incubated at 35°C for 24 h to facilitate growth of a pure culture for confirmation, serogrouping (ongoing), and assessment of antimicrobial susceptibility (ongoing). Broth (TSB) cultures of the isolates in 16% glycerol were frozen in duplicate and stored at -80°C until evaluation of serogroup (ongoing)and antimicrobial susceptibly (ongoing) by the Colorado State University Veterinary Diagnostic Laboratory (Fort Collins, CO).

Sample Processing for Sequencing

In addition to traditional culture-based assessments of *Salmonella* in the SLN, an aliquot of the TSB/SLN homogenate was utilized for assessment of the SLN microbiome. After homogenization, 10 ml from each of the 15 lymph node/TSB suspensions from within each pen were pooled to formulate one composited SLN sample per pen (total of 150 ml). The composited sample was centrifuged (4300 x g, 10 min, 10°C, Sorvall Legend XT-R,ThermoFisher Scientific). Following centrifugation, the supernatant was removed and the remaining pellet stored at -80°C.

DNA Isolation

At the time of DNA isolation, 0.1-0.2 g of the SLN homogenate pellet was weighed to facilitate isolation of DNA using the PowerFecal DNA Isolation Kit (Mo Bio Laboratories, San Diego, CA) with minor modifications to the protocol. The Mini-Beadbeater-16 (Biospec Products, Bartlesville, OK) was used for the bead beating step, where samples were processed

for 3 pulses of 30 s each. During DNA elution from the spin filter, a single 75 µl volume of C6 elution buffer was passed twice through the spin filter used per sample, resulting in a single 75 µl DNA sample.

16S rRNA Gene Sequencing

16S rRNA gene amplification and sequencing was performed by a commercial sequencing company (Novogene Corporation, Beijing, China). Replicates were shipped on ice and analyzed in distinct sequencing runs. The V4 region of the 16S rRNA gene was amplified using the Earth Microbiome Project primer set 515F/806R (Caporaso et al., 2012), with reverse primers containing unique barcode sequences. Library sequencing (paired-end, 2 x 250 base pairs) was performed on an Illumina HiSeq 2500 platform (Illumina, Inc.). Raw data were demultiplexed and quality filtered by Novogene.

Statistical Analysis for Culture Data

Analyses were performed using a commercial statistical software system (R, version 3.3.1) and the car (Fox and Weisberg, 2011), lsmeans (Lenth, 2016), and ggplot2 (Wickham, 2009) packages. A linear model was fit using *Salmonella* percent positive as the response and the data were analyzed as a randomized, complete block design. Fixed effects included treatment (1, 2, 3, and 4) and pen block. Treatments and pen blocks were compared using Tukey adjusted pairwise comparisons using the lsmeans package. For all comparisons, an alpha level of 0.05 was used.

Bioinformatics and Statistical Analysis for Microbiome Data

Novogene trimmed adaptors from samples. Primers were trimmed using cutadapt. Forward and reverse reads for each sample were merged using PEAR v0.9.10 (Zhang et al., 2014). Using Qiime, (Caporaso et al., 2010) raw sequencing reads were categorized into

operational taxonomic units (OTU's) via "open reference" with the default settings. Operational taxonomic units assigned to mitochondria and chloroplasts, and OTUs were assigned to the fraction of 0.05% of the minimum observation count of an OTU for the OTU to be retained, and completely unknown taxa that did not have a Domain assignment were removed. A rarefaction curve was constructed using chao_1 measurements for each sample fastq file to determine whether sequencing depth was adequate to capture diversity.

The "ANOSIM" function in the Vegan Package in R version 3.3.1 was used to assess differences between groups. A Permanova test was performed which uses a permutation test with pseudo F ratios to assess differences between treatment groups. The "ANOSIM" and Permanova tests were used to compare beta diversity across samples. A Kruskal-Wallis test in R was performed to compare alpha diversity between treatment groups. For all comparisons in the study, an alpha level of 0.05 was used.

