Taxonomic Profiling of the Lumen and Epimural Surface of the Duodenum, Rumen and Ileum Using a Cannulated Bovine Model

by

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ABSTRACT

The gut microbiome provides important metabolic functions for the host animal. Bacterial dysbiosis as a result of bacterial, viral, and parasitic gastrointestinal infections can adversely affect the metabolism, productivity, and overall health.

Although there are many peer reviewed studies focusing on the rumen microbiota and the impact on meat and milk production, limited information regarding the microbial populations in the upper small intestine of the bovine is available in the literature, especially studies that were conducted in the live animal.

The ruminant gastrointestinal microbiome grants many physiological and unique functions that are considered essential to maintain overall homeostasis. The author hypothesizes that there are differences in the taxonomic distribution of the commensal microbiome between the lumen and the epimural surface of the bovine duodenum, rumen and ileum. This project involved the surgical fitting of ruminal, duodenal, and ileal indwelling cannulas to allow sample collection using a flexible video-endoscope, with the research units alive and undergoing ordinary husbandry. In the first study, using 16S rRNA gene phylogenetic analysis, the commensal microbiome present in the lumen and the mucosal surface of the duodenum of cattle was characterized and compared providing a detailed descriptive analysis from phylum to genus taxonomic level. Distinct differences in diversity and distribution of the microbiome were found between the luminal and mucosal biopsies at the phylum and lower taxonomic levels.

In the second study, using the same technique, the commensal microbiome present in the lumen and the epimural surface of the rumen epithelium was characterized providing a detailed descriptive analysis from phylum to genus. A great and significant diversity of

microbiota was found between the two locations. In agreement with the literature, the *Firmicutes* and *Bacteroidetes* bacterial phyla composed over 80% of the microbiome in the lumen contents whereas the *Firmicutes*, by itself, composed over 90% of the microbiome in the epimural surface of the rumen. Further analysis at lower taxonomic levels, class, family and genus, showed similar diversity and even distribution between the animals and throughout the study weeks.

The technique validated by the first study, allowed the third study to mature. The objective of this study was to characterize the commensal microbiome present in the lumen and the mucosal surface of the ileum of cattle, as the ileum is the location of where many relevant infectious intestinal diseases occur. *Firmicutes* was the most predominant phyla in luminal and epimural locations followed by *Bacteroidetes*. The *Firmicutes Bacteroidetes* ratio varied between the animals, however, remained relatively consistent across individual cattle throughout the study period. In contrast with the rumen and duodenum, there appears to be no phylogenetic assemblage amongst the two collection sites despite the variation among the animals.

Additionally, the author also hypothesize that the use of a systemic antimicrobial therapy does alter the microbiome characteristics associated with the lumen and epimural surface of the bovine ileum therefore, the microbiome was characterized following on-label administration of an approved systemic antimicrobial commonly used in bovine medicine.

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Chapter 1

INTRODUCTION

The understanding of the microbial composition of the gastrointestinal tract (GIT) is imperative to the long-term health and performance of livestock. The GIT microbiome is responsible for a number of physiological and functional processes, including nutrient digestion and absorption, host metabolism, mucus layer development, barrier function, and mucosal immune responses (Kogut and Arsenault 2016). Disruption of intestinal microbiota homeostasis, termed dysbiosis, can occur as a result of bacterial, viral, and parasitic gastrointestinal pathogenic infections, as well, as due to dietary disorders adversely affecting host metabolism and productivity.

While the characterization of the ruminal and fecal microbiome and its impact on bovine health and production have been previously investigated, the majority of studies examined only intraluminal samples harvested post-mortem. The bovine luminal and mucosal-associated microbiota has not been characterized concomitantly, particularly in the live animal, as this is relevant due to most metabolically-active processes occurring at the mucosal interface.

Chapter 2 of this dissertation reviews the literature pertaining to overall anatomy and basic function of the rumen, duodenum and ileum. In addition, chapter 2 will provide a general review of the most relevant microbiome studies and analysis methods, including metagenomics and microbiological culture, and will conclude with gastrointestinal microbiome peer reviewed studies involving cattle. Chapter 3 presents a profile of the luminal and mucosal microbiome of the rumen. The objectives of this study were to characterize *in vivo*, with the cattle in its normal and expected husbandry, the microbiome present in the lumen of the rumen and in the mucosal surface. Following the same

principles, chapter 4 presents the study where the microbiome associated with the duodenum was determined; chapter 5 describes the commensal microbiome associated with the lumen and mucosal surface of the ileum.

Chapter 6 addresses the potential impact on the microbiome associated with the ileum after the label use of a systemic antimicrobial commonly used for respiratory disease in livestock medicine. The microbiomes of the lumen and mucosal surface were profiled and assessed for changes due to the use of the systemic antimicrobial. Plasma, tissue, and lumen fluid antimicrobial concentrations were determined using HPLC-MS and compared with the microbiome profile during and after treatment. To close, chapter 7 provides conclusions about the study results and suggested directions for future research. The overall goal of this research was to add to the literature knowledge about the GIT microbiome in cattle by utilizing a unique approach and sample collection technique while healthy cattle were undergoing their normal and expected husbandry, as well as, to validate the use of a cannulated bovine model to aid future microbiome studies.

Chapter 2

LITERATURE REVIEW

A. Rumen, Duodenum & Ileum Basic Anatomy & Function

The gastrointestinal (GIT) anatomy of ruminants consists of four compartments: rumen, reticulum, omasum and abomasum. The rumen is known to be the main stomach, often referred as the first compartment, and serves as the primary site for pre-gastric degradation and fermentation of feedstuff. As pre-ruminants, the neonatal rumen develops anatomically in size, structure, and microbial activity as they age and especially as calves adapt from a liquid diet, consisting mainly of milk, to dry forages and concentrate.

In adult cattle, the first forestomach has an average of 50 gallons in volume occupying the left side of the abdominal cavity. Millions of microorganisms are found in the rumen and include bacteria, archaea, viruses, fungi, and protozoa. The ruminal fauna are responsible for digestion of feedstuff by the production of many enzymes that are vital in the breakdown of plant lignocellulolytic and nonstructural carbohydrates, nitrogen compounds, and lipids (Russell and Rychlik 2001). The microorganisms are utilized in a symbiotic host-microbe and microbe-microbe relationship, making the rumen the most important site for microbial activity and fermentation (Weimer 2015).

Microbial fermentation of fiber and non-structural carbohydrates is the mainstay of digestion in ruminants. As a product of a healthy fermentation, volatile fatty acids (VFAs) are the primary end products. The capacity to produce microbial protein from non-protein nitrogen sources and feed proteins are some characteristics unique to ruminants. Symbiotic living of the rumen microbiome is the backbone of all digestive and

fermentation activities within the forestomach and is vital for maintenance of all systems' homeostasis leading to growth, performance, and production (Bath et al. 2013).

The duodenum begins at the pylorus and is the first part of the small intestines receiving the ingesta from the abomasum after undergoing enzymatic digestion. The main unique feature of the duodenum is the presence of Brunner's glands within the submucosal layer. The Brunner`s glands are responsible for secreting mucin glycoproteins, an alkalinizing agent, to neutralize the prevenient acidic gastric secretions from the abomasum. In addition, the duodenum is responsible for mechanical processing and continuing digestion by mixing bile and pancreatic enzymes, as well as, absorption of electrolytes and water-soluble nutrients (Krause 2000).

Aborad from the duodenum is the jejunum and the ileum. The ileum is the last segment of the small intestine and is anatomically separated from the large intestines by the ileocecal valve. The unique feature of the ileum is the presence of Peyer`s patch (aggregated lymphoid nodules) within the mucosal layer, composing the mucosa associated lymphoid tissue (MALT), responsible for induction of immunological tolerance by accessing and transferring antigens independent from systemic immune response (Girard et al. 2001). MALT is involved with bacterial and viral invasion as it is the portal of entry for the most common intestinal pathogens in cattle such as *Salmonella* and *Mycobacteria paratuberculosis*, as well as, the location of viral replication for pestivirus and herpesvirus (Boes and Durham 2017). Aside from this exceptional immunological characteristic, nutritionally the ileum is responsible for enzymatic cleavage of nutrients and absorption of vitamin B12, certain fatty acids, glycerol, and bile salts.

B. Microbiome: Overview

In an article published by Lederberg and colleagues in 2001, the term microbiome was used to name the ecological community of commensal, symbiotic, and pathogenic microorganisms that act as determinants of health and disease (Lederberg and McCray 2001). Similarly, Marchesi and Ravel, describe the term microbiome as the entire habitat, including the bacteria, archaea, lower and higher eurkaryotes and viruses, their genomes and the surrounding environmental conditions. In addition to data collected via clinical and environmental studies, the microbiome can be studied, characterized and analyzed via metagenomics, metabolomics, metatranscriptomics, and metaproteomics (Marchesi and Ravel 2015). Primarily, metaproteomics enables functional activity data to be gained from the microbiome samples, while metabolomics provides analysis into the overall metabolic states with respect to the host–microbiome interactions (Peters et al. 2019). In contrast, metagenomics and metatranscriptomics involve the analysis of genetic materials in a community of organisms. While metagenomics allows to obtain a taxonomic profile of the sample, metatranscriptomics provides a functional profile by analyzing which genes are expressed by the community (Aguiar-Pulido et al. 2016).

The modern taxonomic classification system was originally hypothesized in the mid-1700`s by Carl Linnaeus. The Linnaean classification system suggests that species with similar characteristic traits were more likely to be related and should therefore be placed more closely in a taxonomic rank. Examples of traits include cell wall type and shape, oxygen and energy requirements, motility, reproduction ability, and glucose fermentation activity. Linnaeus' hierarchical system includes seven levels called taxa. The classification categories, from broadest to most specific are: kingdom, phylum, class,

order, family, genus, and species; and is the classification system most commonly used to date (Kuntner and Agnarsson 2006).

The foremost characterized effect and function of the gut microbiome on the host is related to energy extraction from feedstuffs, production of essential by-products such as vitamins, defense against pathogenic organisms, and modulation of local and systemic immune system responses. In order for those to occur, it is imperative to mention that the individual digestive capacity depends on microbiome composition (Krajmalnik-Brown et al. 2012; Turnbaugh et al. 2006).

The composition of the human gastrointestinal microbiome is similar to that of the mouse microbiome. The phylum *Bacteroidetes* and *Firmicutes* are generally dominant in the gut with other phyla comprising 10% or less of the microbiome. Bacteria belonging to the phylum *Bacteroidetes* are characteristically responsible in providing the host with energy harvested from the diet through the fermentation of otherwise indigestible feedstuff. In humans, those are commonly found in the distal intestines and by the digestion of polysaccharides. The production of short chain fatty acids (SCFAs) at up to 10% of daily calories can be supplied to the body when the diet is rich in fiber (McNeil 1984). Conversely in ruminants, the bacteria belonging to these phylum compose more than 90% of the relative abundance in the forestomaches as the primary method of digestion is fermentation of plant-based diets (Magne et al. 2020; Eckburg et al. 2005; Ley et al. 2006). A positive relationship between the two main gastrointestinal bacterial phyla and energy uptake metabolism has been demonstrated as the microbiota influences the lipid metabolism via fatty acid uptake from adipose tissue (Ma, Zhou, and Li 2017; Machado and Cortez-Pinto 2012). Using mice, researchers reported that animals with relative high

abundance of *Firmicutes* have demonstrated an increased capacity to extract energy from feedstuff, whereas mice that were offered a diet limited in carbohydrates or fats showed an increased abundance of bacteria belonging to the phylum *Bacteroidetes* (Ley et al. 2006; Turnbaugh et al. 2006). Bäckhed et al. 2004, used germ free and conventionally-raised mice and allowed them to acquire gut microbiota from birth to adulthood. The results showed the conventionally-raised mice presented more adipose tissue with consequently higher body fat percentage compared to the germ-free group. Furthermore, the group harvested and transplanted cecum contents from the conventionally-raised mice into the adult, germ-free mice, and within a 2 week period, the germ-free mice had a greater than 50% increase in body fat, validating their initial findings (Bäckhed et al. 2004).

Also using mice as a model, Ley and colleagues in 2005, assigned to the study lean mice and genetically obese mice. The latter presented a 50% decrease in *Bacteroidetes* and a similar increase in *Firmicutes* abundance. These significant changes were shown to greatly impact several metabolic and digestive mechanisms associated with energy and transportation of nutrients (Ley et al. 2005).

Interactions between the microbiota and immune system have an evident impact in the host. The gut microbiome affects the innate and adaptive immune systems in humans (Mazmanian et al. 2005). In general terms, the microbiome is responsible for the training and development of major components of the host's innate and adaptive immune system, while the immune system plays a role into the maintenance of vital features of host-microbe symbiosis. This association requires the proper functioning of host immunity to

prevent commensals from over utilizing host resources while maintaining immune tolerance to innocuous stimuli (Zheng, Liwinski, and Elinav 2020).

In mice, the intestinal epithelial cells are replaced by new cells more slowly in germ-free mice than in colonized mice, as bacterial colonization is known to be a modulator of mucosal immune response and cell function (Chu et al. 2004; Guarner and Malagelada 2003). It is clear the influence of the microbiome on abnormal cell proliferation (neoplasia) and the ability to alter the capacity of cells to repair damaged mucosal barriers (Chu et al. 2004; Pull et al. 2005).

The intestinal microbiome in humans has been studied and its characteristics and importance are described in the literature in many ways (Bull and Plummer 2014; Eckburg et al. 2005; Guarner and Malagelada 2003; Human Microbiome Project 2012; Huttenhower et al. 2012; Sekirov et al. 2010). A common conclusion among the literature mentions that the precise characterization of the gut microbiome and its variants is an important step in defining healthy and unhealthy microbiomes and their positive and negative impacts in overall well-being, which largely depends on each circumstance. Intestinal dysbiosis could occur as a result of concurrent bacterial or viral disease, environmental changes, physiological or behavior alterations, or the use of therapeutic medications. Strober (2013) highlighted the role of the gut microbiome and its relationship with certain diseases in humans. The author described that humans with Crohn's disease had a selective loss of *Firmicutes* and *Bacteroidetes* organisms. Those two phyla are reported to possibly be members of the microbiota important in the induction of regulatory cells.

Immunologically speaking, the GI microbiota either controls or prevents intestinal inflammation by inducing the regulatory T cells thus playing a vital role in maintaining the overall intestinal homeostasis. The intestinal homeostasis is dependent on regulatory T cells, induced by commensal microbiota that gain entry into the lamina propria, as well as the presence of innate Toll-like receptor 2. This process occurs under normal conditions and to prevent clinical disease from occurring (Boirivant et al. 2008; Strober 2013).

The mucosa-associated bacteria in the mouse distal colon not only differed significantly from fecal bacteria but also correlated with Toll-like receptor 2 (TLR2) and TLR4 gene expression in colon epithelial cells (Wang et al. 2010). These observations indicate the importance of studying bacterial segregation between mucosal surfaces and digesta throughout the GIT to better understand host-microbe interactions (Malmuthuge, Griebel, and Guan le 2014; Wang et al. 2010).

To complement the understanding of the impact of the commensal bacterial population in the intestinal immune response, another group of researchers analyzed the effect of the viral population of the microbiome, or virome, in patients with inflammatory bowel diseases, such as Crohn`s and ulcerative colitis (Norman et al. 2015). The virome is composed mostly of bacteriophages that play a direct role in intestinal physiology and the alterations associated with the bacterial microbiome (Barr et al. 2013). In the absence of disease, the intestinal bacteriophage population exhibits significant diversity between individuals and are temporally stable (Minot et al. 2013; Norman et al. 2015). Additionally, Norman and colleagues discuss that bacteriophages in the healthy human intestine are predominantly composed by double-stranded DNA (dsDNA) caudovirales or

single-stranded DNA (ssDNA) microviridae that latently infect their bacterial hosts and generate few viral progeny that may infect and kill other bacteria. This principle is important as with dysbiosis due to environmental, physiological or behavior alterations, or the use of therapeutic medications there may be induction of the production of infectious bacteriophages that would lyse their bacterial host and infect neighboring cells bearing specific receptors. The group concludes by speculating if the bacterial microbiome dysbiosis is actually a secondary response to bacteriophage proliferation based on the inverse relationship the group demonstrated between the bacterial microbiome and the enteric virome (Norman et al. 2015).

The microbiota population belonging to the *Bacteroidetes* and *Firmicutes* phylum occupy different functional niches in the gut ecosystem. As a result, differences between individuals in their relative proportion can lead to large differences in function, with relevance for host health. Johnson et.al, 2017, specifically summarized the characteristic of the *Bacteroidetes* phylum and defined its high ability to adapt to life in a rapidly changing environment. The group showed some significant variations in the intestinal microbiota abundance associated with that specific phyla due to diet change. The changes proposed were in reference to either under or over feeding, as well as, different feeding patterns. The group also detailed some associations between certain species of *Bacteroidetes* and the presence of metabolic diseases, such as, diabetes and obesity (Johnson et al. 2017).

Along the same principles, Gagnière et al. 2016, reported the relationship between gut microbiome and colorectal cancer in humans. The group described a positive and important relationship between the evident microbiome variations as a result of such

disease likely due to its impact on several inflammatory, metabolic, and genotoxic mechanisms (Gagnière et al. 2016).

Using surgical biopsies of the colon mucosa of humans with inflammatory bowel disease, a depletion of *Bacteroidetes* and *Firmicutes* in comparison to an increase of bacteria belonging to the *Firmicutes* phylum, *Proteobacteria* and the *Bacillus*, was reported (Frank et al. 2011). Moreover, the abundance of bacteria belonging to the Bacillus genus of the *Firmicutes* phylum was decreased, and the *Proteobacteria* were increased in the small intestines of humans relative to controls; conversely, the *Bacteroidetes* were unchanged (Frank, Robertson et al. 2011).

In animals, specifically ruminants, many studies have been published focusing on the rumen microbiota and the impact on meat and milk production. Rumen microbiota consists of bacteria, archaea, protozoa, and fungi involved in the fermentation of complex carbohydrates, and their composition is influenced by a number of factors (Malmuthuge, Griebel, and Guan le 2015). In contrast with the gastrointestinal tract of humans, the anaerobic bacteria dominate in the rumen of neonatal ruminants by the second day of life (109 CFU/ml of rumen fluid), and specifically the density of cellulolytic bacteria stabilized (107 CFU/ml of rumen fluid) within the first week of life. Also, anaerobic fungi and methanogens appear in the neonatal rumen between 8 and 10 days post-partum while protozoa appear only after 15 days post-partum (Malmuthuge, Griebel, and Guan le 2015; Fonty et al. 1988). The breakdown of carbohydrates and complex plant materials starts in the rumen with nutrient absorption extending from the forestomaches throughout the intestinal tract. As in other species, *Bacteroidetes* and *Firmicutes* are among the primary metabolically-active bacteria with a critical role in breaking down plant wall

compounds and host-derived carbohydrates, including particles attached to the mucins or chondroitin sulfates of the protective mucosal layer of the intestine (Krajmalnik-Brown et al. 2012).

In the adult ruminant, the microbiota composition, specifically the *Firmicutes Bacteroidetes* ratio (F:B), of the rumen varies according to the species, diet, host, age, season and environment. Those two phyla dominate the rumen microbiome responsible for digestion of feed via fermentation and, as a product, the production of volatile fatty acids and microbial protein (Malmuthuge, Griebel, and Guan le 2015). The presence of VFAs (acetate, propionate and butyrate) in the rumen plays an important role in rumen development, especially the development of rumen papillae (Lane and Jesse 1997; Malmuthuge, Griebel, and Guan le 2015).

The composition of the commensal rumen microbiome plays an important role in nutrient and energy extraction and its impact on health, nutrition, and host physiological parameters have been studied (Creevey et al. 2014; Firkins and Yu 2015; Jami and Mizrahi 2012b, 2012a; Krajmalnik-Brown et al. 2012; Welkie, Stevenson, and Weimer 2010).

Changes in the F:B have been demonstrated to affect energy uptake from the diet and energy expenditure which contributes to the development of obesity in pigs, mice, and humans (Krajmalnik-Brown et al. 2012; Pedersen et al. 2013). Aside from variations due to diet changes impacting this ration, which is considered the most common cause, another example published in 2014, was a meta-analysis revealing that the F:B was higher in the populations living at high latitudes, suggesting that extraction of energy from food by the gut microbiota could be greater in these regions, concluding that the gut

microbiota contributes to the adaptation to cold exposure through its ability to harvest energy (Suzuki and Worobey 2014).

Several peer-reviewed studies have been undertaken to analyze the function and describe the GIT bacterial communities in different production animals. The studies were typically conducted in animals shortly after euthanasia, from samples collected at slaughterhouses, from animals reared in sterile laboratory environments, or from animals that received a known, transplanted microbiome. The major limitation to the performance of microbiome studies in production animals has to do with cost (example: laboratory quality animals), sample collection method in the live animal, and even the complete loss of that production unit due to euthanasia and not harvesting for human consumption. Other factors that are known to play a role in microbiota studies are those associated with the potential disruption of the commensal microbiome such as dietary and metabolic changes, infection, and/or inflammatory processes and death as there is a likelihood of changed bacterial populations as a consequence of tissue death (Donaldson and Lamont 2013; Hanning and Diaz-Sanchez 2015).

The most immediate biochemical change that occurs postmortem is a fall in the concentration of oxygen due to absence of circulation. The availability of oxygen in localized regions of the intestine is assumed to have important biological significances. It has been suggested that the aerobic region within the intestines might be related to the outcome of interactions with the gut microbiota, acting as an innate immune barrier to protect the mucosal surface from anaerobic bacteria, while being recognized as a signal to promote invasion by pathogens (Marteyn et al. 2010). This concept supports the

hypothesis that potential differences exist in bacterial abundance between the mucosal surface and the luminal contents of different locations of the GIT.

C. Microbiome analysis methods

i. Culture

Cultivation-based studies on rumen bacteria have made important contributions to describing activities of pure or mixed cultures although with inherent limitations. Cultivation-based studies are needed to definitively determine the metabolism, physiology, and ecology of novel groups characterized on the basis of only rRNA gene sequences (Creevey et al. 2014; Firkins and Yu 2015).

Creevey and colleagues (2014), published a survey of cultured rumen bacteria from culture collections, scientific literature, and public databases. The results noted a diverse population in 88 existing known genera belonging to 9 phyla predominating. *Firmicutes* (45 genera), *Proteobacteria* (20 genera), *Actinobacteria* (11 genera), and *Bacteroidetes* (6 genera) were found. The group also reported that only 146 bacterial cultures are archived in five major international culture collections from the same location. Interestingly, *Bacteroidetes*, was particularly poorly represented in those culture collections.

The main disadvantage of culturing, noted by Eckburg and group, is that even though the techniques involving culturing always involve specific selective media and anaerobic incubation conditions, there is still a large percentage of the microbiota that will not be isolated (Eckburg et al. 2005). However, it is important to make clear that while there are limitations, the main groups of opportunistic bacteria known to be clinically relevant, such as bacteria belonging to the genera *Bacteroides*, *Streptococcus*, *Enterococcus* and

Staphylococcus and the family *Enterobacteriaceae*, can be routinely recovered on culture media.

The ability to isolate pure cultures is still recognized to be the main advantage of this method (Sommer 2015). With the understanding that less abundant GIT bacteria can be overlooked by deep shotgun sequencing, Raymond and colleagues, investigated the gene content recovered from these culture-enriched and culture-independent metagenomes with their taxonomic origin from human. Their analysis identified 187 bacterial species with an assembly size greater than 1 million nucleotides. Of these, 67 were found only in culture-enriched conditions and 22 only in culture-independent microbiomes (Raymond et al. 2019). Similarly, Lagier et al, 2012, using 212 different specific cultivation conditions selected to simulate the gut environment and analyzed fecal samples. The authors isolated over 30,000 colonies comprising over 300 different species. Notably, over half of the species identified in this study had not previously been identified in the human gut, including a number of entirely new species and genera. The authors compared the taxonomy of the cultured isolates to that resulting from 16S rDNA sequencing of uncultured samples and found that only 51 out of a total of 571 species identified overlapped between the two approaches highlighting the need for using both cultivation-based and cultivation- independent approaches to study the gut microbiome (Lagier et al. 2012).

The major take home message from these study examples related to the culture approach was that different metabolic enzymes, associated with certain taxa, and specific culture conditions are necessary for isolation; thus, the benefit of adding a culture-based

approach to metagenomics sequencing appears to be appealing (Lagier et al. 2012; Lau et al. 2016; Raymond et al. 2019).

ii. Metagenomics

The microbial diversity undetermined by culture methods can typically be characterized using molecular techniques. Phylogenetic sequencing is often used to profile host-associated microbial communities. Culture-independent techniques, such as whole metagenome shotgun (WMS) and amplicon 16S rDNA sequencing, are molecular methods capable of providing a direct and detailed understanding of the microbiome composition; however, it is imperative to mention that there is evidence that these methods may lack in the detection of low-abundant organisms (Hiergeist et al. 2015). Metagenomic methods are useful for tracing changes in the microbiome, over time, and identifying differences in bacterial communities in different locations and or potential impact of other variables such as environmental or therapeutic changes.

Function-based and sequence-based techniques are the two known available methods of metagenomics. Function-based metagenomics relies on cloning environmental DNA into expression vectors and propagating them in appropriate hosts. Once the clone is determined, the gene of interest is further analyzed for its biotechnological potential. Sequence-based metagenomics is applied using prior knowledge on proteins, allowing for a screening of genes that are predicted to encode proteins indicative of their functionality (Chistoserdova 2010).

Whole genome shotgun (WGS) sequencing and 16S based metagenomics are generally the method of choice when researchers are looking for a fast and all-inclusive culture-independent analysis of microbial diversity that also correlate with physiological

parameters. A major advantage of WGS sequencing is the ability to sequence broad regions of the genome; while 16S-based methods only sequence a single region of the bacterial genome (Ranjan et al. 2016).

In recent years, amplicon analysis of the 16S ribosomal DNA (rDNA) gene has been the hallmark method to accurately identify bacterial isolates. Most recently, this methodology was used to analyze and collect the microbiome data used by the Human Microbiome Project (Human Microbiome Project 2012).

The 16S rDNA, which codes for the subunit of ribosomal RNA, is present in all prokaryotic cells. The 16S subunit is the most widely used informational macromolecule for bacterial systematic studies at the family, genus, species, and subspecies levels. This type of sequencing is predominantly useful to characterize bacteria that are rare, slow-growing, uncultivable, from culture-negative infections, and display unusual phenotypic trait. A classic example is the *Xylella fastidiosa*, a nutritionally fastidious, pathogenic plant bacteria that is aerobic, Gram-negative, and infects plants by forming a biofilm in its host which compromises several plant defense mechanisms (Chen et al. 2000).

Most recently, 454 pyrosequencing samples using specific bar codes to identify samples have provided more in-depth information about the impact of antibiotics on specific phylogenetic groups of the gut microbiota (Jernberg et al. 2010; Dowd et al. 2008).

Amplicon sequencing of the 16S rRNA gene is the most commonly used strategy to study GIT microbiome in animals. Specifically, in cattle, the rumen microbiome has been characterized in several circumstances using 16S rRNA gene compared with shotgun sequencing. McCann at al. 2017, stated many drawbacks in planning and conducting GIT microbiome studies, such as challenges with sample collection and handling, DNA

extraction bias, primer selection, chimeric sequences, operational taxonomic unit cutoffs, taxonomic assignments, and statistical methodology (McCann, Elolimy, and Loor 2017). The expected development of future technologies will likely replace the current techniques and will fill the gaps between 16S rRNA amplicon sequencing and shotgun metagenomics to allow for a more refined description of microbial communities. Between the current methods and the development of new culture-independent approaches, more information on bacterial diversity, bacterial function, and their interaction will be offer more clarity on how they contribute to the GIT function and protection.

It is vital to mention that even though Polymerase Chain Reaction (PCR) is an essential procedure when performing 16S rRNA analysis to profile microbial communities, it is known to be a common source of systematic and unevenness amplification bias (Silverman et al. 2019). As summarized recently, bias in metagenomics sequencing analysis commonly occurs due to the disproportion in the taxa analyzed and reported over others (McLaren, Willis, and Callahan 2019). To exemplify, the same group, used mocked data to show a significant qualitatively incorrect conclusions associated with the data analysis (McLaren, Willis, and Callahan 2019).

In early 2000`s, a review article detailed the four most common areas PCR bias can occur: (1) differences in the primer used, (2) irregular ratio of mixture of genes in final PCR product regardless of initial ratio of templates, (3) cloning of heteroduplexes where the outcome shows a fake sequence wrongfully increasing the diversity, and (4) the formation of chimeras from an incomplete primer or by primer extensions (Kanagawa 2003). Laboratory assay error, sample handling (Bahl, Bergström, and Licht 2012) and

post sequencing data processing error are other known areas where biases can be introduced.

The addition of calibration curves which allows bias to be characterized directly from the host associated microbial communities without the need to create artificial standards, the utilization of modeling methods, and close monitoring of the PCR product to avoid reproducible events, are some methods used to mitigate biases (Silverman et al. 2019; Lee et al. 2008).

D. *Overview of Bacterial Function*

The GIT commensal microbiota mainly supports the dietary metabolism, prevention of colonization of pathogenic microorganisms and the intestinal immune function (Hooper and Gordon 2001).

Ruminants benefits from the bacterial community in the GIT to degrade otherwise indigestible substrates to produce metabolites, such as, enzymes with essential roles in the breakdown of plant ligno-cellulolitic and nonstructural carbohydrate (starch, sugars), nitrogenous compounds (plant protein, amino acids, urea), and lipids are produced. Additionally is well described in the literature that the gut microbiota is known to protect the host from pathogenic bacteria by reducing availability of substrates produced by certain groups of bacterial communities (Maynard et al. 2012; McCann, Elolimy, and Loor 2017).

Certain bacterial communities do not only affect the host but also each other. This can occur through production of toxins or via the competition for available substrate (Flint et al. 2007). Cross-feeding also occurs when some metabolites, as lactate, are subsequently

utilized by other bacteria in the ecosystem to enhance its survival (Flint et al. 2007; Flint et al. 2012).

The gut microbiota has also been shown to positively impact the lipid metabolism by suppressing the inhibition of lipoprotein lipase activity in adipocytes (Hooper and Gordon 2001). Also, to provide a healthy metabolome, the commensals are in general responsible for increasing the concentrations of pyruvic acid, citric acid, fumaric acid and malic acid in serum, all of which are known to be indicators of higher energy metabolism (Velagapudi et al. 2010).

Certain bacteria belonging to the phyla *Firmicutes* are able to produce butyrate, which at low concentrations have been shown to increase epithelial barrier function, whereas bacteria belonging to the phylum *Bacteroidetes* generally are responsible for the production of lactate and short chain fatty acids (SCFA) (Peng et al. 2007). Specifically, members of the genus *Bacteroides*, are the predominant organisms that participate in carbohydrate metabolism. This type of bacteria are able to accomplish this function due to its capability to express enzymes such as glycosyl transferases, glycoside hydrolases and polysaccharide lyases (Hooper and Gordon 2001).

In the ruminant species, the production the volatile fatty acids (VFA) as a by-product of fermentation is not only important for ensuring availability of energy for microbial growth but also to provide substrates that are essential for gluconeogenesis and lipid metabolism. Microbial metabolism of nitrogen containing compounds is essential for the synthesis of microbial protein that in turn serves a source of amino acids for protein production, such as muscle and milk (McCann, Elolimy, and Loor 2017). In addition to VFA`s, carbon dioxide and hydrogen are also produced as by-products of fermentation

and used as sources of energy by methanogens and acetogenic microbes to synthesize methane gas and acetate respectively (Morgavi et al. 2010).

It is clear the importance of understanding the functions associated with different groups of bacteria. The bacterial diversity in different locations of the GIT in ruminants is substantial, therefore determining the commensal microbiome in vivo and further study their associations with specific metabolic processes is imperative to fully understand the functional significance and their role in the GIT homeostasis.

E. Gastrointestinal Microbiome Studies in Cattle

Enteric diseases in cattle are known to be one of the major contributors, along with bovine respiratory disease, of decreases in feed consumption, slow weight gain, reduction in milk production in dairy cattle, and deaths of youngstock which results in severe economic losses in the dairy and beef industries (Goto et al. 2020). Beef producers attributed 16%, 18%, and 2% of overall mortality to digestive disease in calves less than 3 weeks old, calves older than 3 weeks old, and breeding age cattle, respectively (Firkins and Yu 2015).

A full understanding of the GIT microbiome in cattle is still unrealized. While the characterization of the ruminal and fecal microbiome and its impact on bovine health and production have been previously investigated, the majority of studies examined only intraluminal samples harvested post-mortem (Liu et al. 2016; Mao et al. 2015; Azad et al. 2019).

Various studies have shown the impact bacterial populations have on feed efficiency, growth, and performance of the host animal. Ruminal fermentation is necessary for animal growth and maintenance; hence, the rumen microbiota is essential to the animal's

wellbeing and productivity. Milk fat yield and composition have been highly correlated with F:B, as fat increases with an increase of bacteria belonging to the phylum *Bacteroidetes* in the rumen, and associations of specific rumen microbiota with high and low milk production efficiency (Jami, White, and Mizrahi 2014; Jewell et al. 2015).

With respect to the rumen, the primary phyla found in all cattle regardless of any external factors or individual characteristics consists of bacteria (80-90%) belonging to the phyla *Firmicutes* and *Bacteroidetes*. This finding agrees with current research that used either culture or genomics techniques to determine the core structure and community of the rumen microbiome (Creevey et al. 2014; de Menezes et al. 2011; McCann, Elolimy, and Loor 2017).

The efficiency of nutrient utilization can be determined by the balance of fermentation rate of products such as forages and highly fermentable carbohydrates and the consequent production of volatile fatty acids and microbial protein, which is ultimately controlled by the diverse, but stable, ruminal microbiota. Animal growth and maintenance is directly and positively proportional to a healthy and balanced rumen microbiota thus being essential to the animal's wellbeing and productivity (Hernandez-Sanabria et al. 2012; Jami and Mizrahi 2012a; Jami, White, and Mizrahi 2014; Hurtaud, Rulquin, and Verite 1993).

Research on the diversity of bacterial species between the rumen content phases (solid and liquid) have been conflicting. In a study performed by Pitta et al. 2010, significant changes in bacterial diversity among the different rumen stratifications, solid, liquid, and whole rumen, were seen in fourteen steers surgically fitted with rumen cannulas that were moved from a diet that consisted of Bermuda grass hay for thirty four days to natural

grazing of wheat forage for twenty-eight days. Further, they reported the only bacteria in common found in all fractions of the rumen regardless of diet, belonged to the phylum *Bacteroidetes*, more specifically to the genera *Prevotella* and *Rikenella* (Pitta et al. 2010). Conversely, the genera *Prevotella* and *Tannerella* were overrepresented in the liquid fraction of twelve forage-fed steers, and bacteria belonging to the phylum *Firmicutes*, class *Clostridia*, more specific the genera *Butyrivibrio* and *Blautia* were overrepresented in the solid fraction (Fouts et al. 2012).

In agreement, Henderson et al. 2013, using two different collection methods, also reported the liquid phase of the rumen contents had a higher relative abundance of the family *Prevotellaceae* and a lower relative abundance of the family *Lachnospiraceae* when compared with the total and solid rumen fractions (Henderson et al. 2013).

A study by de Menezes et al. 2011, used a rarefaction analysis to determine that the bacterial diversity was higher in the liquid phase compared to the solid phase of rumen contents. The group focused on the family level and determined a higher abundance of the *Fibrobacteraceae* (phylum *Fibrobacteres* – previously *Bacteriodetes*) in total mixed ration solid samples and the members of the propionate-producing *Veillonelaceae* (phylum *Firmicutes*) in pasture samples. The group concluded that the rumen bacteria were clearly associated with specific diets and indicated how relevant the knowledge of ruminal rumen microbial ecology impacts the feed management of livestock (de Menezes et al. 2011).

Microbial fermentation degrades feedstuff and produces end products such as short-chain volatile fatty acids, carbon dioxide, methane, and ammonia. Metabolic energy is used in the synthesis of cellular components needed for microbial growth and other functions

whereas the volatile fatty acids are largely absorbed and used as the main energy source by the host. These fermentation products have a direct effect on the host physiological parameters, such as milk production and quality (Hurtaud, Rulquin, and Verite 1993; Nagaraja et al. 1997).

Prevotella bryantii 25A is a specific strain of bacteria that is fast grower with the ability to compete for starch and is known to produce organic acids, other than lactate. Succinate is an example of a salt prevenient from succinic acid, produced by *P. bryantii* 25A, that is rapidly metabolized in the rumen to propionate. Using twelve surgically cannulated cows in early lactation, a group of researchers administered *P. bryantii* in the rumen. The results were as expected, and they reported an increased concentration of fermentation products which indicated an increased rate in feed digestion without impacting feed intake between treated and control. With respect to milk production, the group did not report an increase in production; however, they did see a positive relationship between milk fat concentrations with the treated group. These findings were in accordance with the increased acetate and butyrate concentrations in the rumen of treated cows (Chiquette, Allison, and Rasmussen 2008).

Interestingly, a few years later, Jami and colleagues (2014), did not find the same impact in milk fat caused by the *Prevotella* bacteria. Actually, they reported a negative effect; however, the authors noted that this effect was caused by the inoculation of that one specific strain and did not reflect a general modulation by the genus *Prevotella* (Jami, White, and Mizrahi 2014; Turnbaugh et al. 2006).

Nonetheless, this genus of bacteria is known to play an important nutritional role due to its versatility as it takes advantage of several different sugars, amino acids, and small

peptides that can be used to support its growth. *Prevotella* has also been found in higher abundance in the rumens of dairy cows producing high levels of vitamin B12 (Chiquette, Allison, and Rasmussen 2008; Franco-Lopez et al. 2020; Indugu et al. 2017).

Using genome sequencing method, Jami et al. (2012), characterized and compared the rumen microbiota of cattle. This group suggested the existence of a core microbiome in the bovine rumen, and even though the variability was great, the authors demonstrated a high phylogenetic correlation among the described genera with 51% similarity in bacterial taxa across samples. Taxonomic investigation showed that the dominant ruminal bacterial phyla, were *Firmicutes* and *Bacteroidetes*, representing 42% and 51% of total operational taxonomic units (OTUs), respectively, totaling over 90% of the bacterial reads. In addition to approximately 5% of the reads being attributed to the phylum *Proteobacteria*, *Actinobacteria* and *Tenericutes* represented just under 1% of bacterial reads. An interesting finding was the identification of the genus *Prevotella* (phylum *Bacteroidetes*) being present in the overall rumen bacterial community across all the samples. The *Prevotella* accounted for an average of 52% of all rumen bacterial genera, 80 OTUs out of 157 OTUs shared by all samples. This finding was in agreement with other studies that identified this type of bacteria as an important player in energy production and metabolism (Jami and Mizrahi 2012b).

In another study, the same researchers examined the rumen microbiome in lactating cows. Using automated ribosomal intergenic spacer analysis (ARISA) and quantitative real-time PCR analysis of specific bacterial 16S RNA genes, they analyzed the similarity of bacterial populations from 16 animals with surgically-fitted rumen cannulas. The results were consistent with those of the first study, as they also demonstrated the

presence of a core microbiome in the rumen, highlighting the fact that 32% of the OTUs found were shared by at least 90% of the animals in the study and 19% of the OTUs were common to 100% of the animals (Jami and Mizrahi 2012b, 2012a).

A study in 2014, showed a positive relationship between the rumen microbiome and certain physiological parameters in the lactating dairy cow. The group reported a strong correlation between milk fat yield and the *Firmicutes* to *Bacteriodetes* ratio (F:B) present in the ruminal contents. Such results agree with studies that show a similar relationship to affect energy harvesting and body fat in humans and mice. This finding mirrors that in mice, where a decreased amount of *Bacteroidetes* in the microbiota was correlated with increased fat in the blood and tissue (Ley et al. 2006; Turnbaugh et al. 2006; Jami, White, and Mizrahi 2014)

Earlier in 2005, it was reported that members of genus *Rosburia* are affected by changes in pH in the rumen (Walker et al. 2005). The same was found by Jami et al., where they reported a great variation in ruminal pH between cows and significant positive correlations between four genera, all belonging to the order *Coriobacteriales*, and milk-lactose content and positively correlating with average milk yield (Jami, White, and Mizrahi 2014). Considering variations between anatomical locations and sampling times, Li revealed a high similarity, over 90%, in the rumen microbiome within an individual cow across different sampling times and anatomical location; however, a lower similarity, approximately 85% between the different cows sampled on a controlled diet (Li et al. 2009).

Looking at different dietary sources, Tapio et al. 2017 studied the effects of forage to concentrate ratio and sunflower oil supplement on the ruminal bacteria, archaea, ciliate

protozoa, and fungi communities in dairy cattle simultaneously. Plant oils are known to be an effective feed additive to mitigate methane emissions in livestock. In agreement with previous publications, both forage to concentrate ratio and oil supplements have been demonstrated to alter protozoa, change methanogen diversity, and affect bacterial or fungal community structure; however the literature makes it clear that details on how lipids affect the entire rumen microbial community is still unknown (Ivan et al. 2001; Popova et al. 2011; Tapio et al. 2017).

Using 16S rRNA gene sequencing of rumen digesta, Snelling et.al 2019 looked into the effect of high concentrate diets, typically fed to finishing beef cattle, on the microbial diversity in the rumen, finding a positive correlation between composition and feeding scheme between animals fed basal diets that are largely driven by the reduction of fiber degrading microbial groups and specifically an increased relative abundance of an unclassified Gammaproteobacteria OTU in the high concentrate fed animals. Interestingly, the group also measured the response and stability of the microbial community over the time course of the experiment, and it appears that there is a continuing adaptation of the microbial community up to 25 days in the high concentrate groups (Snelling et al. 2019).

Kumar et al., 2015 demonstrated diet and age effects on fungal, bacterial, and archaeal taxa co-occurrence in dairy cows, suggesting that biotic and abiotic factors affecting rumen microbial community function still need to be better understood (Kumar et al. 2015).

Because of the rumen's known contribution to nutrient availability and methane production, many studies have concentrated on the rumen and less research has been

conducted on the remainder of the GI tract. Very little is known about the bovine small intestine intestinal microbiome, especially at the mucosal level, and particularly in the live animal; this is relevant since most metabolically-active processes occur at the mucosal interface.

Most recently, Freetly and colleagues, characterize the microbiota differences throughout the digestive tract of finishing beef cattle that differed in average daily gain (ADG) with a similar feed intake. Rumen, duodenum, jejunum, ileum, cecum, and colon digesta were collected at slaughter for microbiome analysis and compared with each animal`s performance. From rumen contents, in agreement with other studies, the authors filtered OTUs belonging to *Bacteroidetes* (42%), *Firmicutes* (30%), or *Proteobacteria* (28%). In addition, OTUs sequenced from the duodenum belonged to the *Firmicutes* (86%) and *Actinobacteria* (7.2%). Although they did not report the abundance of the phylum *Bacteroidetes*, at the family level the most abundant reported was *Prevotellaceae* at a little under 90%. Interestingly, nearly 40% of the *Firmicutes* in the duodenum were unassigned to a family level indicating the lack of understanding and information regarding the microbial populations in the upper small intestine of the bovine. Further into the small intestines, the characterization of the microbiome from ileal contents revealed 55% of *Firmicutes* and 38% *Bacteroidetes* followed by *Proteobacteria* and *Actinobacteria* in smaller richness (Freetly et al. 2020).