Results

Culture Results

The overall prevalence of *Salmonella* in the SLN was 84.6% (95% CI, 0.7859379 to 0.9073954); however, *Salmonella* prevalence did not differ ($P = 0.8402$) between treatment groups, indicating that the feed additives in this study—or their exclusion—did not influence the prevalence of *Salmonella* within the SLN (Table 1). However, large differences ($P < 0.001$) were noted between blocks. Block two had a lower prevalence of *Salmonella* (48.25% [95% CI, 0.3593585 to 0.6056415]) in comparison to the other blocks (66.75%- 100%). There were 1514 isolates recovered from the SLN that were plated on the BGS agar and there were 1405 isolates recovered from the XLT4 agar. There was a total of 2,919 isolates from the SLNs.The antimicrobial susceptibility and serogroup results are currently pending.

16S rRNA Results

Due to low biomass of the lymph node samples, only nine of the samples could be successfully sequenced. Although the number of samples that could be sequenced was not ideal, among those sequenced, every treatment was represented at least twice. Sixteen phyla were represented in the samples sequenced (Figure 1). The three most relatively abundant phyla present were Proteobacteria (67.3%), Actinobacteria (10.2%), and Acidobacteria (9.5%). The family level of classification was dominated by Enterobacteriaceae, making up 42.6% of the samples. Pseudomonadaceae (6.8%) was the second most abundant in the SLNs at the family level. The genus could not be resolved for the most abundant Enterobacteriaceae (18.9%), which was followed closely by *Citrobacter* (17.6%), and then *Trabulsiella* (6.1%) (Figure 2). It was not unexpected that the genus could be not resolved for the most abundant Enterobacteriaceae because it is difficult to differentiate closely related species, such as Enterobacteriaceae, that may only have a few nucleotides different at the V4 region (Jovel et al., 2016 and Vetrovsky and Baldrian 2013). As a result, these closely related species could not be classified at the lowest taxa level. *Salmonella* was identified by culture methods to be present in the SLNs, so it is probable that it was found in the 16S samples, however, the genus could not be resolved.

Alpha diversity (within sample diversity) was examined using a Kruskal-Wallis test to compare Shannon alpha diversity scores among treatment groups. There was not a difference in alpha diversity between treatment groups ($P > 0.3108$; Figure 3, Table 2). Similarly, beta diversity (differences in species composition across samples) did not differ by treatment groups ($P = 0.267$ [ANOSIM] and $P = 0.223$ [PERMANOVA]). A principal coordinate analysis plot (PCoa) was constructed with unweighted UniFrac distances (Figure 4) to aid in the visualization of beta diversity across samples. Each dot represents a composited SLN pen sample, and each

dot was scaled to show overlapping of samples. Principal coordinate one accounted for 98.54% of the variability in the sample microbiomes. There was clustering observed in the PCoA that could not be attributed to treatment effects. There was not a difference in composition between treatment groups.

The relationship between treatment groups with and without tylosin (control, treatment 3, and treatment4) was analyzed. Alpha and beta diversity did not differ between Tylosin and no Tylosin treatment groups ($P > 0.05$). There was clustering in the PCoA plot, but differences between treatment groups were not significant (Figure 5). There was no difference in microbial composition between tylosin and no tylosin treatment groups. Nonetheless, due to the small sample size, it is difficult to make inferences about the entire SLN microbial community in the study.

Alpha diversity varied among SLN samples, which was probably the driving factor in the clustering seen on the PCoA plot (Figure 4). SLN composites 18, 40, and 38 had a greater Shannon alpha diversity index (Table 2, Figure 2) than the other samples, which accounted for their clustering. SLN sample 24 had the lowest Shannon alpha diversity index at 1.812, and was dominated by a genus that could not be resolved for Enterobacteriaceae (making up 80% of the sample) (Table 2, Figure 2). SLN samples 20, 4, 21, 16, and 5 had a similar Shannon alpha diversity index, with many similar taxa being represented across all samples. There was a possible indication that Tylosin and Treatment 4 influenced alpha diversity (Figure 6). SLN samples 16 and 5 from Treatment 4 and both Tylosin samples clustered on the PCoA with Shannon alpha diversity. The control SLN samples clustered with Treatment 3 SLN 38, indicating a similar alpha diversity (Figure 6). Treatment 3 had two samples with different alpha diversities and were distant from another on principle component 1, which accounted for 98.54%

of the variability (Figure 6). SLN samples 40 (control), 18 (control), 38 (Trt 3), and 24 (Trt 4) were similar in that they were clustering by principal component one. Samples from the same treatments groups did not always have the same characteristics. A larger samples size would be needed to understand treatment effects, if there are any present. Samples with high alpha diversity also had the largest proportion of rare taxa (taxa that were not included in the top 25 most common taxa in the samples).