With a different approach in reference to ADG data collection, the authors were in agreement with recent rumen microbiome studies. The ones that looked into the relationship between feed efficiency and intestinal microbiome (Jami and Mizrahi 2012b; Jami, White, and Mizrahi 2014; McGovern et al. 2018; Paz et al. 2018; Jami and Mizrahi

2012a) suggest that even though their results did not support the hypothesis, the shift of phyla seen from rumen to lower GI tract may be associated with animal performance (Freetly et al. 2020).

Synthesis of cyanocobalamin (Vitamin B12) in the rumen is largely influenced by diet composition. Franco-Lopes et al. 2020 examined the microbiome of the bovine rumen, feces, and milk and attempted to understand how the bacterial communities at each site affected the metabolism of vitamin B12. To study the topic, the authors collected 92 rumen, blood, and fecal samples and 71 milk samples from 50 Holstein dairy cows fitted with rumen cannulas. As expected, and in agreement with other published studies, the phyla *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* were identified in different abundances for each location. In lactating cows that had high ruminal concentration of cyanocobalamin, the genus *Prevotella* was found at an increased abundance, while the phylum *Bacteroidetes*, the family *Succinivibrionaceae*, and the genera *Ruminiclostridium*, *Butyrivibrio*, and *Succinimonas* were each identified at higher abundances in animals with low vitamin B12 concentrations (Franco-Lopez et al. 2020). As discussed previously, in the rumen, *Prevotella* displays direct and indirect impact on energy and nutritional resourcefulness where several different sugars, amino acids, and small peptides can be used to support its growth (Chiquette, Allison, and Rasmussen 2008; Franco-Lopez et al. 2020; Jami, White, and Mizrahi 2014).

In a clinical setting, transfaunation is a common medical therapeutic practice in food animal medicine to treat gastrointestinal related diseases such as simple indigestion, over eating disease, and anorexia due to septicemia in ruminants that may lead to considerable shifts in the microbiota profile of rumen contents. Transfaunation includes a broad

spectrum of microorganisms including bacteria, protozoa, fungi, and archaea that are transferred from the rumen of a donor to the rumen of a recipient (Azad et al. 2019; DePeters and George 2014; Pitta et al. 2016). In 2018, a group from Canada, investigated the impact of transfaunation on the rumen microbiome. Steers were selected using criteria pre-selected by the researches and fitted with rumen cannulas. While transfaunation has shown to have a positive clinical outcome (Rager et al. 2004), the results of this study showed the bacterial profile recovery patterns and extent at genus level varied among steers and each identified bacterial genus responded to transfaunation differently within each host. Further, the group showed that after the transfaunation was accomplished, the recipient's fermentation parameters remained stable indicating that the transfer procedure in the current study was completed without interfering with normal rumen functions (Zhou et al. 2018; Rager et al. 2004; Shanks 2012).

Little is known regarding the microbial populations in the upper, small intestine of the bovine. Recently, Freetly et al. 2020, noted that the ileum has the majority of the OTUs classified as *Firmicutes* followed by *Bacteroidetes* (Freetly et al. 2020). Using the same analytical methods as Freetly, Gong and associates 2019, compared the duodenal mucosal microbiota with the gastric mucosal microbiota from duodenal biopsies of human patients with intestinal metaplasia. The group stated that the mucosal microbiota might play a crucial role in gastrointestinal health as dysbiosis of the small intestinal microbiota has been found in humans with different intestinal conditions. The results showed the microbiome associated with the gastric and duodenal mucosal belonged to the phyla of *Firmicutes*, *Proteobacteria*, and *Actinobacteria* with major differences in abundance at the genus level. The group concluded that in healthy patients the gastric

mucosal microbiota was similar to their duodenal mucosal microbiota, whereas, the gastric mucosal microbiota of diseased patients differed from their duodenal mucosal microbiota indicating a potential role for duodenum microbiota in intestinal pathology (Gong et al. 2019).

In contrast with the human study, Derakhshani et al. 2016, analyzed the microbial dysbiosis of twenty-eight *Mycobacterium avium* subsp. *paratuberculosis* (MAP) positive and five control calves using 16S rRNA profiling of ileal mucosa and fecal microbiota samples collected at slaughter from dairy calves (Derakhshani et al. 2016). The ileal mucosa and fecal microbiota of either group did not differ in richness and biodiversity of microbial communities which in general terms is in disagreement with studies that showed microbial dysbiosis in cases of inflammatory bowel diseases (Norman et al. 2015; Walker et al. 2011). The authors speculate the lack of severe inflammatory responses during subclinical stages of Johne's disease due to MAP, influenced the likelihood of seeing a positive impact on the microbial population. Conversely, they were able to see positive associations between microbiota belonging to the genus *Clostridium*, as it is understood that commensal species within that class are known to be an inflammatory modulator by stimulating expression of regulatory T cells (Atarashi et al. 2011; Boirivant et al. 2008; Strober 2013).

The bovine luminal and mucosal-associated microbiota has not been characterized concomitantly, particularly in the live animal, as this is relevant due to most metabolically active processes occurring at the mucosal interface. The authors of this study hypothesize that under normal husbandry, the luminal and mucosal associated microbiota of the bovine rumen, duodenum and ileum will differ significantly in their

overall composition, as well as in their respective proportion. Further, the authors hypothesize changes in the luminal- and mucosal-associated microbiota of the bovine ileum will occur after an on-label administration of an approved systemic antimicrobial.

STATEMENT OF RESEARCH OBJECTIVES

The primary objective of this dissertation was to systematically examine the taxonomic distribution of the commensal microbiome of the gastrointestinal tract of the bovine. The research project involved the surgical fitting of ruminal, duodenal, and ileal indwelling cannulas to allow sample collection *in vivo* with the research cattle undergoing normal and expected husbandry. Description and comparison of the commensal microbial diversity was also analyzed between the luminal space and epimural surface of the anatomical locations mentioned above. Additionally, the microbiome of the lumen and mucosal surface of the ileum, specifically, was characterized following on-label administration of an approved systemic antimicrobial commonly used in bovine medicine for the treatment of respiratory disease.

Chapter 3

In Vivo Microbiome Profiling of the Luminal and Mucosal Surface of the Duodenum Using a Cannulated Yearling Bovine Model

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Introduction

The microbiota refers to the entire population of microorganisms that colonizes a specific location of the body, and includes bacteria, fungi, archaea, viruses, and protozoans (Sekirov et al. 2010). The gastrointestinal (GIT) population of bacteria, in particular, plays an important role in the dietary metabolism of the host, including nutrient metabolism and utilization. Disruption of intestinal microbiota homeostasis, termed dysbiosis, can occur as a result of bacterial, viral, and parasitic gastrointestinal pathogenic infections adversely affecting host metabolism, productivity, and overall health (Krajmalnik-Brown et al. 2012).

Enteric diseases are known to be one of the major contributors, along with bovine respiratory disease, to decreases in feed consumption, weight gain, reduction in milk production in dairy cattle, and deaths of youngstock, resulting in severe economic losses in the dairy and beef industries (Goto et al. 2020). The impact of such diseases extends to human health via the increased use of antimicrobial medications, risk of development of antimicrobial resistance, and the potential microbial contamination of human food products. Diarrhea accounted for 57% of deaths in unweaned dairy heifers according to the most recent National Animal Health Monitoring System USDA 2010 survey (Firkins and Yu 2015). Likewise, beef producers attributed 16%, 18%, and 2% of overall

mortality to digestive disease in calves less than 3 weeks old, calves older than 3 weeks old, and breeding age cattle, respectively (Firkins and Yu 2015).

In ruminants, specifically cattle, the composition of the rumen microbiota and its impact on health, nutrition, and host physiological parameters has been studied (Creevey et al. 2014; Firkins and Yu 2015; Jami and Mizrahi 2012b, 2012a; Welkie, Stevenson, and Weimer 2010). As mentioned above, metabolism of nutrients is key in the symbiotic relationship between the host and the microbiota. The intestinal microbiota is generally responsible for breaking down and metabolizing complex carbohydrates. Specifically, in ruminants, the breakdown of carbohydrates and complex plant materials starts in the rumen with nutrient absorption extending from the forestomaches throughout the intestinal tract. *Bacteroidetes* and *Firmicutes* are among the primary metabolically-active bacteria with a critical role in breaking down plant wall compounds and host-derived carbohydrates, including particles attached to the mucins or chondroitin sulfates of the protective mucosal layer of the intestine (Flint et al. 2012; Krajmalnik-Brown et al. 2012). Changes in the *Firmicutes Bacteroidetes* ratio (F:B) has been demonstrated to affect energy uptake from the diet and energy expenditure, contributing to the development of obesity in pigs, mice, and humans (Krajmalnik-Brown et al. 2012; Pedersen et al. 2013).

Several peer-reviewed studies have been undertaken to analyze the function and/or describe the GIT bacterial communities in different production animals. The studies were typically conducted in animals shortly after euthanasia, from samples collected at slaughterhouses, from animals reared in sterile laboratory environments, or from animals that received a known transplanted microbiota (Derakhshani et al. ; Freetly et al.).

However, these studies have multiple limitations including the following: cost (example: laboratory quality animals), sample collection method in the live animal, and complete loss of a production unit due to euthanasia and not harvesting for human consumption. Another factor known to influence the outcome of studies of the microbiota is the potential for disruption of the commensal microbiota through dietary changes, infection, and/or inflammatory processes. Additionally, tissue death had been demonstrated to alter bacterial populations (Donaldson and Lamont 2013; Hanning and Diaz-Sanchez 2015). A full understanding of the GIT microbiota in cattle is still unrealized. While the characterization of the ruminal and fecal microbiota and its impact on bovine health and production has been previously investigated, the majority of studies examined only intraluminal samples harvested post-mortem (Liu et al. 2016; Mao et al. 2015). To date, the bovine mucosal-associated microbiota has not been characterized, particularly in the live animal. This is relevant due to most metabolically-active processes occurring at the mucosal interface. The authors hypothesize that under normal husbandry, the luminal- and mucosal-associated microbiota of the bovine duodenum will differ significantly in their overall composition, as well as in their respective proportion. The purpose of this study was to provide a detailed analysis of the enteric mucosal microbiota *in vivo* through the use of serial mucosal biopsy and luminal samples collected endoscopically through a transabdominal-duodenal cannula surgically fitted in yearling cattle.

Materials and Methods

Animals

The study was conducted at the Auburn University College of Veterinary Medicine following approval of all procedures by the campus Institutional Animal Care and Use Committee (PRN 2015-2676). Six dairy, crossbred, healthy steers approximately 12 months of age and having an average body weight of 249 kilograms (range: 240 - 277 kilograms) were selected for inclusion in this study. All the study animals were housed in a one-acre pasture and followed a strictly controlled diet consisting of one flake of Bermuda grass hay and five pounds of soy hull pellets per head twice daily, and fresh water ad libitum.

Cannulation Model Technique

Three months prior to sample collection, the animals enrolled in the study had a T-shaped 2.5 centimeters intestinal cannula surgically fitted in the duodenum as previously outlined by Komarek (Komarek 1981). Briefly, with the animal standing and restrained in a livestock chute, analgesia of the right paralumbar fossa was achieved by regional infusion of 2% lidocaine hydrochloride. A standard laparotomy was performed followed by exposure of the pylorus to allow visualization and exteriorization of the duodenum. Approximately six centimeters aborad to the pylorus, a five-centimeter anti-mesenteric incision was made in the duodenum. The duodenal cannula was inserted through the enterotomy site and the duodenal incision was closed over the cannula using an inverted closing pattern. A 15-centimeter incision in the body wall was then made caudoventral to the last rib in order to exteriorize and secure the duodenal cannula to its final location.

The laparotomy incision was then closed using routine methods. A 7.5-centimeter rumen cannula was also surgically fitted in the rumen as previously described (Laflin and Gnad 2008) at the same time as duodenal cannulation for a concomitant rumen microbiota study. Post-operative treatment consisted of ceftiofur hydrochloride administered subcutaneously (2.2. mg/kg) once daily for five days and meloxicam administered orally (1.0 mg/kg) once daily for five days. A three-month recovery period was observed following surgery to allow complete healing of the surgical sites, ensure appropriate drug withdrawal periods were met, and provide research animals a consistent diet prior to study initiation and sample collection. Following the recovery period, all cattle were housed in the same pasture throughout the length of the study without fence-to-fence contact with other animals, and were fed a diet that remained consistent throughout the sample collection period.

Sample Collection

In order to provide consistency and assess potential variation due to individual, environmental, and bacterial factors, each animal was sampled three times over a six-week period. The order of sample collection was randomly assigned and is shown in Table 1.

For sample collection, each individual calf was haltered and restrained in a livestock chute. The duodenal cannula was opened by manually removing the compression plug. A sterile 20-centimeter Foley urinary catheter was inserted completely through the cannula aborally to facilitate the collection of 0.5 to 1 milliliters of duodenal contents; these samples were designated as lumen samples (L). Next, a flexible video-endoscope[e] was

inserted through the cannula and advanced aborally 51.1 centimeters on average (range: 35 - 70 centimeters). Three mucosal biopsy samples (B), with a total average weight of 14.7 grams (range: 0.33 - 26.4 grams), of the epimural surface were taken from each calf at each designated collection time point. All samples were placed in 750 µl of RNAlater immediately after collection, to preserve RNA integrity during storage at 4°C until processed.

Calf ID	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
69	X			X		X
70	X		X		X	
71	X		X		X	
7		X	X			X
10		X		X		X
50		X		X	X	

Table 1. Timeline for sample collection

Sample Processing

DNA Isolation

A total of 18 luminal samples and 18 mucosal biopsy samples were collected for analysis and subsequent sequencing. Isolation of DNA from all samples was extracted using a commercial kit (E.Z.N.A® Stool DNA, Omega bio-tek®, Norcross, GA) according to the manufacturer`s guidelines for DNA extraction in tissue, using glass beads, and for fluid samples. The pathogen detection protocol allows rapid and reliable isolation of purified DNA using a combination of reversible nucleic acid-binding properties of HiBind® matrix and spin column technology to allow the elimination of humic acid, polysaccharides, phenolic compounds, and enzyme inhibitors. The extracted DNA was

eluted into 100 µl of sterile elution buffer and stored at −20 °C until the time of DNA sequencing and bioinformatics analysis.

16S rDNA sequencing and bioinformatic analysis

The bacterial microbiome was analyzed using 16S rRNA gene V4 variable region PCR primers 515/806 in a single-step 30 cycle PCR using a commercially available kit following the protocol outlined by Dowd et al., 2008. Sequencing was performed on an Ion Torrent PGM (Personal Genome Machine) following the manufacturer's guidelines and processed using a proprietary analysis pipeline at MR DNA laboratory. Sequences were de-multiplexed and sequence adaptors were removed prior to QIIME analysis (Bolyen et al. 2019). Bacterial composition was assessed using the Quantitative Insights into Microbial Ecology (QIIME) suite, QIIME2 version 2019.4. Reads were filtered for length and quality and chimeras were removed. Sequences were clustered into operational taxonomic units (OTUs) with a 97% identity threshold. Taxonomic assignment was performed using BLASTn classifier (trained by the SILVA database, release version 132) (Quast et al. 2013). OTUs with an abundance below 20 and present in less than five samples were not included in the downstream analysis. Remaining OTUs were consolidated into an OTU network for all individual samples using QIIME2 and this was imported into RStudio for downstream analysis.

Data and Statistical analysis

Individual samples from each group were used to assess microbial abundance and variation for both sampling strategies. Alpha diversity was assessed through rarefaction

graphs constructed with QIIME2. Relative abundance was used to calculate means and standard deviations of each group at each time point using the statistical program R (Team 2017). Using the RStudio statistical platform, t-tests were performed to identify significant difference in relative abundance of microbial taxa. Non-metric multidimensional scaling (nMDS) ordination was generated in RStudio using the *vegan* package (Dixon 2003). To generate the nMDS, raw bacterial hits were used to compute a sample dissimilarity matrix using the Bray-Curtis dissimilarity index. This matrix was then used to compute an ordination of the samples in two dimensions. The *vegan* package was also used to calculate Shannon's Diversity Index scores. Then, the Pielou's Evenness Index was calculated by dividing the Shannon's Diversity Index score by the log of unique species amount. Significance reported for any analysis is defined as $p<0.05$.

Results

After rigorous quality sequence curation, 1,444,966 sequences were parsed and then clustered. A total of 1,434.061 sequences identified within the Domain Bacteria were utilized for final microbiota analyses. The average reads per sample was 19,917.
The analysis of the bacterial diversity is a function of sequencing effort and represented as individual samples by the color-coded lines. The positive assessment of richness for each sample collected is determined by the fact that each color-coded line achieved its maximum peak and plateau consistently with each other signifying adequate depth of sampling and alpha diversity (Figure 1).

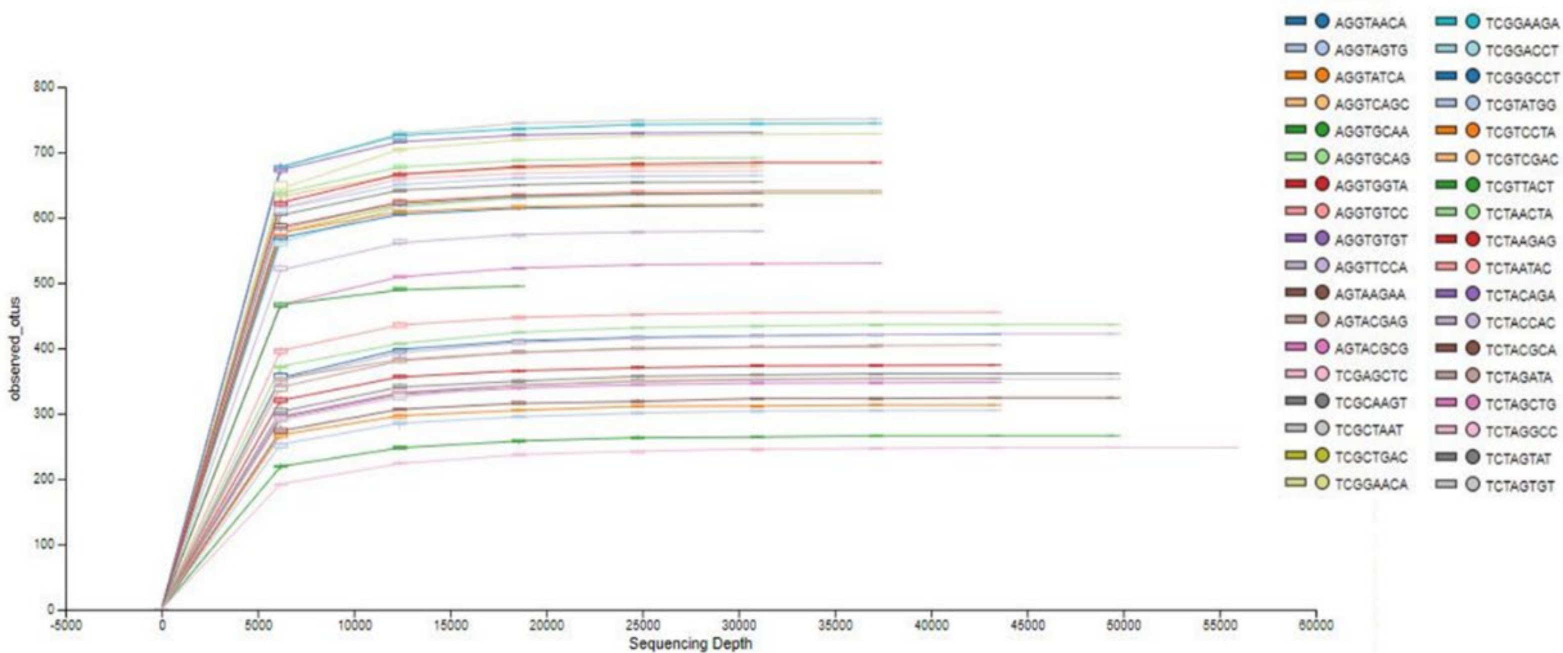


Figure 1: Phylogenetic rarefaction curves estimating species richness. The rarefaction curves produce smoother lines facilitating full dataset comparison by reaching a clear asymptote

Species richness between the two locations, mucosal surface and lumen contents, was measured using the Shannon-Wiener Index, while evenness was measured utilizing Pielou's Evenness Index (Figure 2). Throughout the experiment, minimal change was observed in the diversity and evenness within the microbiota for both locations sampled. This is confirmed by the lack of statistical significance of the Shannon index reporting a p value equal to 0.49 for the mucosal surface samples and 0.64 for the lumen contents, and for the evenness trend at 0.59 and 0.54 for the mucosal surface and the lumen contents respectively.

Next, to determine the amount of dissimilarity seen in the microbiota associated with the lumen and mucosal surface, an nMDS ordination plot utilizing a Bray-Curtis dissimilarity index was generated (Figure 3). Figure 3 demonstrates a clear separation of samples in the ordination plot, suggesting the microbiota between the two locations are dissimilar to each other as displayed by two distinct clusters of the same samples.

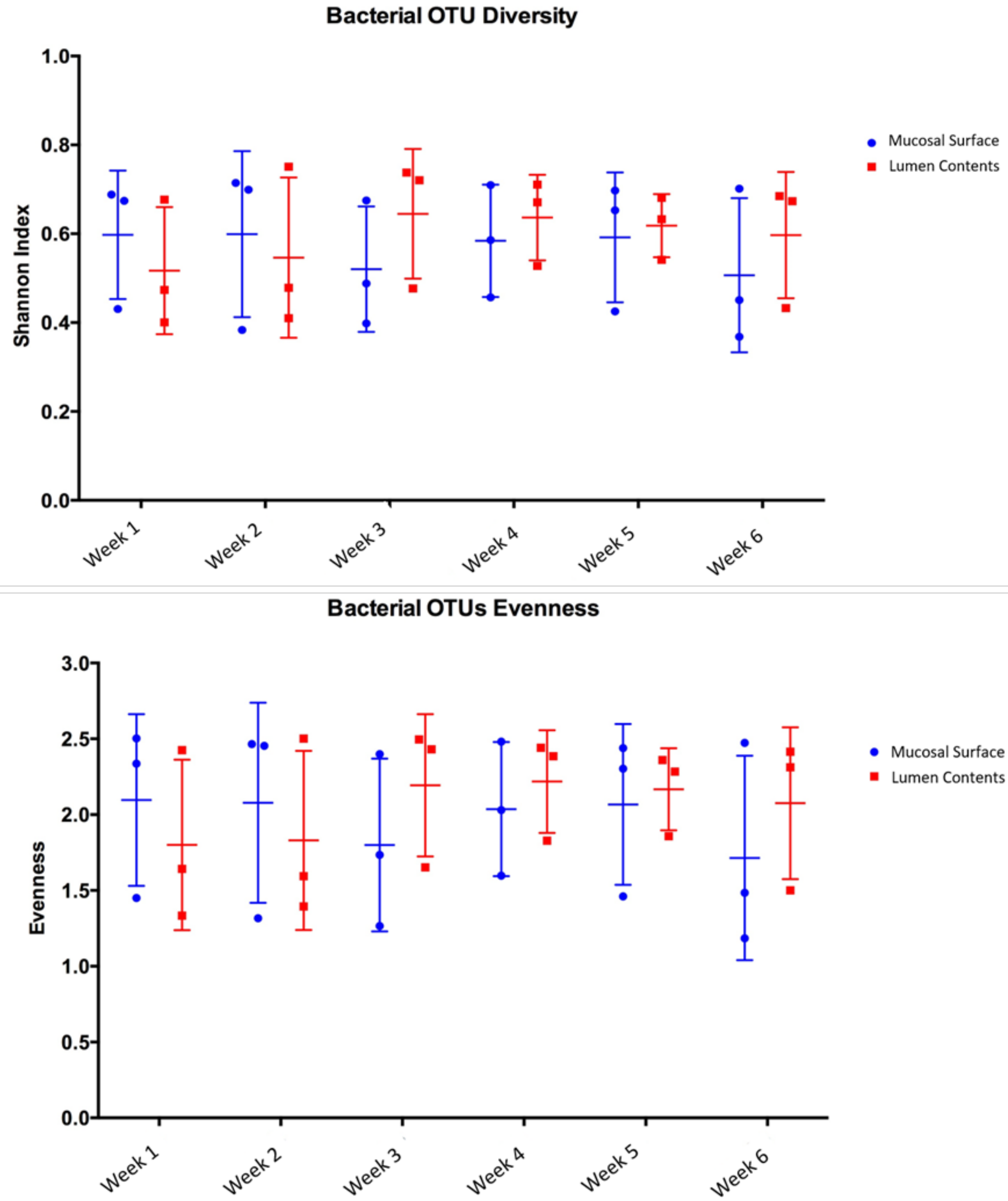


Figure 2: Comparison of bacterial OTU`s Shannon index diversity and Pielou's evenness for the mucosal surface and lumen contents for each week sampled.

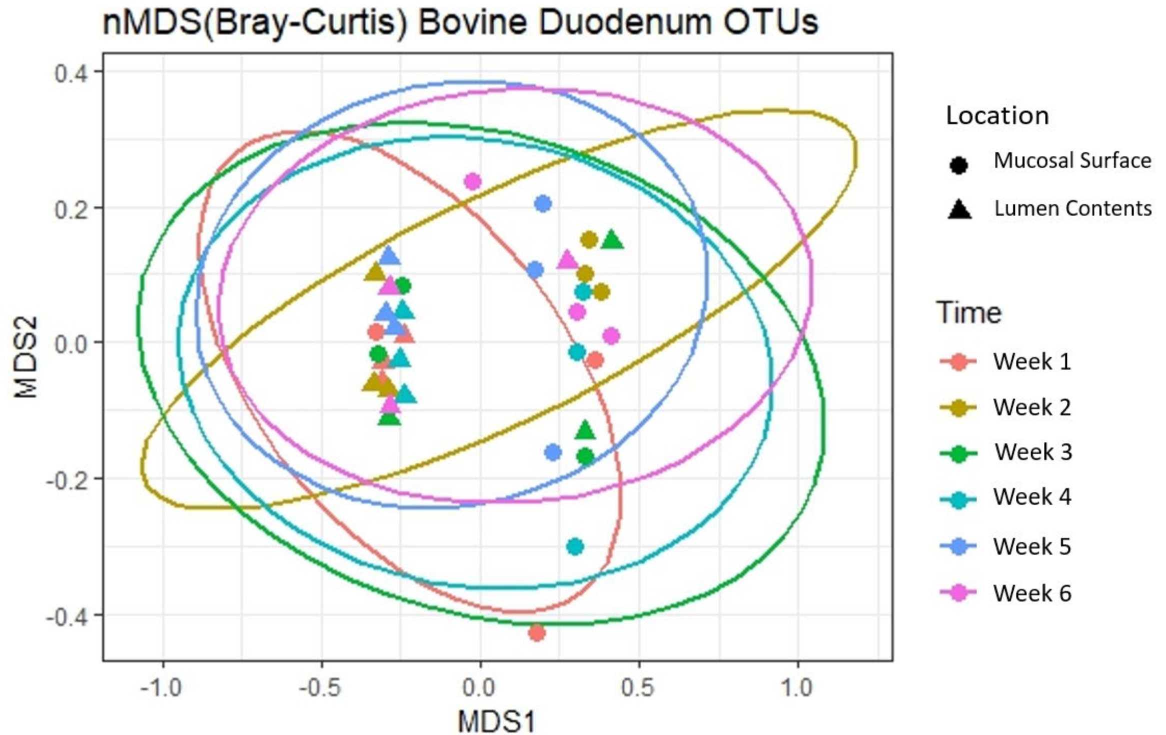


Figure 3: Multidimensional scaling plot (MDS) of bacterial lineages in the mucosal surface and lumen contents.

At the phylum level, *Firmicutes* (63%) and *Bacteroidetes* (21%) composed over 80% of the microbiome present in both sample locations. The relative abundance of *Firmicutes* was greater in the mucosal biopsy samples (75%) compared to the samples from the lumen contents (52%) for all cattle, whereas *Bacteroidetes* were mostly populated in the lumen contents (32% vs. 10%). The abundance of *Proteobacteria* and *Actinobacteria* was similar in total abundance, among the two locations (Figure 4). Overall, the F:B in the mucosal biopsy samples was significantly higher relative to the samples collected from the luminal contents especially on weeks 2, 4, and 5 (p = 0.005, p = 0.04 and p = 0.01

respectively), whereas on weeks 1, 3, and 6 the statistical significance varied between *p*=0.27 and 0.65.

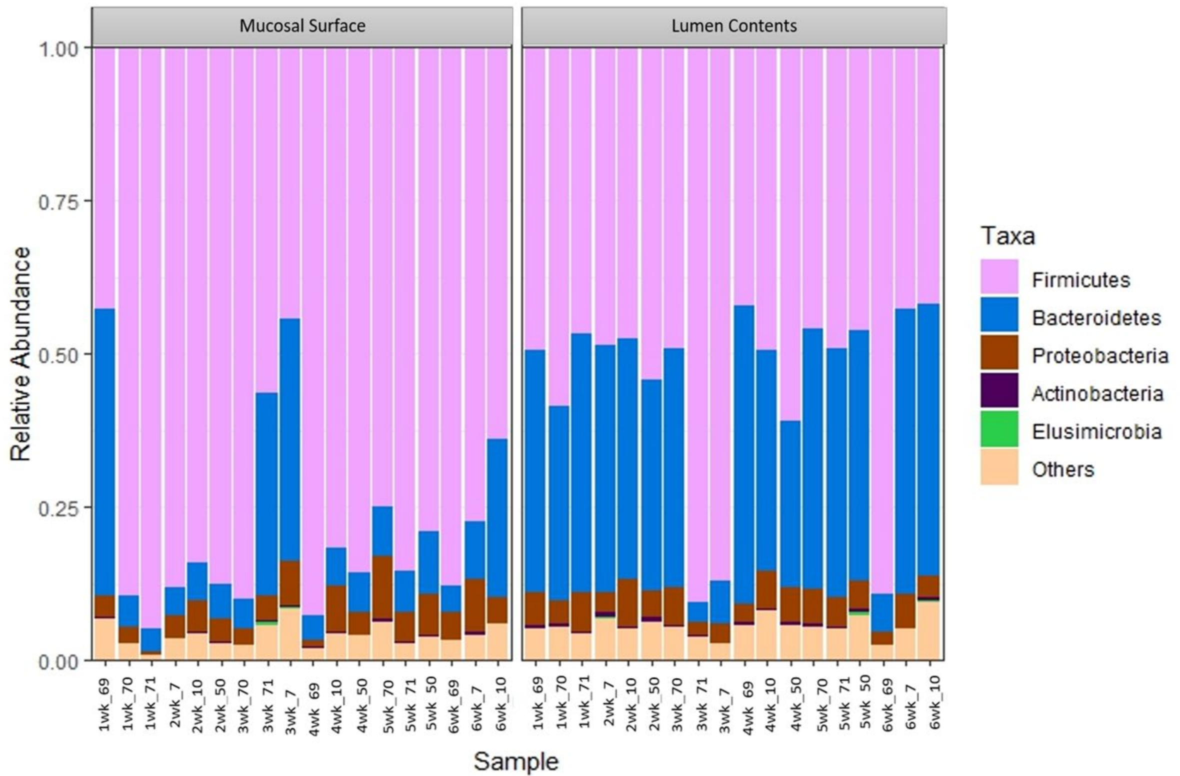


Figure 4: Bacterial Phylum. Stacked bar chart representing the abundance of the top 5 phyla microbiota in the mucosal biopsy surface and lumen contents across the sampled weeks for each animal.

To further determine what populations are driving the dissimilarity between the two groups, the relative abundance at the taxonomic level of class was calculated (Figure 5). A significantly high abundance of *Bacilli* in the mucosal biopsy surface was observed (*p*= 0.02 – week 2, *p*= 0.001 – week 4 and *p*= 0.001 – week 5), whereas *Clostridia* and *Bacteroidia* were more abundant in the samples of luminal contents. Statistical

significance was found during the same weeks as described above. (*Clostridia* - p= 0.06, 0.001 and 0.02; *Bacteroidia* – p= 0.003, 0.03 and 0.002).

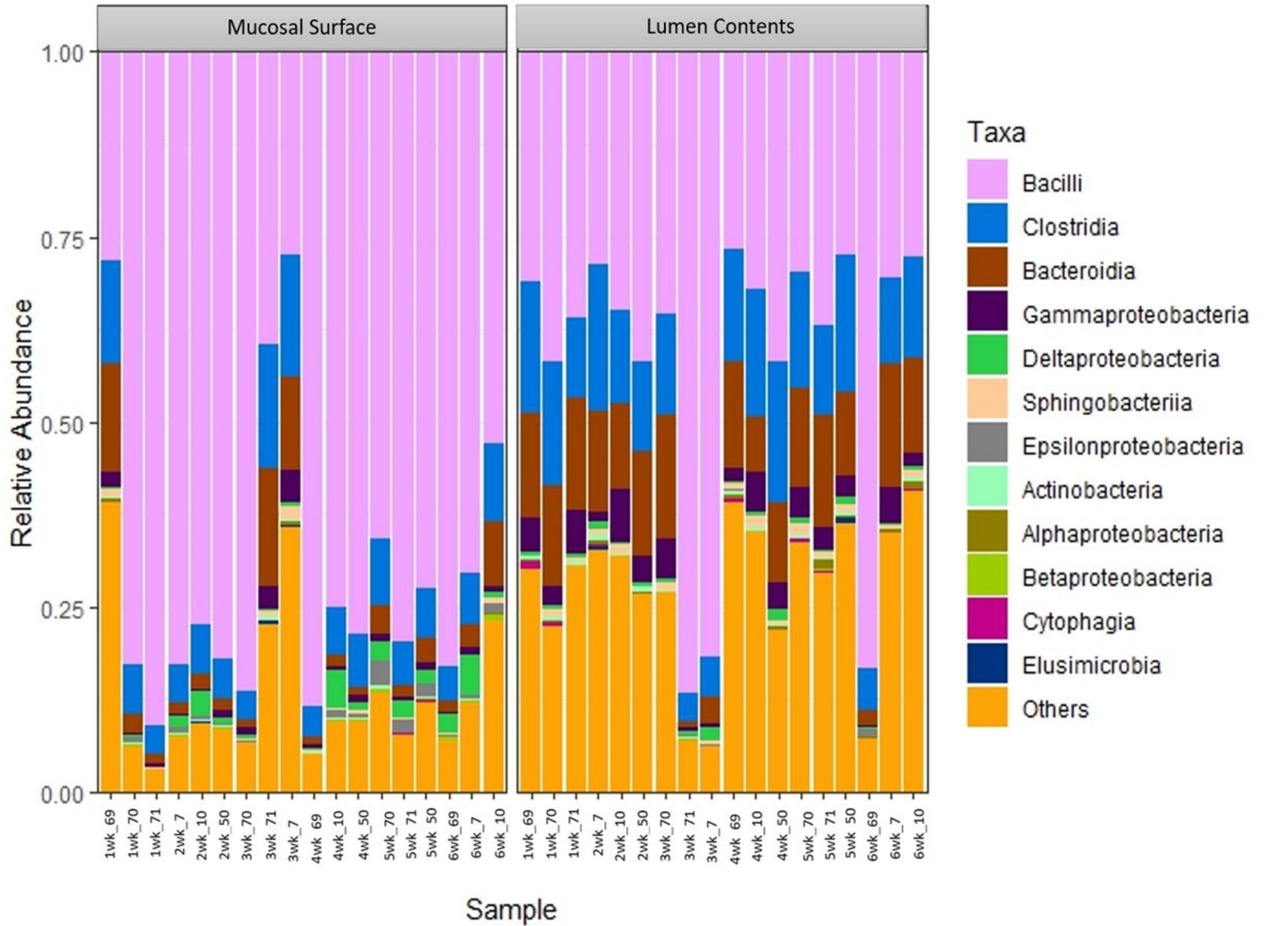


Figure 5: Bacterial Class. Stacked bar chart representing the abundance of the microbiota at the class taxonomic level in the mucosal biopsy surface and lumen contents across the sampled weeks for each animal

The same distribution between the two locations is seen at lower taxonomic level, at the family and genus, however it appears that the microbiota derived from the *Bacteriodetes* is predominant in the lumen contents, representing a shift from a mostly *Clostridia*

abundance whereas no specific change or shifts were seen at the mucosal biopsy surface, as bacteria belonging to the class *Bacilli* predominates throughout (Figure 6 & 7).

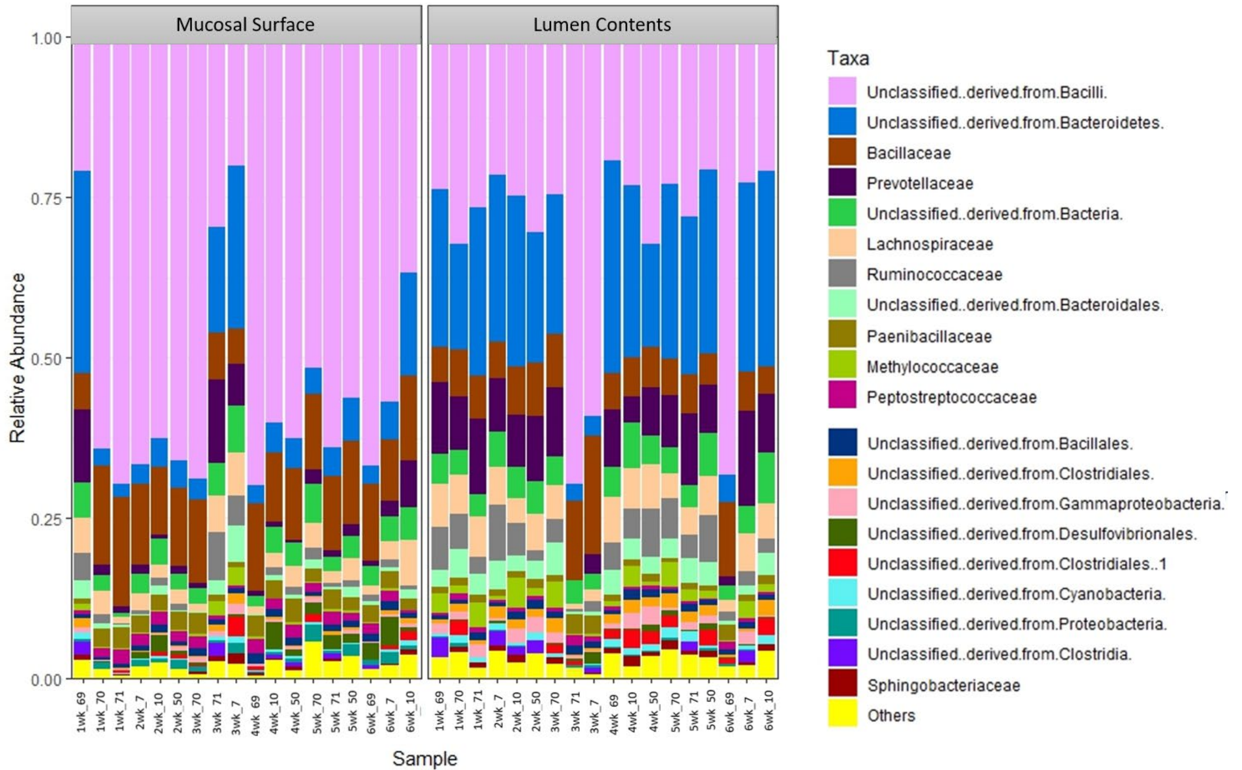


Figure 6: Bacterial Family. Stacked bar chart representing the abundance of the microbiota at the family taxonomic level in the mucosal biopsy surface and lumen contents across the sampled weeks for each animal

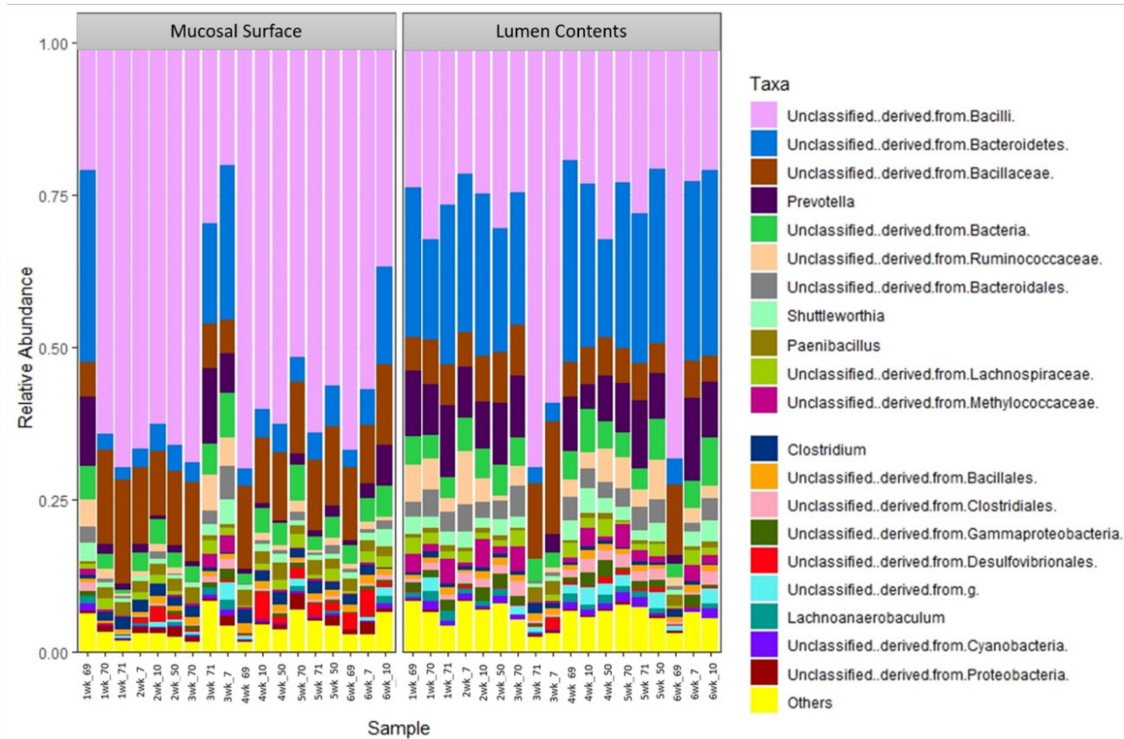


Figure 7: Bacterial Genus. Stacked bar chart representing the abundance of the microbiota at the genus taxonomic level in the mucosal biopsy surface and lumen contents across the sampled weeks for each animal

Discussion

Significant differences were observed between luminal and mucosal biopsy bacterial populations in the bovine duodenum in the current study. The method by which the duodenal mucosal biopsies were collected in this study is unique. A series of three endoscopic biopsy samples per animal per location were collected over a six-week period via the surgically fitted duodenal cannula. This technique and approach allowed the collections to be executed in real time in the live animal undergoing normal husbandry. Target gene sequencing using the Ion Torrent PGM 16S rRNA phylogenetic analytical method was used. Genome sequencing using the 16S rRNA method is widely used

among microbiome studies. This technique has a wide range of uses, including the characterization of a comprehensive variety of microbial diversity, taxonomical analysis, and species identification (Human Microbiome Project 2012; Chen et al. 2000; Ranjan et al. 2016). Using a culture-based analysis, Creevey et al 2014, reported the existence of nine phyla in the rumen; in decreasing order of abundance the top four phyla reported were *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteriodetes*. In contrast, the main phyla found in duodenal samples in this study were *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* in different abundance which varied by sample location. This indicates that although some phyla are conserved in different parts of the GIT, the exact abundance of the microbiome in different regions differs (Creevey et al. 2014). Also, using 16S rRNA pyrosequencing of the ruminal DNA, Jami and colleague 2012, characterized and compared the rumen microbiota of cattle. This group suggested the existence of a core microbiome in the bovine rumen, and even though the variability was great, the authors demonstrated a high phylogenetic correlation among the described genera (Jami and Mizrahi 2012a). In another study, the same researchers examined the rumen microbiome in lactating cows (Jami and Mizrahi 2012b). The results were consistent with those of the first study, in which they demonstrated the presence of a core microbiome in the rumen. Specifically, they reported a bacterial population with 32% of the OTUs shared by at least 90% of the animals in the study and 19% of the OTUs common to 100% of the animals. Similarly, in the study reported here the commensal duodenal microbiota is also represented by a core microbiome with variability; with bacteria belonging to the phyla *Firmicutes* and *Bacteroidetes* representing 80% of the bacteria phylum present. However, the F:B in the mucosal biopsy samples was

significantly higher relative to samples collected from the lumen. The same similar pattern was seen at the other taxonomic levels.