Discussion

This study suggested that addition of a feed additive does not influence the microbiome (composition and diversity) or the prevalence of *Salmonella* in the subiliac lymph nodes of beef feedlot cattle. The high prevalence of *Salmonella* in SLNs is in agreement with previous research indicating *Salmonella* lymph node prevalence is affected by both region and season, and tends to be higher in the southern region of the United States during the warmer months (Gragg et al., 2012 and Haneklaus et al., 2012).

The microbial community of the lymph node is dependent on the microorganisms that are currently trying to infect the host, and will vary from animal to animal for this reason. This variability among animals could account for the differences in microbial communities of the different lymph nodes, since the differences could not be attributed to treatments. Additionally, it is likely that differences in the microbial community of the lymph node are probably influenced by immune status and general health of the animal.

This study, to our knowledge, was the first of its kind to assess not only the microbiome of the lymph node, but also the effect of immune modulators on the host microbial community. In that regard, we encountered several challenges which limit our study results. Although compositing of lymph nodes prior to sequencing was performed due to monetary constraints, the

potential for individual animal bias in the composite limits our interpretation of the data. One sick or immune challenged animal in the pen with a large biomass in the SLN could have dominated the sample, and eliminated the ability of the composite to represent the pen. Further, as the biomass of the SLN was relative low, using targeted extractions kits could have increased our success in sequencing the SLN samples. Nonetheless, this study provided information that is useful to understanding the complexity of microbiological approaches to assess immune system organs. Future studies, with a larger sample size, will be useful to identify the influence of pre-harvest management strategies on the lymph node microbiome

Table 1. The impact of pre-harvest feeding strategies[1] on the prevalence of *Salmonella enterica* in the subiliac lymph nodes of feedlot beef cattle.[2]

Treatment	Percentage (%) of Subiliac Lymph Nodes positive for *Salmonella enterica*
Tylosin	86.00
No Tylosin	83.33
Treatment 3	86.67
Treatment 4	82.00

[1] Finishing ration with tylosin, finishing ration without tylosin, finishing ration without tylosin, but with an essential oil, and finishing ration without tylosin but with Diamond V-Prototype
[2] Prevalence did not differ among treatment groups ($P = 0.8402$).

Table 2. The Shannon diversity index of pen composited subiliac lymph nodes[1]

SLN	Treatment	Shannon diversity
4	3	3.846
5	4	4.049
16	4	4.795
18	2	6.410
20	1	3.978
21	1	5.131
24	4	1.812
38	3	6.525
40	2	6.532

[1] Percentage did not differ among treatment groups ($P = 0.3108$).