A study in swine used a similar method to successfully compare microbial populations in the mucosa and luminal microbiota in the colon of pigs, with and without dysentery, at necropsy (Burrough, Arruda, and Plummer 2017). The authors demonstrated significant differences in the microbiome population of the gastrointestinal tissue and luminal ingesta between diseased and not diseased pigs. Furthermore, they also reported, at the genus level, the colonic bacterial population itself had changed in pigs with dysentery for both mucosal and luminal samples whereas a different population (*Clostridiales*, *Erysipelotrichales*, and *Fusobacteriales*) was seen in the luminal samples only. Those findings were comparable to the current study which demonstrated significant differences of the commensal population at all taxonomic levels between the mucosal biopsy and luminal sites in healthy animals. Thus, future studies of the microbiome must take into account population differences between sampling sites as, most likely, study results will vary as a direct effect of sampling location, technique, and potential disease processes.

In addition, de Rodas et al. 2018, published the microbial profile from different anatomic sites of the GIT over time at different ages from farrow to finish using 16S rRNA V4 region sequencing with Illumina MiSeq. The group was able to observe shifts in the microbiome as the animals aged, as well as a positive correlation between several bacteria at the genus level and pig weight (De Rodas et al. 2018). In contrast, while the current study found a highly diverse population between the duodenal mucosa and lumen, a significant change in the microbiome profile over the 6-week sampling period was not observed, suggesting the duodenal microbiome is relatively stable over a short period of

time. Microbiome studies of longer duration in cattle would be valuable to determine the impact that aging, diet, and other factors have on the microbiome profile. The commensal microbiome plays an important role in its interaction with the immune system, allowing the host to distinguish commensal and pathogenic bacteria. The higher species abundance observed for the mucosal communities suggests their core importance metabolically and immunologically to the host. Results of the current study are consistent with a previous study characterizing the GIT microbiome of pre-weaned calves, where significant differences were found in the bacterial populations of the mucosal surface and luminal contents (Malmuthuge, Griebel, and Guan le 2014). In that study, the authors proposed that the core metabolically active epimural bacterial population could survive mucosal immune defense mechanisms, and can be crucial for priming the host mucosal immune system. Therefore, the understanding of the commensal microbiota in different parts of the host, *in vivo*, is imperative (Hanning and Diaz-Sanchez 2015).

The results of the current study showed the F:B in the duodenum mucosal biopsy samples was significantly higher relative to the samples collected from the lumen. This is consistent with previous reports that have analyzed F:B in mice and humans, where imbalances in the ratio in the GIT have been demonstrated to affect obesity and the capability of the host to harvest energy (Krajmalnik-Brown et al. 2012; Pedersen et al. 2013). The microbiome present in obese hosts demonstrated greater capacity to harvest energy from the diet. Therefore, obesity in the host was supported and even exacerbated by the imbalanced bacterial populations (Ley et al. 2006; Turnbaugh et al. 2006). Similarly, a correlation between pig weight and bacterial profiles has been demonstrated

further supporting the idea that the microbiome is not an incidental finding, but an active player in the host's metabolism and health (De Rodas et al. 2018).

Using terminal restriction fragment length polymorphism (T-RFLP) analysis and quantitative PCR (qPCR) in conjunction with a clone library, Reti and colleagues, analyzed and examined the bacterial communities associated with mucosa and within digesta throughout the intestinal tract of beef cattle (Reti et al. 2013). In their study, jejunal mucosal-associated bacterial communities consisted of mainly *Proteobacteria*, and differed conspicuously from those in the ileum and large intestine and mucosa-associated populations of the ileum, cecum, and descending colon where *Firmicutes* was the primary phylum identified. In contrast with the present study, *Proteobacteria* were only the third most common phylum observed in both the mucosal biopsy and luminal samples representing approximately 6% of the population.

The authors speculate that the difference seen between the current study and the one published by Reti, is threefold. First, the sequencing method used was different, as Ion Torrent is more accurate than T-RFLP. Secondly, dietary differences were present between the two studies. Lastly, the methodology used to collect samples was different. Reti and colleagues, collected the study samples at slaughter versus *in vivo* and it is possible that *Proteobacteria* proliferates faster post-mortem and thus slaughter samples do not accurately reflect the *in vivo* populations.

The ruminant gastrointestinal microbiome grants many physiological and unique functions that are considered essential to maintain overall homeostasis. Significant differences in the bacterial populations of the lumen and mucosal surfaces of the bovine duodenum were identified in this study. This is consistent with other mammalian GIT

microbiota studies by characterizing the presence of the three dominant phyla, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*. Results of this study indicate the duodenal microbiota of cattle is composed primarily of *Firmicutes* and *Bacteroidetes*. A much higher abundance of *Firmicutes* was observed in the mucosal surface than the luminal contents, and such pattern was also observed at lower taxonomic levels. This result is not unexpected as the active and controlled metabolism is believed to occur at the mucosal level. An important finding of this work was that all sampled animals shared the same primary group of bacterial classes, family and genus; however, their respective abundance was significantly different between the sample locations. It has been suggested that the aerobic region within the intestines might be related to the outcome of interactions with the gut microbiota, acting as an innate immune barrier to protect the mucosal surface from anaerobic bacteria, while being recognized as a signal to promote invasion by pathogens (Marteyn et al. 2010). This concept may explain the standardized differences in bacterial abundance when mucosal biopsy and luminal contents are contrasted. Facultative aerobic Firmicutes, which have colonized the mucosal surface, may have readily available oxygen from the host essential for bacterial survival or as an advantage to growth, whereas the anaerobic environment of the lumen perhaps benefits the survival or enhanced growth of the *Clostridia* bacterial class. This principle is also observed with the Bacteroidetes in the results of this study; a larger and significant concentration of this phylum of bacteria is observed in the lumen versus the mucosal surface.

Characterizing the gastrointestinal microbiome *in vivo* is imperative. This study documents the presence of significantly different compositions of the bacterial

populations in two distinct locations of the duodenum in live cattle undergoing normal and expected husbandry. This novel approach is crucial as many metabolically and biochemical changes in all body tissues are believed to be altered upon death (Donaldson and Lamont 2013). While this study demonstrates the differences in bacterial populations in different sites within the bovine duodenum and increases the understanding of the bovine duodenum microbiome, characterization of population differences between mucosal and luminal microbiota in different areas of the gastrointestinal tract remains to be described.

Chapter 4

Characterization and Comparison of the Rumen Luminal and Epithelial Microbiome Profiles using Metagenomics Sequencing Technique

Introduction

The ruminal bacterial population plays an important role in the dietary metabolism of the host, including nutrient consumption and utilization, and consequently, manipulation of the rumen microbiota is known to affect animal performance, production, sustainability and ultimately profitability (McCann, Elolimy, and Loor 2017). The commensal microbiome plays an important role in nutrient and energy extraction and energy regulation (Krajmalnik-Brown et al. 2012). In ruminants, specifically cattle, the composition of the rumen microbiome and its impact on health, nutrition, and host physiological parameters have been studied (Creevey et al. 2014; DePeters and George 2014; Firkins and Yu 2015; Freetly et al. 2020; Jami and Mizrahi 2012b, 2012a; Jami, White, and Mizrahi 2014; Snelling et al. 2019; Tapio et al. 2017; Welkie, Stevenson, and Weimer 2010).

The 16S rRNA gene sequencing technique is a more commonly used strategy to study the rumen microbiome. A plethora of publications can be found that use this method to study the microbiome, however, methodology and analysis of the taxonomic data collected are still known difficulties encountered by microbiome researchers (McCann, Elolimy, and Loor 2017).

The commensal microbiota composition of the rumen is largely determined by dietary factors. However, age, breed, and the ruminant species in question are also known factors

that impact rumen health (King et al. 2011; Kittelmann et al. 2013). Enzymes necessary for digestion via fermentation of the diet consumed by ruminants are provided by the commensal rumen microbiome. Also, the microbiota is responsible for the synthesis of amino acids and vitamins that are later absorbed in the small intestine to fulfill the host requirements (Cammack et al. 2018).

The characterization of the ruminal and fecal microbiome and its impact on bovine health, production has been investigated (Jami and Mizrahi 2012a; Jami, White, and Mizrahi 2014; Liu et al. 2016). Milk yield and composition were found to be highly correlated with the abundance of various bacterial members of the rumen microbiome, specifically the impact between the F:B on milk-fat yield (Jami, White, and Mizrahi 2014). On a later date, another group investigated the composition of bacterial microbiota in the rumen content, epithelium and feces of dairy cattle. They were able to demonstrate a remarkable compositional differences among the three locations suggesting that bacterial communities are specific and adapted to the environment (Liu et al. 2016).

At the phylum level, *Bacteroidetes* and *Firmicutes* are among the primary metabolically-active bacteria with a critical role in breaking down plant wall compounds and host-derived carbohydrates, including particles attached to the mucins or chondroitin sulfates of the protective mucosal layer of the intestine (Krajmalnik-Brown et al. 2012). The *Firmicutes Bacteroidetes* ratio has been demonstrated to affect energy uptake from the diet and expenditure leading to obesity in pigs, mice and humans (Krajmalnik-Brown et al. 2012; Pedersen et al. 2013).

The rumen microbiome profile is dependent on the composition of substrate that has been offered, such as the proportions of cellulose, hemicellulose, pectin, starch, and amino

acids. Further into the taxonomic analysis, it is reported that *Butyrivibrio fibrisolvens*, *Prevotella ruminocola, Ruminococcus flavefaciens,* and *Ruminococcus albus* are known to be responsible for the digestion of hemicellulose and cellulose rich diets, such as, forages (Puniya 2015). The digestion of grain based (high starch) diets is accomplished by *B. fibrisolvens, Prevotella ruminocola, Fibrobacterer succinogenes, Clostridium species, Streptococcus bovis, Ruminobacter amylophilus, Succinimonas amylolytica,* and *Selenomonas ruminantium*. Further, aminoacids are readily fermented by bacteria belonging to the genus *Prevotella* to produce adenosine triphosphate (ATP) (Puniya 2015).

Gaps in knowledge are present with respect to the rumen epithelium and its unique interaction between host and microbial metabolism. Biopsy sampling techniques of the rumen epithelium have been used to analyze the effects of dietary transition on ruminal epithelial gene expression and the effects of diet on rumen epithelial development (Lin et al. 2019; Novak et al. 2019). However, full understanding of the true commensal microbiome in cattle is still limited, especially with respect to what ensues at the epithelial surface of the rumen when cattle are undergoing normal husbandry.

The aim of this project was to characterize the commensal microbiome present in the lumen and the epimural surface of the rumen of cattle. It was hypothesized that due to metabolic processes and/or host properties, there are differences in the natural microbiota present in the epimural surface and luminal contents of the adult bovine major forestomach.

Materials and Methods

Animals

The study was conducted at the Auburn University College of Veterinary Medicine following approval of all procedures by the campus Institutional Animal Care and Use Committee (PRN 2015-2676). Six dairy crossbred steers weighing an average of 249 kilograms, ranging between 240 – 277 kilograms (530-610 pounds) were used in this study. The cattle were housed in grass pasture, fed one flake of Bermuda grass hay and five pounds of soy hull pellets per head twice daily, and offered water ad libitum. In order to achieve the goals of the study, all steers were surgically fitted with a three-inch rumen cannula (Bar Diamond® #8C). The surgical procedure was performed as described by Laflin and Gnad, 2008. Post-operative treatment consisted of 2.2 milligrams per kilogram of ceftiofur hydrochloride (Excenel® RTU EZ) administered subcutaneously once daily for five days and one milligram per kilogram of meloxicam (Meloxicam 15mg, Cipla USA, Inc., Miami, FL) administered orally once daily for five days in addition to standard daily cleaning procedure. A three-month recovery period was observed following surgery to allow complete healing of the surgical sites, ensure appropriate drug withdrawal periods were met, and provide research animals consistent diet prior to study initiation and sample collection. Once the recovery period elapsed, the cattle were housed in the same pasture throughout the length of the study without fence-to-fence contact with other animals, and were consistently fed five pounds of a 50:50 mixture soy hull and corn gluten pellets plus one flake of Bermuda grass hay (approximately 3 pounds) per head per day. To ensure consistence and eliminate dietary

bias, this nutritional scheme remained the same until all samples from all the steers were collected.

Study Timeline and Sample Collection

In order to optimize consistency and still assess potential variation due to individual, environmental, and bacterial factors, each animal was sampled once a week in random days over a three week period (Table 2).

For sample collection, each individual animal was haltered and restrained in a livestock chute system. The rumen cannula was opened by manually removing its cap. Using a sterile double-gloved sleeve, the sample collector entered the rumen and manually palpated the cranial pillar and ruminoreticular fold. A sample of the ingesta from the cranial sac of the rumen (located between the two locations described above) was collected using a snap cap collection vial. After collection, the cap was closed inside the cranial sac of the rumen before removal to minimize potential contamination of samples. Such samples were designated as lumen contents samples. Next, epithelial biopsy samples were collected from the cranial sac of the rumen using a 54 cm Jackson uterine biopsy forceps (Jorgensen Labs INC.). Using a new sterile double-gloved sleeve, the sample collector entered the rumen with the forceps covered by a sterile sleeve. Once the cranial sac was located and the biopsy site identified, the “push through” technique was used to expose the forceps allowing the biopsy of the rumen epithelium to be taken. The forceps was pulled back in the sleeve before removal from the rumen by the sample collector. All samples were placed in 750 µl of RNAlater immediately after collection and stored at 4°C until processed.

	Week 1	Week 2	Week 3
Monday			50, 69, 70
Tuesday		93, 10, 50	
Wednesday	71, 70, 93		93, 10, 71
Thursday		70, 69, 71	
Friday	10, 69, 50		

Table 2. Timeline for sample collection

DNA Isolation and 16S rDNA sequencing

A total of 18 luminal samples and 18 mucosal biopsy samples were collected for isolation and subsequent sequencing over the three-week collection period. Isolation of DNA from all samples was extracted using a commercial kit (E.Z.N.A® Stool DNA,Omega bio-tek®, Norcross, GA) according to the manufacturer`s guidelines for DNA extraction in tissue, using glass beads, and for fluid samples. The pathogen detection protocol allows rapid and reliable isolation of purified DNA using a combination of reversible nucleic acid-binding properties of HiBind® matrix and spin column technology to allow the elimination of humic acid, polysaccharides, phenolic compounds, and enzyme inhibitors. The extracted DNA was eluted into 100 µl of sterile elution buffer and stored at −20 °C until the time of DNA sequencing and bioinformatics analysis.

The bacterial microbiome was analyzed using 16S rRNA gene V4 variable region PCR primers 515/806 in a single-step 30 cycle PCR using a commercially available kit following the protocol outlined by Dowd et.al, 2008. Sequencing was performed on an Ion Torrent PGM (Personal Genome Machine) following the manufacturer’s guidelines and processed using a proprietary analysis pipeline at MR DNA laboratory.

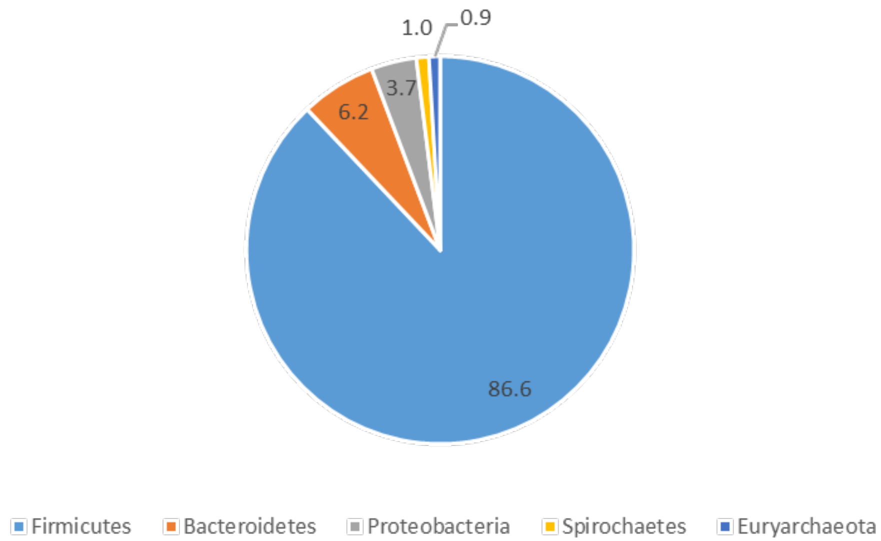
Data Analysis

Data was evaluated in a multivariate manner to profile the microbiome in each group based on the relative abundance of target specific genera identified by the lab. The microbial community structure was analyzed using weighted UniFrac distance matrices. Principal coordinate analysis plots were used to visualize the data in these matrices, and pairwise analysis of similarities (ANOSIM) was utilized to determine if there were any significant differences between the microbial communities. To provide a visual overview combined with the analysis a dual hierarchal dendrogram was used to display the data for the predominant Genus with clustering related to the different groups.

Results

After stringent quality sequence curation, a total of 2,239,622 sequences were parsed and 2,074,523 were then clustered. A total of 2,071,427 sequences identified within the Bacteria and Archaea domains were utilized for final microbiota analyses. The average reads per sample was 51,785.

At the phylum level, *Firmicutes* (86.6%) and *Bacteroidetes* (6.2%) followed by smaller percentages of *Proteobacteria* (3.7%), *Spirochetes* (1%), and *Euryarchaeota* (0.9%) were the top 5 most abundant bacteria in the epimural biopsy samples. In contrast, *Firmicutes* (55.3%), *Bacteroidetes* (30.7%), *Proteobacteria* (6.7%), *Fibrobacteres* (1.3%) and *Tenericutes* (1.3%) were the five most abundant bacteria present in the luminal contents (Figure 8).



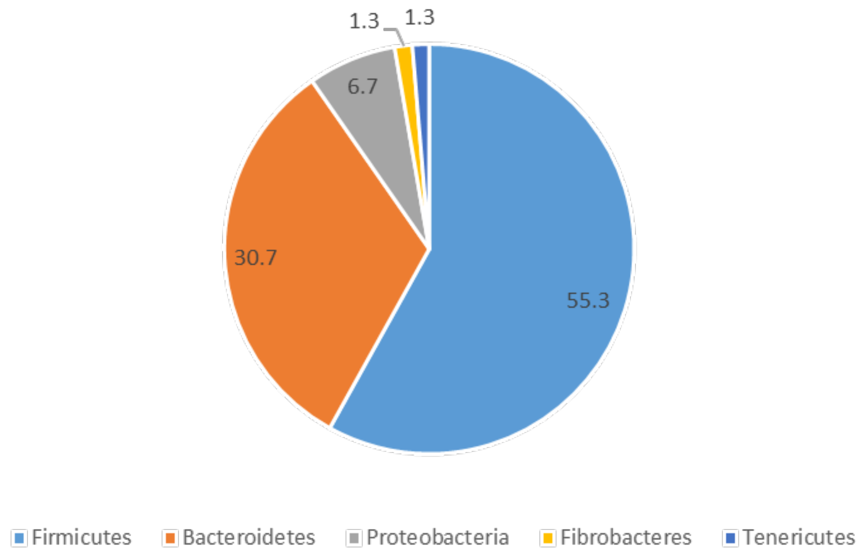


Figure 8: Pie Composition, by phylum, of the top five most abundant bacterial populations present in the epimural surface and lumen contents across all samples collected as determined by Ion Torrent PGM sequencing of the 16 S rRNA gene.

When the proportion and comparison of different phyla in each sample collected were evaluated, the percentage abundance was generally similar between each sample collected and between time points for each individual with very small variability (Figure 9).

Figure 9: Phylum-level composition and abundance of microbiota associated with epimural surface and the luminal fluid of the rumen of each animal, for the three different sampling times.