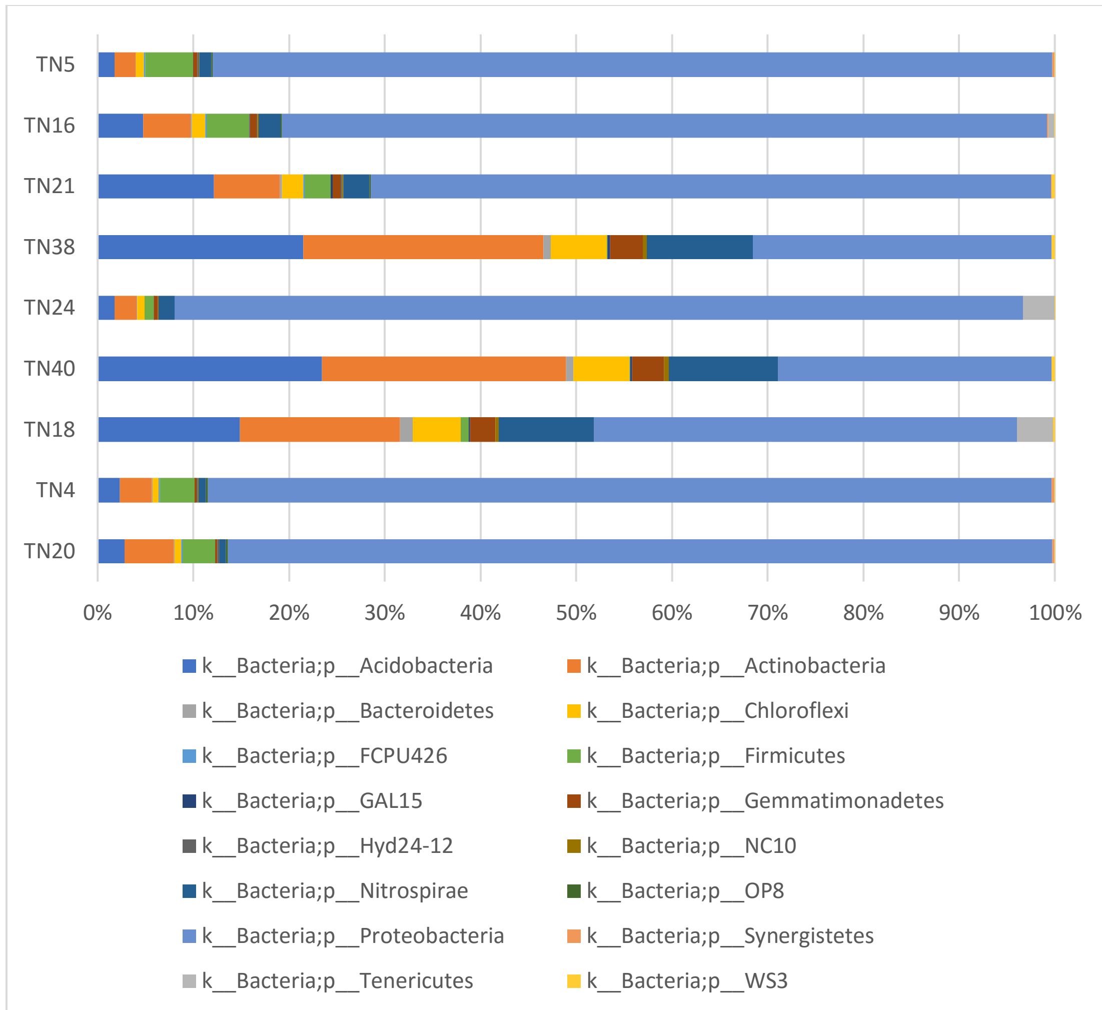

Figure 1. The phylum-level taxa plot summary of nine lymph nodes derived from feedlot cattle fed one of four feed supplements: finishing ration with tylosin, finishing ration without tylosin, finishing ration without tylosin, but with an essential oil, and finishing ration without tylosin but with Diamond V-Prototype.

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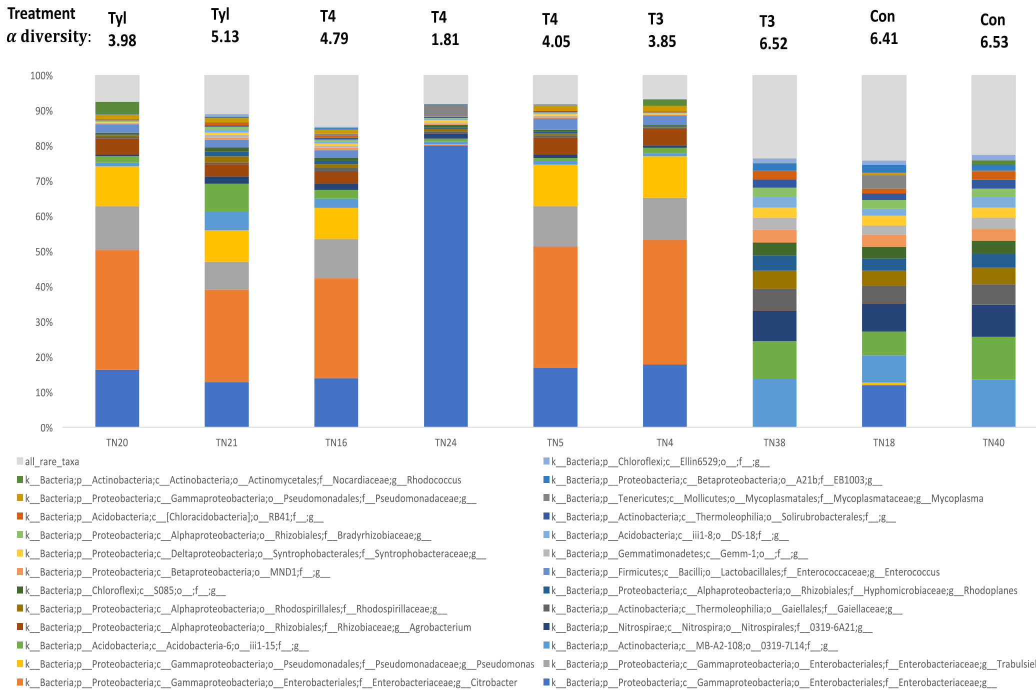