Firmicutes and *Bacteroidetes* composed over 85% of the microbiome of the rumen in both sample locations. The distribution of these two predominant phyla was further explored and their ratios represented by Figure 10. The consistent and low variation among the 3 weeks the study took place, with the *Firmicutes* phylum being predominant in the epimural surface and the *Bacteroidetes* in the lumen contents is clearly noted.

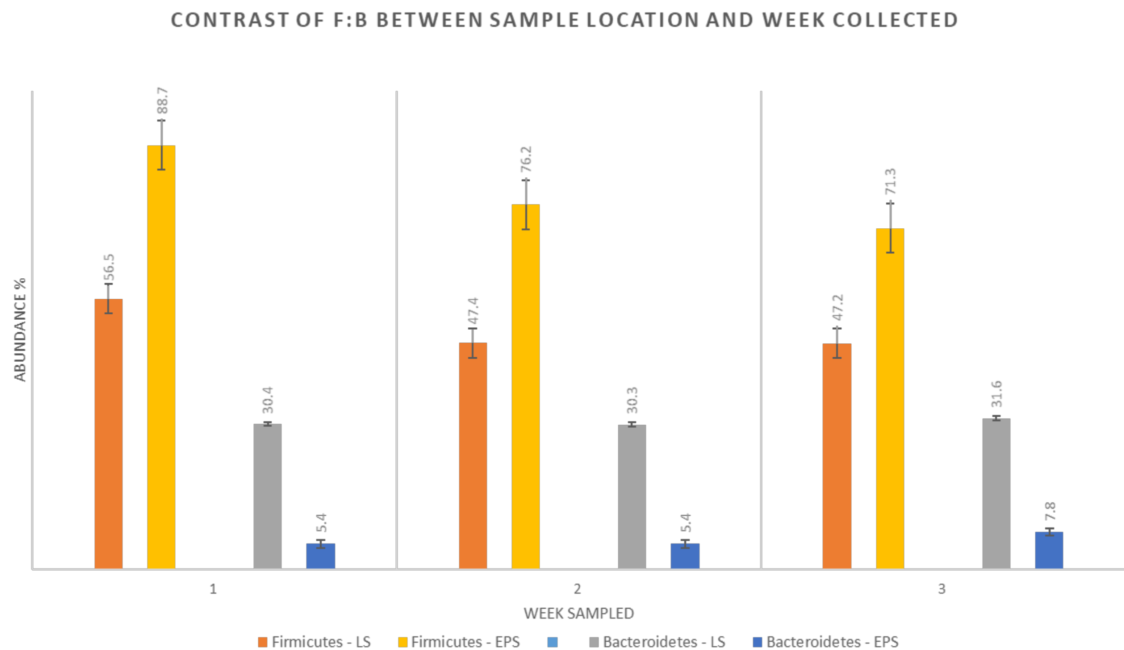


Figure 10: Variability and notable numerical differences between the percentage of overall OTUs for the phylum Firmicutes and Bacteroidetes throughout the length of the study.

To further study the distribution of these two predominant phyla at a lower taxonomic level, we investigated the distribution of microbial populations at the Class, Order,

Family and Genus levels for the most abundant phyla. At the Class level, in the lumen contents, the vast majority of the *Firmicutes* in the lumen contents were represented by *Bacilli* (30.5%) and *Clostridia* (20.9%) constituting over 90% of the 55% *Firmicutes* present, while in the epimural surface, *Bacilli* (76.5%) and *Clostridia* (9.1%) were the top classes represented (>95%) within that phylum. Conversely, minimal variation was observed for the *Bacteroidetes* phylum regardless of the location of the sample, with over 25% represented by *Bacteroidia* in the lumen versus 5% in the biopsy. At the Order level, *Lactobacillales* represented about half of the abundance (~27%) within the *Firmicutes* phylum, in contrast with over 65% in the epimural surface. *Bacteroidales* was the most abundant bacteria population (~25%) in the lumen versus a little less than 5% in the biopsy samples. The same abundance pattern was observed at the Family and Genus levels with the *Enterococcaceae* and *Enterococcus* representing over 25% in the lumen samples versus over 65% in the epimural surface, whereas the *Prevotellaceae* and Genus *Prevotella* is present in over 14% of the samples associated with the lumen in only a little over 2% of samples associated with the epimural surface of the rumen.

Phylogenetic assemblage amongst the epimural surface sample was significantly different (p=0.001) from the lumen contents samples. Primary vector explains 88.5% of the variation between the groups. The first 3 vectors together exhibit 93.1% of the variation among the groups, p= 0.001 (Figure 11).

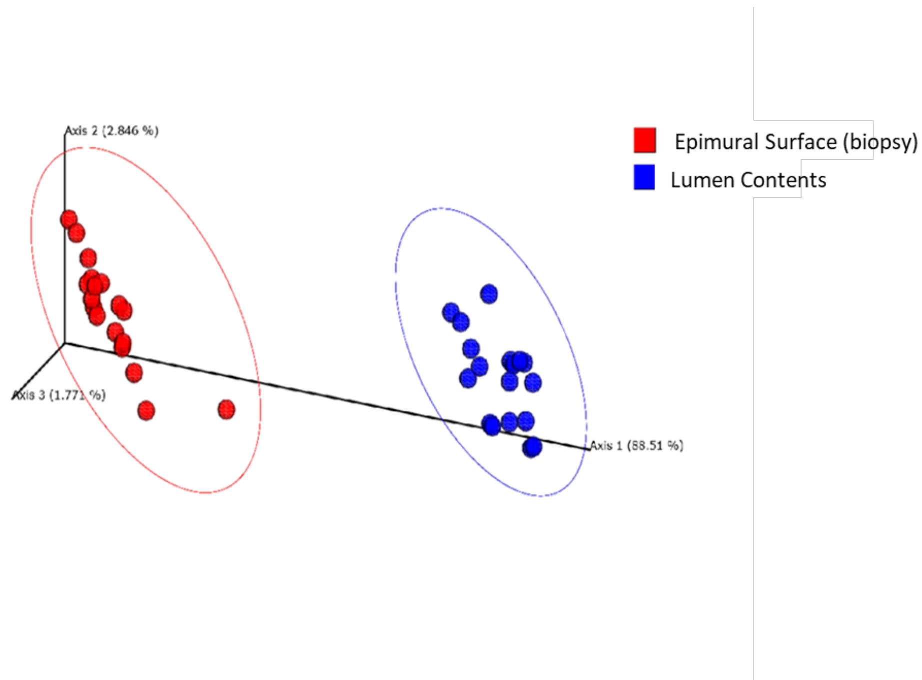


Figure 11: Principal coordinate plot of weighted UniFrac data

Based on the ANOSIM R value in Table 3, we can confidently indicate the most similar samples are in the same group (R=0.99).

Group 1	Group 2	Sample size	R	*p*-value
Epimural Surface	Lumen Contents	36	0.999	0.001

Table 3. Pairwise ANOSIM of weighted UniFrac distance matrix.

Discussion

In this study, significant differences obtained via target gene sequencing were demonstrated between luminal and epimural bacterial populations in the bovine rumen. Although the rumen microbiome has been investigated using different methods, the novelty of this study is the characterization of the microbiota present in two locations of

the rumen concomitantly *in vivo* with the animals studied were undergoing customary husbandry.

The maintenance of healthy and stable ruminal fermentation is known to be critical for ruminants to preserve their rumen bacterial populations and functional fermentation and digestion. Metabolism of nutrients is key in the symbiotic relationship between the host and microbiome

Significant differences obtained via pyrosequencing were observed between luminal and epimural bacterial populations in the bovine rumen. The higher species abundance observed for the epimural communities suggests their core importance metabolically and immunologically to the host. These findings are in agreement with a study published by Malmuthuge et al. (2014) suggesting that the core metabolically active epimural bacterial population can survive mucosal immune defense mechanisms and may be crucial for priming the host mucosal immune system.

Using cultivation-based analysis, Creevey et al. (2014), reported the existence of nine phyla in the rumen, with *Firmicutes*, *Proteobacteria* and *Actinobacteria* comprising 90% of the cultures. Similarly, the top 3 phyla reported in this manuscript associated with the luminal samples were *Firmicutes* (55.3%), *Bacteroidetes* (30.7%) and *Proteobacteria* (6.7%) which made up the top 90%, whereas, Firmicutes alone composed over 85% of the microbiota present on the epimural surface. The variation of lesser abundant bacteria, beyond the anticipated core microbiome, is speculated to be related to the dietary uniqueness of the individual. In addition, the significant abundance of *Firmicutes* at the phylum level and *Bacilli* (~75%) at the Class taxonomic level found on the epimural

surface was expected as those bacteria play an active role in ruminants with respect to carbohydrate metabolism (Ottman et al. 2012).

Previous research suggested the existence of a core microbiome in the bovine rumen, and even though variability was great, the authors demonstrated a high phylogenetic correlation among the described genera (Jami and Mizrahi 2012b, 2012a; Creevey et al. 2014). In another study, the same researchers examined the rumen microbiome in lactating cows (Jami and Mizrahi 2012b). The results were consistent with those of the first study also in that both studies demonstrated the presence of a core microbiome in the rumen (Jami and Mizrahi 2012b, 2012a). Specifically, they reported a bacterial population with 32% of the operational taxonomic units (OTUs) shared by at least 90% of the animals in the study and 19% of the OTUs common to 100% of the animals. . Similar to previous studies, the samples evaluated in the current study over a 3 week period demonstrated constant taxonomic characteristics, also representing a core rumen microbiome with minimal variation between animals and weeks, however with significant variation existing between locations.

The importance of the ratio of Firmicutes to Bacteroidetes has been analyzed in mice and human studies, where imbalances in the ratio in the GIT has been demonstrated to affect obesity and the capability of the host to harvest energy (Krajmalnik-Brown et al. 2012; Pedersen et al. 2013). The microbiome present in obese hosts demonstrated greater capacity to harvest energy from the diet. Therefore, obesity in the host was supported and even exacerbated by the imbalanced bacterial populations (Ley et al. 2006; Turnbaugh et al. 2006). Since healthy animals were used in this study, consistency in the ratio throughout the project between the two locations was appreciated, therefore the authors

speculate this finding as a positive result as habitual and consistent husbandry was undertaken during the study time.

A positive relationship between the rumen microbiome and certain physiological parameters in the lactating dairy cow has been identified (Jami, White, and Mizrahi 2014). The group reported a strong correlation between milk fat yield and the F:B present in the ruminal contents. The specific presence of Prevotella bacteria, up to 72% in some samples, negatively affected the milk fat yield in the same group of cattle (Jami, White, and Mizrahi 2014). The current study reported that Prevotella was found with greater abundance in the lumen samples compared to the epimural surface, approximately 14% versus 2% respectively. This finding is consistent with the fact that Prevotella, in the rumen is physiologically responsible for the prevention of the colonization of acid-producing bacteria which are known to disrupt the overall digestive processes in ruminants. This finding likely explains the higher abundance of *Prevotella* spp in the luminal contents as compared to the epimural location more closely associated with the host (Margolis and Fredricks 2015).

The ruminant gastrointestinal microbiome grants many physiological and unique functions that are considered essential to maintain overall homeostasis. In general, the present study demonstrates that microbiota associated with the rumen of cattle exhibit relative abundances of *Firmicutes* and *Bacteroidetes*. A much higher abundance of *Firmicutes* was observed in the epimural surface than the luminal contents. This result is not unexpected as the active and controlled metabolism is believed to occur at the mucosal level. An important finding of this work was that all sampled animals shared the same group of bacterial class, order and family; however, their respective abundance was

significantly different between the sample locations. Marteyn et al., suggested that the aerobic region within the intestines might be related to the outcome of interactions with the gut microbiota, acting as an innate immune barrier to protect the mucosal surface from anaerobic bacteria, while being recognized as a signal to promote invasion by pathogens (Marteyn et al. 2010). Perhaps this concept explains the difference in bacterial abundance in all levels between the epimural surface and the lumen samples with respect to the active *Firmicutes*. *Firmicutes* that have colonized the epimural surface may have readily available oxygen from the host essential for bacterial survival or as an advantage to growth, whereas the anaerobic environment of the lumen perhaps benefits the survival and a more balanced concentration of the *Clostridia* and *Bacilli* bacterial class. This observation could also apply to the *Bacteroidetes*, where a larger concentration of this phylum of bacteria was observed in the luminal samples as compared to the epimural surface.

Characterizing the gastrointestinal microbiome in vivo is important to mimic as close to normal physiologic processes as possible. This study has established the presence in different components and concentrations of the microbiota in two distinct location of the rumen in live cattle. This approach is crucial as many metabolically and biochemical changes in all body tissues is believed to be alterated upon death (Donaldson and Lamont 2013). Similar collection methods could be used in different locations of the gastrointestinal tract, allowing further investigation of the core commensal microbiome in vivo to study the impact of medical therapy and or environmental influences in the concentration of the metabolically-active circulating gastrointestinal bacteria in ruminants.

Chapter 5

In Vivo Profiling of the Commensal Microbiome of the Luminal and Mucosal Surface of the Bovine Ileum

Introduction

The microbiota refers to the entire population of microorganisms that colonizes a specific location of the body, and includes bacteria, fungi, archaea, viruses, and protozoans (Sekirov et al. 2010). The gastrointestinal tract (GIT) bacterial population of bacteria plays an important role in the dietary metabolism of the host, including nutrient metabolism and utilization. Disruption of intestinal microbiota homeostasis, termed dysbiosis, can occur as a result of bacterial, viral, and parasitic gastrointestinal pathogenic infections adversely affecting host metabolism, productivity, and overall health (Krajmalnik-Brown et al. 2012).

Enteric diseases are known to be one of the major contributors, along with bovine respiratory disease, to decreases in feed consumption, weight gain, and reductions in milk production in dairy cattle, and deaths of youngstock, resulting in severe economic losses in the dairy and beef industries (Goto et al. 2020). Diarrhea accounted for 57% of deaths in unweaned dairy heifers according to the most recent National Animal Health Monitoring System USDA 2013 survey. Likewise, beef producers attributed 16%, 18%, and 2% of overall mortality to digestive disease in calves less than 3 weeks old, calves older than 3 weeks old, and breeding age cattle, respectively (Firkins and Yu 2015; National Animal Health Monitoring 2013). The impact of such diseases extends to human health via the increased use of antimicrobial medications, risk of development of

antimicrobial resistance, and the potential microbial contamination of human food products.

In ruminants, specifically cattle, the composition of the rumen microbiota and its impact on health, nutrition, and host physiological parameters has been studied (Creevey et al. 2014; Firkins and Yu 2015; Jami and Mizrahi 2012b, 2012a; Welkie, Stevenson, and Weimer 2010). Metabolism of nutrients is key in the symbiotic relationship between the host and the microbiota. The intestinal microbiota is generally responsible for breaking down and metabolizing complex carbohydrates. Breakdown of carbohydrates and complex plant materials starts in the rumen with nutrient absorption extending from the forestomaches throughout the intestinal tract. *Bacteroidetes* and *Firmicutes* are among the primary metabolically-active bacteria with a critical role in breaking down plant wall compounds and host-derived carbohydrates, including particles attached to the mucins or chondroitin sulfates of the protective mucosal layer of the intestine (Flint et al. 2012; Krajmalnik-Brown et al. 2012). Changes in the *Firmicutes Bacteroidetes* ratio (F:B) has been demonstrated to affect energy uptake from the diet and energy expenditure, contributing to the development of obesity in pigs, mice, and humans (Krajmalnik-Brown et al. 2012; Pedersen et al. 2013).

Several peer-reviewed studies have been undertaken to analyze the function and/or describe the GIT bacterial communities in different production animals. The studies were typically conducted in animals shortly after euthanasia, from samples collected at slaughterhouses, from animals reared in sterile laboratory environments, or from animals that received a known transplanted microbiota (Derakhshani et al. 2016; Freetly et al. 2020). However, these studies have multiple limitations, including cost (example:

laboratory quality animals), sample collection methods in the live animal, and complete loss of a production unit due to euthanasia and not harvesting for human consumption. Another factor known to influence the outcome of studies of the microbiota is the potential for disruption of the commensal microbiota through dietary changes, infection, and/or inflammatory processes. Additionally, it has been shown that tissue death alters bacterial populations (Donaldson and Lamont 2013; Hanning and Diaz-Sanchez 2015). A full understanding of the GIT microbiota in cattle is still unrealized. While the characterization of the ileal and fecal microbiota and its impact on bovine health and production has been previously investigated, the majority of studies examined only intraluminal samples harvested post-mortem (Liu et al. 2016; Mao et al. 2015). To date, the bovine mucosal-associated microbiota has not been characterized, particularly in the live animal. This is relevant due to most metabolically-active processes occurring at the mucosal interface. Therefore, the purpose of this study was to characterize the ileal mucosal microbiota *in vivo* through the use of serial mucosal biopsy and luminal samples collected endoscopically through a transabdominal-ileal cannula surgically fitted in yearling cattle. Our working hypothesis is that the luminal- and mucosal-associated microbiota of the bovine ileum will differ significantly in their overall composition, as well as in their respective proportion in cattle undergoing consistent husbandry.

Materials and Methods

Animals

The study was conducted at the Auburn University College of Veterinary Medicine following approval of all procedures by the campus Institutional Animal Care and Use

Committee (PRN 2015-2676). Six healthy dairy-breed, steers that were approximately 14 months of age with an average body weight of 425 kilograms were included in this study. All the study animals were housed in a pasture and followed a strictly controlled diet consisting of one flake of Bermuda grass hay and five pounds of soy hull pellets per head twice daily, and fresh water ad libitum.

Ileum Cannulation Technique

Three months prior to when the study took place, study cattle had a T-shaped 2.5 centimeters intestinal cannula surgically fitted in the ileum as previously outlined (Allen et al. 2009).

Briefly, with the animal standing and restrained in a livestock chute, analgesia of the right paralumbar fossa was achieved by regional infusion of 2% lidocaine hydrochloride using the distal paravertebral approach. A standard right flank laparotomy was performed. The cecum and distal ileum palpated and exteriorized allowing clear visualization of the ileocecal junction. An approximate 3 cm incision on the anti-mesenteric side of the distal ileum, about 10-15 cm from the ileocecal junction, was made for insertion of the cannula. To ensure fixation of the cannula to the ileum a purse string suture pattern was placed followed by closure of the enterotomy site a using an inverted closing pattern. A 15 cm incision in the body wall was then made caudoventral to the last rib in order to exteriorize and secure the ileal cannula to its final location. The laparotomy incision was then closed using routine methods. Post-operative treatment consisted of ceftiofur hydrochloride, as an antibiotic, administered subcutaneously (2.2. mg/kg) once daily for five days and meloxicam, as an anti-inflammatory, administered orally (1.0 mg/kg) once daily for five

days. A three-month recovery period was observed following surgery to allow complete healing of the surgical sites, to ensure appropriate drug withdrawal periods were met, and provide research animals a consistent diet prior to study initiation and sample collection. Following the recovery period, all cattle were housed in the same pasture throughout the length of the study without fence-to-fence contact with other animals, and were fed a diet that remained consistent throughout the sample collection period.

Sample Collection and Processing

In order to provide consistency and assess potential variation due to individual, environmental, and bacterial factors, each animal was sampled three times over a four week period. Day of the week and time of the day were assigned randomly.

For sample collection, each individual steer was haltered and restrained in a livestock chute. The ileal cannula was opened by manually removing the compression plug. A sterile 20-centimeter Foley urinary catheter was inserted completely through the cannula aborally to facilitate the collection of 0.5 to 1 milliliters of ileal contents; these samples were designated as luminal content samples. Next, a flexible video-endoscope was inserted through the cannula. Air was used to insufflate the intestinal lumen to facilitate advancement of the scope. Following visual examination, a sterile biopsy instrument was inserted via the endoscope port to remove 3 biopsy samples from the epimural surface of the ileum mucosa. The biopsies were taken from areas consistently within 15 centimeters of the cannula. All samples were placed in 750 µl of RNAlater immediately after collection, to preserve RNA integrity during storage at 4°C until processed.

DNA isolation and sequencing

A total of 18 luminal samples and 18 mucosal biopsy samples were collected for isolation and subsequent sequencing over the four-week collection period. Isolation of DNA from all samples was extracted using a commercial kit (E.Z.N.A® Stool DNA,Omega bio-tek®, Norcross, GA) according to the manufacturer`s guidelines for DNA extraction in tissue, using glass beads, and for fluid samples. The pathogen detection protocol allows rapid and reliable isolation of purified DNA using a combination of reversible nucleic acid-binding properties of HiBind® matrix and spin column technology to allow the elimination of humic acid, polysaccharides, phenolic compounds, and enzyme inhibitors. The extracted DNA was eluted into 100 µl of sterile elution buffer and stored at −20 °C until the time of DNA sequencing and bioinformatics analysis.

The bacterial microbiome was analyzed using 16S rRNA gene V4 variable region PCR primers 515/806 in a single-step 30 cycle PCR using a commercially available kit following the protocol outlined by Dowd et.al, 2008. Sequencing was performed on an Ion Torrent PGM (Personal Genome Machine) following the manufacturer's guidelines and processed using a proprietary analysis pipeline at MR DNA laboratory.

Data Analysis

Data was evaluated in a multivariate manner to profile the microbiome in each group based on the relative abundance of target specific genera identified by the lab. The microbial community structure was analyzed using weighted UniFrac distance matrices. Principal coordinate analysis plots were used to visualize the data in these matrices, and pairwise analysis of similarities (ANOSIM) was utilized to determine if there were any

significant differences between the microbial communities. To provide a visual overview combined with the analysis a dual hierarchal dendrogram was used to display the data for the predominant Genus with clustering related to the different groups.

Results

After rigorous quality sequence curation, a total of 1,891,919 sequences were parsed and 1,759,325 were then clustered. A total of 1,757,838 sequences identified within the Bacteria and Archaea domains were utilized for final microbiota analyses.

At the phylum level, *Firmicutes* (49.5%) and *Bacteroidetes* (30.9%) followed by smaller percentages of *Spirochetes* (5.7%), *Proteobacteria* (4.6%) and *Fusobacteria* (1.6%) were the top five most abundant bacteria in the epimural biopsy samples. In contrast, Firmicutes (60.2%) and *Bacteroidetes* (26.4%) followed by smaller percentages of Spirochetes (3.4%), *Proteobacteria* (3.1%) and *Fusobacteria* (2.3%) composed the lumen contents of the ileum (Figure 12).

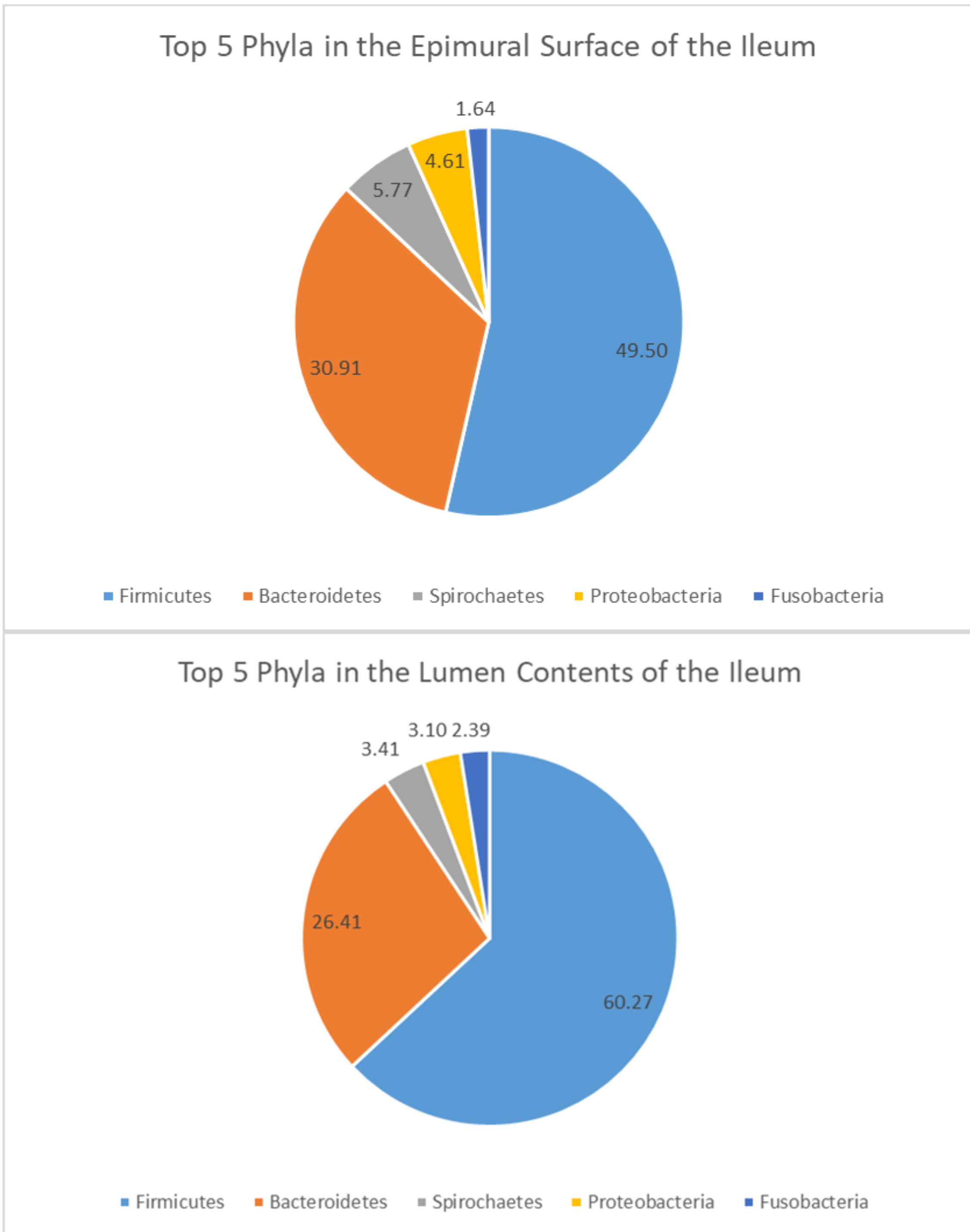


Figure 12: Composition, by phylum, of the top five most abundant bacterial populations present in the epimural surface and lumen contents across all samples collected as determined by Ion Torrent PGM sequencing of the 16 S rRNA gene.

Firmicutes and *Bacteroidetes* comprised over 70% of the microbiome of the ileum in both the epimural luminal sample locations. The F:B for each individual animal of these two predominant phyla was further explored (Figure 13). Variation was observed among animals with respect to F:B; however, the ratio was consistent in individual animals over time. *Firmicutes* phylum is the predominant bacteria regardless of the location, with the *Bacteroidetes* showing the greatest variability.

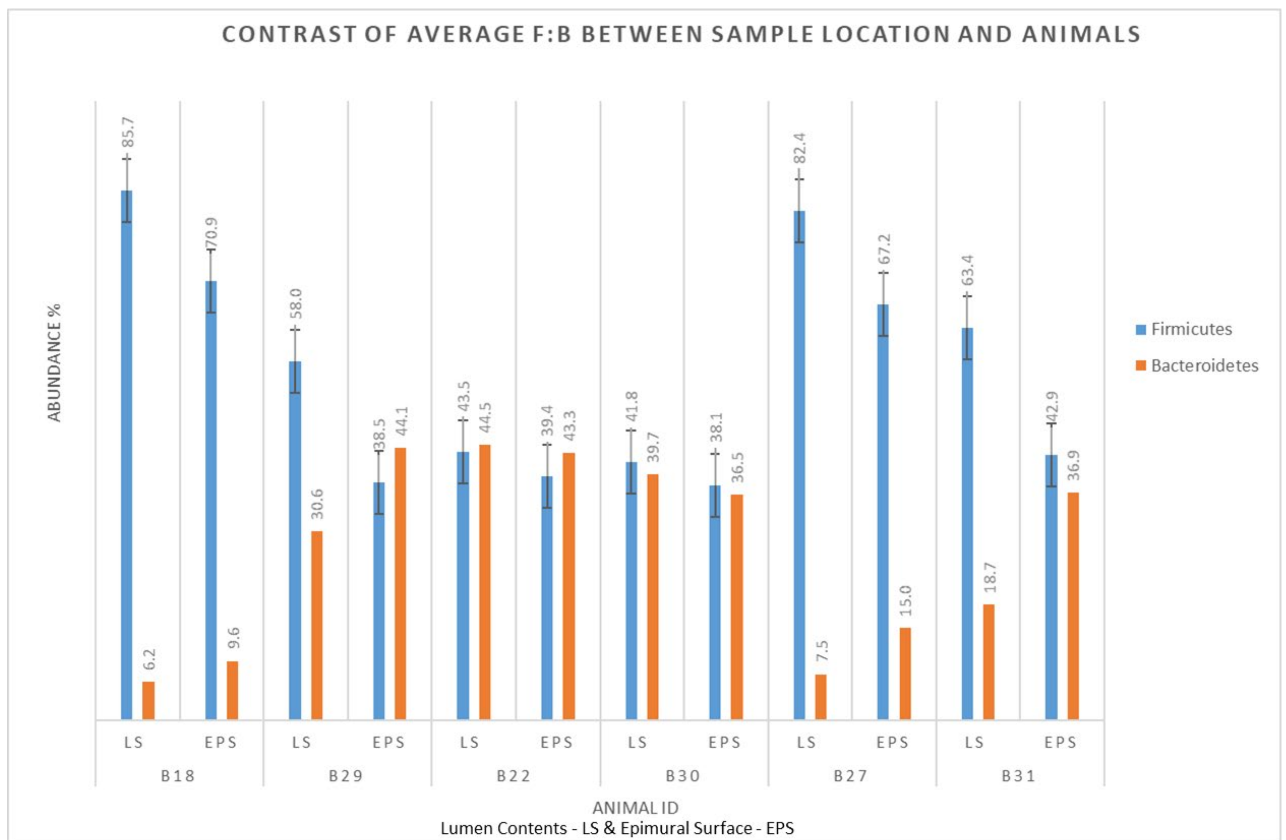


Figure 13: Variability and notable numerical differences between the percentage of overall OTUs for the phylum *Firmicutes* and *Bacteroidetes* throughout the length of the study.

A small cluster of samples seems to be exclusively from for the lumen samples, however it does not appear to have a clear distinction between the two collection sites (Figure 14).

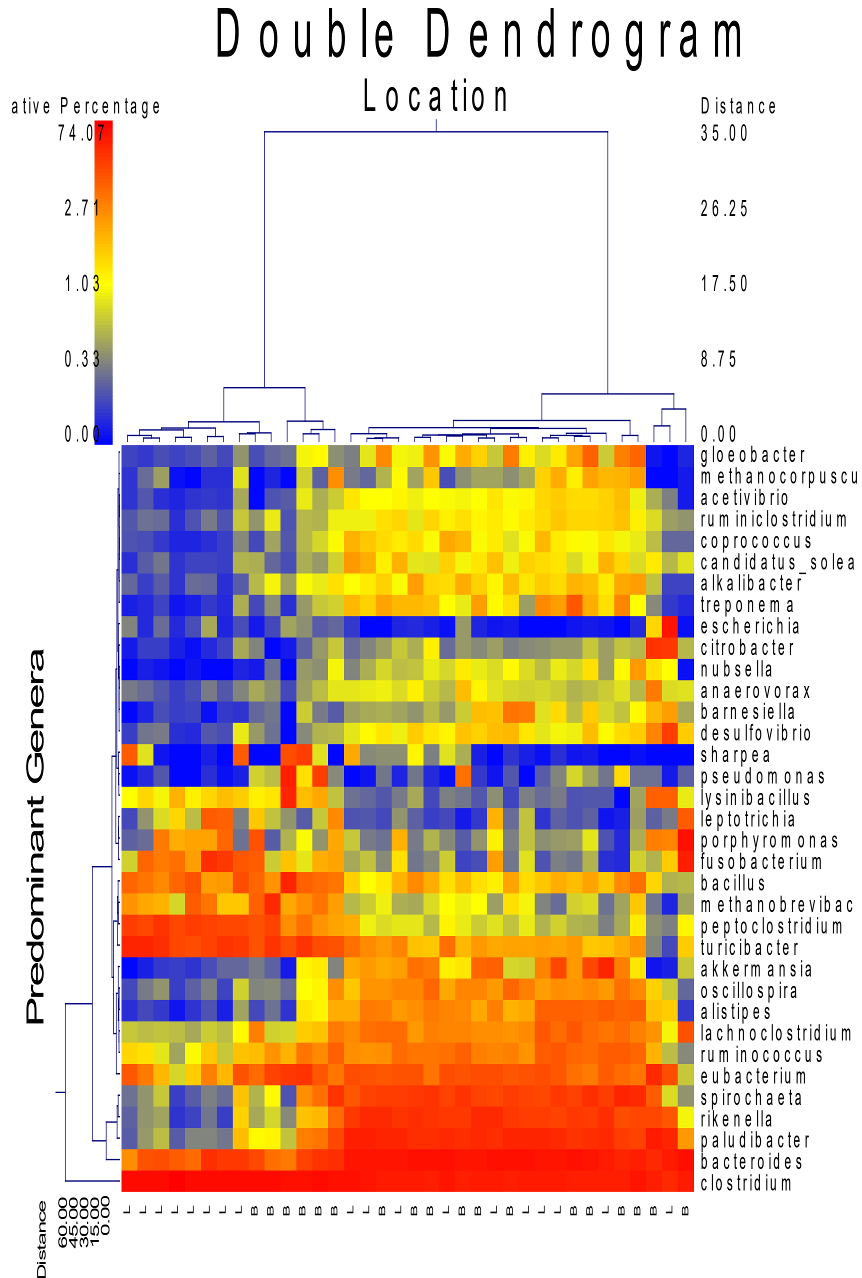


Figure 14: Dual Hierarchal dendrogram evaluation of the taxonomic classification data, with each sample clustered on the X-axis labeled based upon the treatment. Samples with more similar microbial populations are mathematically clustered closer together.

The genera (consortium) are used for clustering. Thus the samples with more similar consortium of genera cluster closer together with the length of connecting lines (top of heatmap) related to the similarity, shorter lines between two samples indicate closely matched microbial consortium. The heatmap represents the relative percentages of each genus. The predominant genera are represented along the right Y-axis. The legend for the heatmap is provided in the upper left corner.

In Figure 15, although it seems to have a tendency, there appears to be no significant phylogenetic assemblage amongst the two collection sites. The primary vector explains 8.4% of the variation between the groups. The first 3 vectors together exhibit 85.3% of the variation among the groups, p=0.06.

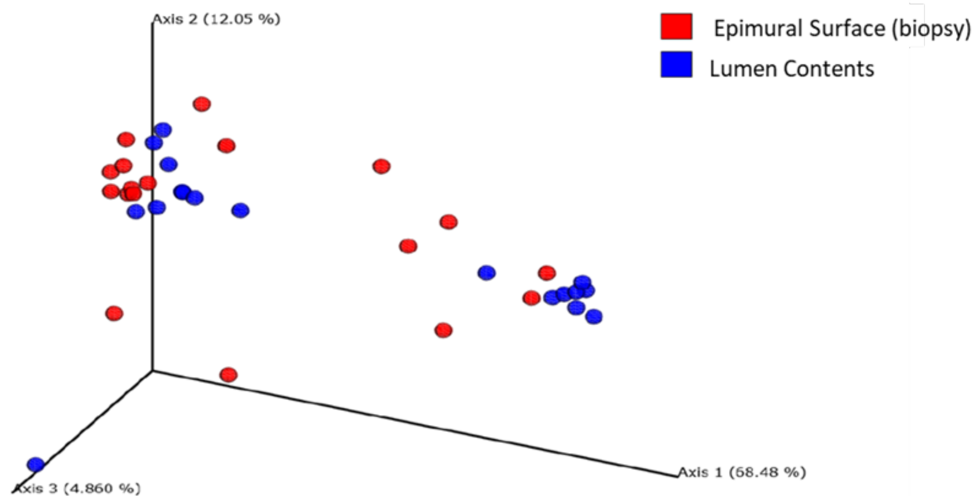


Figure 15: Principal coordinate plot of weighted UniFrac data

Based on the ANOSIM R values in Table 4, we can conclude that there is no relationship observed between similar and dissimilar samples (R=0.077).

Group 1	Group 2	Sample size	R	*p*-value
Epimural Surface	Lumen Contents	36	0.077	0.06

Table 4. Pairwise ANOSIM of weighted UniFrac distance matrix

Discussion

The ileal cannulation model allowed the characterization of the microbiome of the bovine ileum in vivo. Variations between luminal and mucosal biopsy bacterial populations was observed among the study animals. For microbiome analysis, the method by which the ileal mucosal biopsies were collected in this study is unique. A series of three endoscopic biopsy samples per animal per location were collected over a four-week period via the surgically fitted ileal cannula.

Target gene sequencing using specifically Ion Torrent PGM 16S rRNA phylogenetic analytical method was used. Genome sequencing using the 16S rRNA method is widely used among microbiome studies (Freetly et al. 2020; Gong et al. 2019; Jami and Mizrahi 2012b, 2012a; Paz et al. 2018; Zhou et al. 2018; Derakhshani et al. 2016; Yousif et al. 2018). This technique has a wide range of applications, including the characterization of a comprehensive variety of microbial diversity, taxonomical analysis, and species identification (Human Microbiome Project 2012; Chen et al. 2000; Ranjan et al. 2016). Using terminal restriction fragment length polymorphism (T-RFLP) analysis and quantitative PCR (qPCR) in conjunction with a clone library, Reti and colleagues, analyzed and examined the bacterial communities associated with mucosa and within

digesta throughout the intestinal tract of beef cattle (Reti et al. 2013). In their study, jejunal mucosal-associated bacterial communities consisted of mainly *Proteobacteria*, and differed conspicuously from those in the ileum and large intestine and mucosa-associated populations of the ileum, cecum, and descending colon where *Firmicutes* was the primary phylum identified. In agreement, the present study also showed *Firmicutes* as the most common phylum observed in the ileal mucosal biopsy and luminal samples representing approximately 50% of the population. Interestingly, Proteobacteria was the 4th most common bacteria in both locations of the ileum. The authors speculate that such difference seen between this study and the one published by Reti and colleagues, has to do with the fact that they collected the study samples at slaughter versus *in vivo* and it is possible that *Proteobacteria* proliferates faster post-mortem and thus slaughter samples do not accurately reflect the in vivo commensal populations. Sampling methods used in the present study could have allowed for analysis closer to natural conditions, hence the continued predominant abundance of *Firmicutes*.

Similar analytical methods were used to compare microbial populations in the mucosa and luminal microbiota in the colon of pigs, with and without dysentery, at necropsy (Burrough, Arruda, and Plummer 2017). The authors demonstrated significant differences in the microbiome population of the gastrointestinal tissue and luminal ingesta between diseased and disease-free pigs. At the phylum level the relative abundance of *Firmicutes* was greater in the luminal samples of pigs with dysentery whereas *Proteobacteria* and *Fusobacteria* were more abundant in the mucosal scrapings of diseased pigs. The bacterial population found in pigs were comparable to the present study, suggesting the

importance of the commensal microbiome of the ileum in protecting against associated diseases.

De Rodas et al. (2018), analyzed the microbial profile from different anatomical sites of the GIT over time at different ages from farrow to finish using 16S rRNA V4 region sequencing with Illumina MiSeq. The group was able to observe shifts in the microbiome as the animals aged, as well as a positive correlation between several bacteria at the genus level and pig weight. In contrast, while the current study found a highly diverse population between the ileal mucosa and lumen, no significant change in the microbiome profile between the animals was observed, suggesting the ileal microbiome is relatively stable in each individual and the impact is likely dependable on individual metabolic processes. Microbiome studies of longer duration in cattle would be valuable to determine the impact that aging, diet, and other factors have on the microbiome profile. The commensal microbiome plays an important role in its interaction with the immune system, allowing the host to distinguish commensal and pathogenic bacteria. The higher species abundance observed for the mucosal communities suggests their core importance metabolically and immunologically to the host. Results of the current study are consistent with a previous study characterizing the GIT microbiome of pre-weaned calves, where significant differences were found between the bacterial populations at the mucosal surface and within luminal contents (Malmuthuge, Griebel, and Guan le 2014). In that study, the authors proposed that the core metabolically active epimural bacterial population may survive mucosal immune defense mechanisms, and may be crucial for priming the host mucosal immune system. Therefore, understanding the commensal

microbiota in different anatomic locations under normal rearing conditions is critical to understanding GIT metabolism and immunity (Hanning and Diaz-Sanchez 2015).

An interesting finding, which appears to be unique to the ileum, is the presence of *Spirochaetes* as the third most abundant phylum in the bovine ileum with 5.7% versus 3.4% in the epimural surface and lumen respectively. This group of bacteria includes both aerobic and anaerobic species, and both free-living and parasitic forms. In ruminants, *Treponema* spp reside in the rumen where they metabolize cellulose and other difficult-to-digest plant polysaccharides. The function of this group of bacteria in the ileum of healthy cattle is unclear and further investigations at lower taxonomic levels are warranted to study this finding.

The ruminant gastrointestinal microbiome grants many physiological and unique functions that are considered essential to maintain overall homeostasis. Noteworthy findings in the bacterial populations of the lumen and mucosal surfaces of the bovine ileum were identified in this study. Results of this study indicate the ileal microbiota of cattle is composed primarily of *Firmicutes* regardless of location. Many intestinal diseases in cattle occur primarily at the ileum, therefore this result is understandable as the *Firmicutes* bacteria play a profound effect on various aspects of the host's physiology, immune, metabolic, and endocrine functions (Martin et al. 2019; Derakhshani et al. 2016). An important finding of this work was that all sampled animals shared the same primary group of bacteria, their respective abundance was in particular numerically different between the individuals and not between the two locations studied.

It is clear the importance of the microbiome in several physiological processes, including digestion and energy utilization, synthesizing vitamins and essential amino acids, the

immune system, and maintaining the integrity of the gut mucosal barrier (Krajmalnik-Brown et al. 2012), therefore, characterizing the gastrointestinal microbiome *in vivo* is imperative. The microbiome of the ileum from live cattle undergoing normal and expected husbandry was evaluated during this study. This novel approach is crucial as many metabolically and biochemical changes in all body tissues are believed to be altered upon death (Donaldson and Lamont 2013). While this study demonstrates the existence of differences in bacterial populations between individual animals enrolled in the study, significant differences in microbial populations was not observed between the luminal and epimural sampling sites within the bovine ileum. Future research examining perturbations of the ileal microbiome by antimicrobial therapy, dietary changes and or different diseases processes are warranted.

Chapter 6

Response of the Ileal Microbiome of Cattle to a Systemic Macrolide Administration

Introduction

The use of antimicrobial therapy is considered the mainstay for treatment of infectious bacterial and fungal diseases. Negative effects on GIT microbiota and host health during antimicrobial therapy are currently undetermined. The most recent National Animal Health Monitoring System (NAHMS) survey (2012), has indicated that, approximately 10% of cattle operations use antibiotics in feed to prevent respiratory disease in replacement heifers and other weaned calves, and roughly 60% of operations use antibiotics in the feed of replacement heifers weaned but not yet calved to prevent respiratory disease. Further, smaller operations are more likely to use antibiotics in the feed of pre-weaned calves to prevent respiratory disease compared larger beef cattle farms (National Animal Health Monitoring 2012).