Figure 2. The genus-level taxa plot summary, and Shannon alpha diversity, of the nine lymph nodes derived from feedlot cattle fed one of four feed supplements: finishing ration with tylosin, finishing ration without tylosin, finishing ration without tylosin, but with an essential oil, and finishing ration without tylosin but with Diamond V-Prototype.

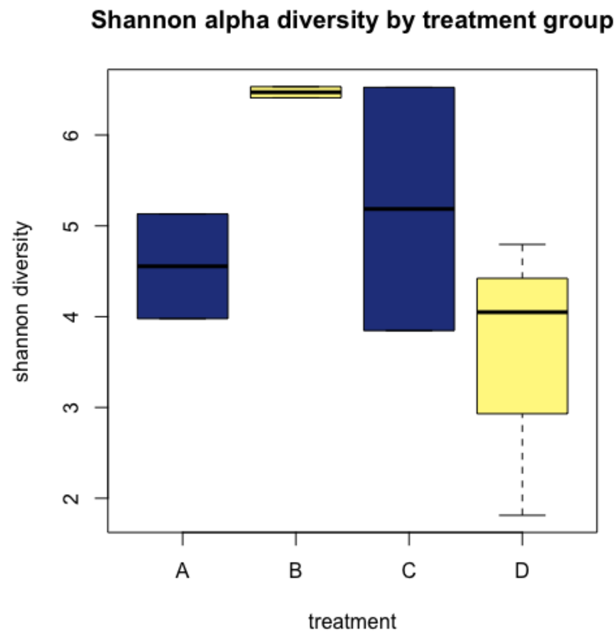


Figure 3. A boxplot of Shannon alpha diversity of lymph nodes derived from feedlot cattle fed one of four feed supplements: finishing ration with tylosin, finishing ration without tylosin, finishing ration without tylosin, but with an essential oil, and finishing ration without tylosin but with Diamond V-Prototype

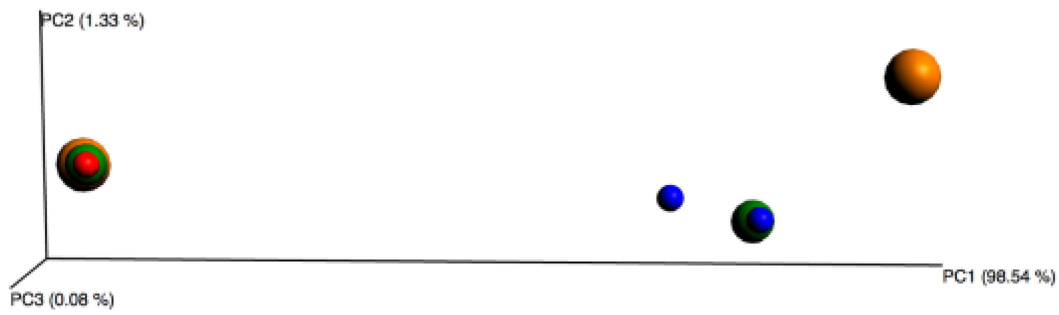
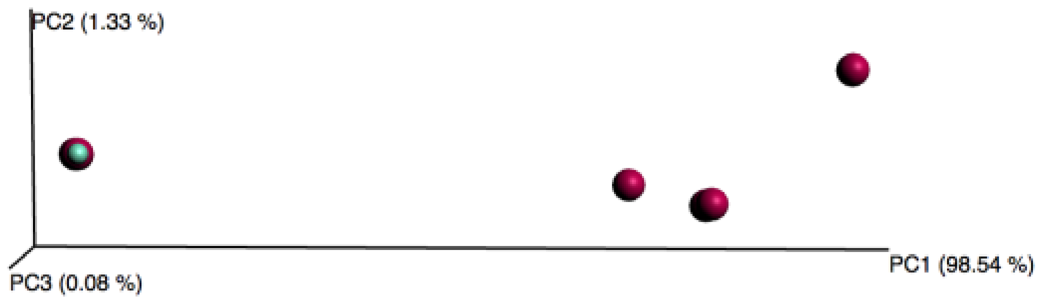


Figure 4. Principal coordinate analysis plot of lymph nodes derived from feedlot cattle fed one of four feed supplements: finishing ration with Tylosin, finishing ration without tylosin, finishing ration without tylosin, but with an essential oil, and finishing ration without tylosin but with Diamond V-Prototype, scaling is used to show overlapping samples.



= Tylosin

= No Tylosin

Figure 5. Principal coordinate analysis plot of tylosin and no tylosin (finishing ration without tylosin, finishing ration without tylosin, but with an essential oil, and finishing ration without tylosin but with Diamond V-Prototype) treatment groups, scaling is used to show overlapping samples.

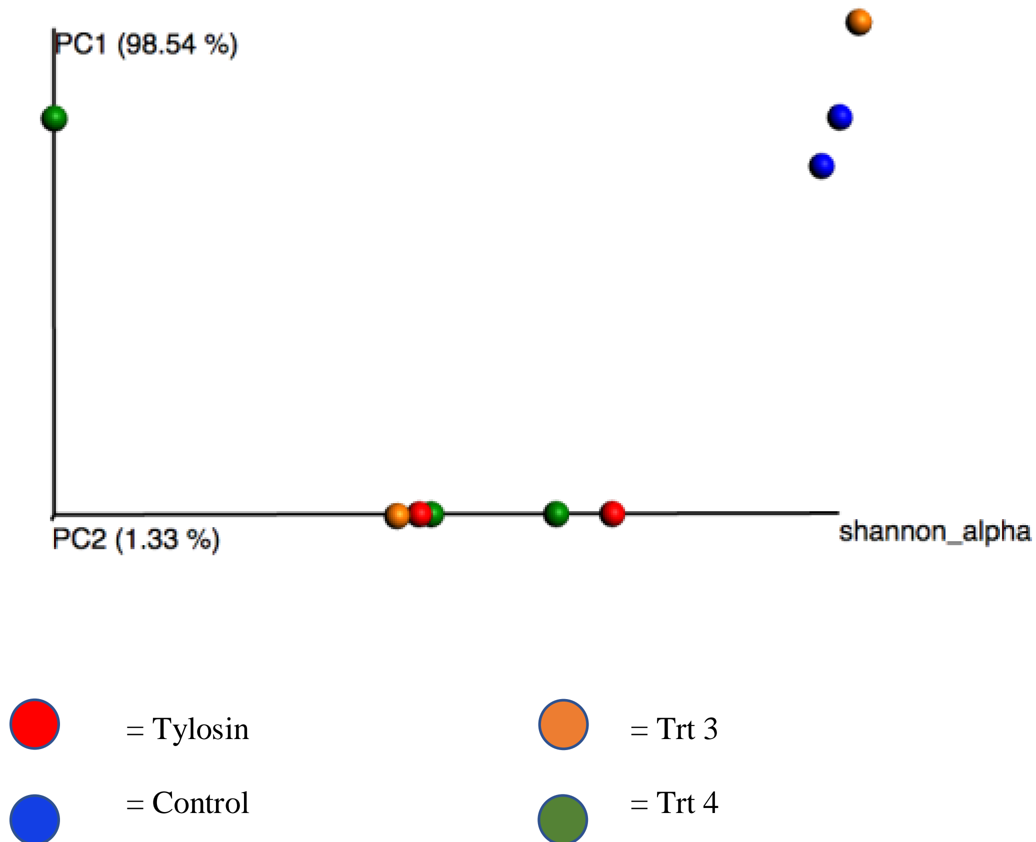


Figure 6. Principal coordinate analysis plot of lymph nodes derived from feedlot cattle fed one of four feed supplements: finishing ration with tylosin, finishing ration without tylosin, finishing ration without tylosin, but with an essential oil, and finishing ration without tylosin but with Diamond V-Prototype including Shannon alpha diversity

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