As far as treatment of individual diseased animals, the survey reported that larger beef cattle operations (>50 head) were more likely to use oral or injectable antibiotics to treat replacement heifers for respiratory disease. Of the animals that were affected with BRD, over 95% of them were treated with injectable antibiotics. Overall, roughly 20% of operations used injectable antibiotics to treat animals for respiratory disease. Macrolide antibiotics were the primary injectable medication used by 2.7% of operations, preceded only by tetracyclines (~8%) and florfenicols (~4.5%). (National Animal Health Monitoring 2012). Prudent use of antimicrobials in food animals is imperative. The guidance and regulations regarding the use of antimicrobials in food producing animals is set by the Food and Drug Administration Center for Veterinary Medicine. The Title 21,

Code of Federal Regulations, Part 530 (21 CFR 530) provides details on how lawfully licensed veterinarians may or may not use antimicrobials in food animals and provides a list of drugs that are specifically prohibited for use. In 1994, the FDA created The Animal Medicinal Drug Use Clarification Act (AMDUCA) which permits veterinarians to prescribe extra-label uses of certain approved, new animal drugs and approved, human drugs for animals under certain conditions. It also provides guidance with respect to labeling, compounding, and veterinary client patient relationship (VCPR).

As discussed in previous chapters, great variability of the microbiome exists within different areas of the GIT, and the microbiome plays a very important role in several physiological processes, including digestion and energy utilization, synthesizing vitamins and essential amino acids, the immune system, and maintaining the integrity of the gut mucosal barrier (Krajmalnik-Brown et al. 2012). Many studies have shown that antimicrobials can result in microbial dysbiosis, and the disruption of the GIT microbiota in neonates and adults contributes to the development of diseases such as diabetes, obesity, inflammatory bowel disease, asthma, rheumatoid arthritis, depression, autism, and superinfection in critically ill patients (Ley et al. 2005; Machado and Cortez-Pinto 2012; Sekirov et al. 2010; Zhang and Chen 2019).

A recent report published in 2019 summarized relevant concepts related to direct and indirect effects of antimicrobials on the GIT microbiota (Zhang and Chen 2019). It is clear, based on the literature, that different antimicrobials or their combinations cover different bacterial spectra and, therefore, will likely result in different changes to the microbiome (Zhang and Chen 2019).

Antibiotics can affect gut microbiota through direct or indirect mechanisms. Direct mechanisms is defined by antibiotics that were given with the intent to decrease pathogenic bacteria, however due to its pharmacological characteristics, a subset of commensal microbiota are also inhibited or exterminated.

An example of direct effect was explained by Zhang and colleagues (2019), when vancomycin decreased fecal microbial diversity and the absolute number of bacteria belonging to the phylum *Firmicutes*, whereas amoxicillin did not change total bacterial numbers and microbial diversity significantly. Further, a combination of different classes of antibiotics such as ampicillin, gentamicin, metronidazole, neomycin, and vancomycin not only reduced the total number of bacteria but also dramatically shifted the composition of gut microbiota.

Indirectly, symbiosis and codependency are universal among different subsets of the GIT microbiota. Heinken et al (2015) suggested that anoxic conditions in the large intestine drive mutualistic cross feeding, leading to the evolvement of an ecosystem more complex than that of the small intestinal microbiota (Heinken and Thiele 2015). Further, it is important to also mention that some metabolites accumulated in the GIT may be toxic to other microbes, and microbial biotransformation of these toxic metabolites may be restricted to specific species (Zhang and Chen 2019).

A notable example is the conjugated bile acids that can inhibit the growth of bacteria in the duodenum and jejunum (Rowland et al. 2018). Deconjugation by *Lactobacilli*, *Bifidobacteria*, *Clostridium*, and *Bacteroides* is the key step in reducing the toxicity of bile acid. The loss of specific populations of microbiota can lead to the alteration of

metabolites and therefore can greatly impact homeostasis of the GIT (Rowland et al. 2018).

With regards to *Clostridiodes difficile,* formerly named *Clostridium difficile* (Lawson et al. 2016) a major cause of severe enteritis in humans and often associated with the use of antimicrobials, a study in 2015, reported the impact of the combination of antimicrobials, such as, cefoperazone, clindamycin, and vancomycin is associated with the loss of *Lachnospiraceae* and *Ruminococcaceae* families. This dysbiosis then results in reduced transformation of primary bile acids to secondary bile acids in the large intestine which knowingly alters the transformation of amino acids, especially proline, which in turn increases the risk of *C. difficile* infection (Buffie et al. 2015).

With respect to the GIT immunological system, lymphoid cells (tissue resident group 3 innate lymphoid cells) that are prevalent in the intestinal lamina propria, are known to be essential for retaining resident microbes in the GIT lumen and preventing bacteria translocation via an interleukin 22 (IL-22) dependent pathway. Thus, the use of antimicrobials could deplete the GIT microbiota and create a great impact on group 3 innate lymphoid cells activity. This will result in reduction of IL-22 production which could render the host more susceptible to invasion of pathogens through the GIT (Becattini, Taur, and Pamer 2016).

In food animals, there is a plethora of studies that investigated the impact of antimicrobial use in the overall health and effects on different production levels. Holman et al, 2019, studied the impact of oxytetracycline and tulathromycin on the fecal and nasopharyngeal microbiota of beef cattle that were transported to a feedlot. Those two antimicrobials as used frequently in cattle production systems. The group concluded that a single injection

of each of those antibiotics resulted in significant changes in the nasopharyngeal and fecal microbiota during the first 5 days after treatment, where the nasopharyngeal location, overall, appeared to be more sensitive to antibiotic treatment than the fecal microbiota (Holman, Yang, and Alexander 2019). Along the same principles, Foditsh and colleagues studied the changes in the fecal microbiome composition and function after a single treatment of enrofloxacin or tulathromycin administered to dairy calves (Foditsch et al. 2019). They determined that very few significant changes in the microbiota composition were seen, and the small differences over the first weeks resumed back to baseline by day 112 among all study groups concluding that enrofloxacin or tulathromycin had minimal impacts on the microbial composition and genetic functional microbiota of dairy calves (Foditsch et al. 2019).

The impact of feeding milk replacer with or without a combination of different antibiotics, on the gut microbiota of pre-weaning calves was investigated (Yousif et al. 2018). Three study groups were evaluated including a group fed milk replacer without antibiotics, a group fed a milk replacer containing a combination of antibiotics (penicillin, streptomycin, tetracycline and ceftiofur), and a group fed milk replacer treated with only ceftiofur. The group fed the milk replacer with the antibiotic combination had significant changes at different taxonomic levels in the gut microbiota compared to other groups. Changes were associated with decreased abundance of *Enterobacteriaceae*, especially *E. coli* in the ileum as well as a reduction in the relative abundance of *Acidaminococcaceae* in the rectum. The group that received the diet with the single antibiotic showed a significant increase in the abundance of bacteria belonging to the class *Bacilli* in the colon and rectum and a reduced abundance of *Comamonas*, a Gram- negative bacteria

from the genus of *Proteobacteria*, in the ileum. An increase in *Prevotellaceae*_Ga6A1_ group in the colon was also observed in the single treatment group (Yousif et al. 2018). Conversely, with respect to *E.coli*, another group reported that in-feed antibiotics led to an increase in the abundance of pathogenic bacteria like *E.coli* in calves and pigs (Looft et al. 2012; Xie et al. 2013). However, such comparisons must be interpreted carefully as different studies use different antimicrobials which may increase the likelihood of discordant results as the pharmacokinetics of different drug classes differs greatly from one another.

It is a common practice to feed "hospital milk", term used for non-salable milk that originated from lactating dairy cattle. Feeding raw milk with drug residues to calves from birth to weaning showed significant differences in bacterial population only at the genus level with a decrease in the relative abundance of *Clostridium spp* and *Streptococcus spp* but not at the phylum, class, order or family levels. The authors speculated that drug residues in milk distinguished the microbial diversity in the gut of young dairy calves (Van Vleck Pereira et al. 2016).

In pigs, an antimicrobial-dependent shift in the composition of fecal microbiota has been shown to occur over time (Zeineldin et al. 2018a). Similarly, another study demonstrated that the shifts in fecal microbiota structure caused by neonatal antimicrobial administrations is minimal and limited to certain microbiota. In addition, the researchers speculated the early administration of procaine penicillin G and tulathromycin may promote the selection of antimicrobial resistance genes in herds (Zeineldin et al. 2019). The same group evaluated the potential changes in the nasal microbiota of pigs after administration of commonly used antibiotics. Similar to the GIT, the nasal microbiota of

pigs was dominated by *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*. It was concluded that the impact of systemic antibiotics on the deep nasal swab microbiota of pigs is variable and has a considerable impact in modulating the nasal microbiota (Zeineldin et al. 2018b).

In dogs, a minimum of a four-week effect after treatment with metronidazole on fecal microbiome has been demonstrated. Metronidazole altered the microbiome composition by decreasing species richness, with an observed abundance of *Fusobacteria spp* and *Clostridium hiranonis*. Also fecal dysbiosis index was significantly increased as determined by increased fecal total lactate, decreased secondary bile acids and lithocholic acid (Pilla et al. 2020).

Tylosin, a macrolide antibiotic commonly used in feed of feedlot cattle to aid the prevention of liver abscesses, was studied. The investigators compared the microbiome and antimicrobial resistance genes in feces of feedlot cattle. They concluded that tylosin did not influence the resistance genes or fecal microbiome composition (Weinroth et al. 2019). Using tulathromycin, a different but related macrolide to tylosin, metaphylaxis treatment was performed at arrival to the feedlot and fecal microbiome assessed. No differences were seen in the microbiome between groups, in fact, it was determined that changes in diet, geography, conspecific exposure, and environment associated with movement of cattle to the feedlot may cause greater impact on the fecal microbiome than treatment itself (Doster et al. 2018). Several formulations of macrolides are available in the USA for treatment of diseased cattle and/or for metaphylaxis, more specifically bovine respiratory disease. A NAHMS survey indicated that the macrolide tulathromycin

was used as metaphylaxis in 45.3% of feedlots at time of arrival and oxytetracycline in 17.4% (National Animal Health Monitoring 2013).

The most recent literature shows conflicting results associated with the impact of macrolides on the microbiome associated with the GIT of cattle. The small intestines, in general, and the ileum, in particular, is the location of significant microbial processes in both metabolic (e.g. digestion) and pathologic events. The ileum is responsible for the absorption of vitamin B12, bile salts and other products of digestion not absorbed in the proximal small intestines. Lesions of paratuberculosis, a chronic bacterial enteropathy of ruminants with clinical signs and pathogenesis similar to Crohn's disease in humans, are centered in the ruminant ileum. Alterations in the microbiome are suspected to play key roles in the pathogenesis and clinical progression of Crohn's disease as well as chronic enteropathies in dogs (Cassmann et al. 2016; Naftali et al. 2016). Consequently, appropriate balance of the ileal microbiome is thought to be important to the health of the individual in both the healthy and diseased states.

For the study described in this chapter, the authors hypothesized that animals treated with a systemic macrolide antimicrobial used for bovine respiratory disease (BRD) in bovine medicine, gamithromycin, undergo significant changes in the microbiome due to antimicrobial concentration in the lumen contents and mucosal surface of the ileum which is likely to cause a negative impact on the commensal microbiota of the bovine ileum. The novelty of this study was the use of an indwelling ileal cannula which allowing the study to be conducted in vivo.

Pharmacological Characteristics of Gamithromycin

Gamithromycin is a novel semi-synthetic macrolide labeled for the treatment and prevention of BRD. Gamithromycin has both bacteriostatic and bactericidal action mediated through the disruption of bacterial protein synthesis. The broad spectrum antimicrobial activity of gamithromycin has allowed for label claims against pathogenic bacterial including *Mannheimia haemolytica*, *Pasteurella multocida* and *Histophilus somni*, the bacterial pathogens most commonly associated with BRD. This compound achieves high concentration in peripheral tissues, such as respiratory, tonsillar, and reproductive tissues, when compared to plasma. The epithelial lining fluid (ELF) is known to be a target region where this macrolide accumulates in the host polymorphonuclear leukocytes and macrophages cells with the goal to eradicate bacterial pathogens as efficiently as possible.

Ionization

Antibiotics are either weak organic acids or bases and exist in solution in both the non-ionized and the ionized forms. Non-ionized drugs are lipid soluble and cross membranes rapidly in order to achieve equilibrium with the adjacent compartment. The ionized or charged form of a drug is water soluble and does not cross lipid-rich cell membranes as easily. The electrical charge on a drug molecule is, therefore, very important in determining the rate of absorption across cell membranes and tissue barriers. Ionization state depends on the pH of the drug's environment and the pKa (pH at which 50% of the drug is ionized) of the drug. In the case of an acidic drug, the drug becomes ionized when the pKa is less that the ambient pH. A basic drug is ionized when the pKa is greater than

the ambient pH. Basically, the consequences of a shift in pH away from equilibrium is opposite for weak acids and weak bases. Thus, at a pH less than pKa, the non-ionized, more lipid soluble form of a weak acid is favored, whereas it is the ionized, less soluble form of a weak base that is present in excess. In some cases, due to differences in pH, a non-ionized drug becomes ionized upon reaching its target tissue; consequently, ionization causes the drug to remain in the tissue (Ziv 1980a, 1980b).

Macrolides in general are potent weak bases that become ion-trapped within acidic intracellular compartments such as lysosomes and phagosomes. A beneficial consequence of macrolide accumulation within cells is an increase in activity against intracellular pathogens. Its basic characteristics allow macrolides to concentrate heavily in other target tissues, such as the epithelial lining fluid (ELF) and the alveolar macrophages, rather than stay concentrated in the central compartment (plasma) (Giguère et al. 2011).

Plasma Protein Binding and Distribution

The rate and extent of penetration of a drug into most sites outside the vascular space are also determined by the drug's molecular charge and size, lipid solubility, extent of plasma protein binding, and blood flow at the site of infection (Giguère et al. 2011).

The degree of protein binding of a drug has marked effects on its distribution, elimination, and pharmacological effect. Within the blood stream, drugs are associated with constituents such as albumin, α ß, and γ globulins, lipoproteins, and erythrocytes. Drugs can bind to a single or multiple proteins, but the most significant protein involved in drug binding is albumin (Ziv 1980a). Unbound or free drug molecules can penetrate membranes and the walls of intact blood vessels, whereas protein-bound molecules

cannot. Thus, highly protein-bound drugs are less effective therapeutically if they cannot reach the site of infection. On the other hand, protein binding allows drugs to persist in the body, thereby providing a reservoir from which free drug is slowly released to perform its antimicrobial activity.

Gamithromycin's high volume of distribution (~25 L/kg), long half-life (~45 hrs) and very low protein binding (~26%) confers its ability to be rapidly absorbed after subcutaneous administration and reach the target tissue promptly. Because only 26% of gamithromycin binds to bovine plasma protein, more unbound drug is available for distribution, which is an important factor to the large volume of distribution and rapid accumulation in lung tissue (Huang et al. 2010). Evidently, drug distribution varies greatly between antimicrobial agents of different classes because of their distinct chemical nature, blood flow to tissues, and ability of a drug to penetrate cellular barriers. With very low protein binding, gamithromycin, tends to absorb and concentrates at sites of infection very effectively (Huang et al. 2010). With these features, in vivo clinical efficacy is better studied when gamithromycin concentration is analyzed at the site of infection or target tissue rather than utilizing the comparison of plasma concentration and minimum inhibitory concentration for the pathogens in question (FOI NADA 141-328).

Research Project

Concomitantly to the study in Chapter 5, the research team also characterized the ileal lumen and the mucosal surface associated microbiome, in vivo, from six, 12-14 month old dairy steers with a surgically-fitted, indwelling ileal cannula following administration

of a single 6 mg/kg dose subcutaneously of gamithromycin 150 mg/mL according to the manufacturer label.

Sample collection and timeline is detailed on Table 5. The methodology was accomplished as described for the other studies detailed in this dissertation, but briefly, the ileal cannula was opened by manually removing the compression plug. A sterile 20-centimeter Foley urinary catheter was used to collect luminal contents followed by the insertion of a flexible video-endoscope[e] aiding in biopsy sample collection at each designated collection time point. All samples were placed in 750 µl of RNAlater™ immediately after collection to preserve RNA integrity during storage at 4°C until processed. Blood samples were collected via the coccygeal vein using a serum separator blood collection tube and vacutainer.

Animal ID	Day 0		Day 3	Day 8	Day 15	Day 35 *	Day 70^
	Groups	Samples Collected					
22	Tx	L & P	L, B & P	L, B & P	L, B & P	L, B & P	L & P
31	Tx	L & P	L, B & P	L, B & P	L, B & P	L, B & P	L & P
29	Tx	L & P	L, B & P	L, B & P	L, B & P	L, B & P	L & P
18	Tx	L & P	L, B & P	L, B & P	L, B & P	L, B & P	L & P
27	Control	L & P	L, B & P	L, B & P	L, B & P	L, B & P	L & P
30	Control	L & P	L, B & P	L, B & P	L, B & P	L, B & P	L & P

*Labeled meat withdrawal for gamithromycin in cattle
^ 1 ½ times labeled meat withdrawal
Tx: 6mg/kg subcutaneously once of 150mg/ml of gamithromycin @ day 0 of the study
L: lumen sample for microbiome and antimicrobial analysis
B: biopsy of mucosal surface for microbiome and antimicrobial analysis
P: blood collection for antimicrobial analysis

Table 5. Ileal microbiome followed antibiotic administration study timeline

For the characterization of the bacterial microbiome 16S rRNA gene V4 variable region PCR primers 515/806 in a single-step 30 cycle PCR using a commercially available kit following the protocol outlined by Dowd et.al, 2008 was used. (Dowd et al. 2008) Sequencing was performed on an Ion Torrent PGM (Personal Genome Machine) following the manufacturer's guidelines and processed using a proprietary analysis pipeline at MR DNA laboratory.

Analysis of gamithromycin

Antimicrobial concentration analysis was performed using high performance liquid chromatography (HPLC) at the Kansas State University College of Veterinary Medicine Department of Anatomy & Physiology Pharmacology Laboratory. This method is quantitative, compound specific, and ideal for confirming positive screening test results and for use in research studies.

All chemicals were liquid chromatography – mass spectrometry (LC-MS) grade and were purchased from Fisher Scientific (Hampton, NH). Gamithromycin was purchased from Cayman Chemical Company (Ann Arbor, Michigan) and erythromycin (internal standard) was purchased from Millipore Sigma (Burlington, MA).

Analytical parameters for gamithromycin: LC analysis was performed on a Vanquish UHPLC (Thermo Fisher Scientific, San Jose, CA) consisting of a vacuum degasser, a binary pump, a thermostated autosampler, and a thermostated column compartment. The sampler temperature was set at 8 °C and the column compartment temperature at 55 °C. A UPLC column Eclipse Plus C18 column (1.8, 100 x 2.1 mm) from Agilent

Technologies (Santa Clara, CA)was used to separate the compounds of interest with a mobile phase composed of 0.1% of aqueous formic acid (A) and acetonitrile (B). The gradient elution was set as follows: 0.0 min the initial mobile phase composition contained 2% B, then from 0–0.5 min linear gradient from 2 to 30% B; 0.5–4 min linear gradient from 30% to 50% B; 4.00–4.50 min linear gradient from 50 to 100%A followed by a 2 min wash with 100% B and back to 2% B and equilibration of the column for 2 min. The total run time was 8.50 min. The flow rate was set at 0.4 mL/min. The sample injection volume was 10 mL.

The UHPLC system was interfaced to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with a heated electrospray ionization (HESI) source. The optimized parameters were as follows: capillary temperature, 365°C; auxiliary temperature, 400°C; electrospray voltage, 3.0 kV (positive mode); sheath gas, 50 arbitrary units; auxiliary gas, 15 arbitrary units; sweep gas flow, 3 arbitrary units, S lens RF level, 50. Analyses were carried out using TraceFinder 4.0 software (Thermo Fisher Scientific, San Jose, CA). The analyses were acquired in Full MS-ddMS2 (full scan data-dependent acquisition) in positive mode at a resolving power of 35,000 FWHM at m/z 200. To improve the sensitivity of detection, the scan range was set at m/z 360-410 for gamithromycin from 1.5-2.5 min and at m/z 680-730 for erythromycin between 2.8-3.5 min. The automatic gain control (AGC) was set at 1e6, with an injection time of 50 ms. The isolation window of the quadrupole that filters the precursor ions was set at m/z 1.2. The fragmentation of precursor was optimized at three values of normalized collision energy (NCE) (25, 35 and 45 eV). The detection was based on calculated [M+2H]2+ for

gamithromycin and [M+H] + for erythromycin with an accuracy of 5 ppm, retention time and fragments match (m/z and intensity).

Analysis of gamithromycin in bovine serum

Calibration standards: Calibration standards working solutions were prepared freshly in aqueous formic acid 1% at the following concentrations: 2.5, 5, 10, 25, 50, 100, 250 ng/mL. On the day of analysis, 0.1 mL of working standard and 0.1 mL erythromycin 50 ng/mL were added to 0.1 mL of negative control bovine serum and 0.3 mL of aqueous phosphoric acid 4% in a 1.0 mL microcentrifuge tube. After mixing for 5 seconds, the tubes were centrifuged at 13,000 g for 5 minutes.

Negative control: 0.2 mL of aqueous formic acid 1% was added to 0.1 mL of serum sample and 0.3 mL of aqueous phosphoric acid 4% in a 1.0 mL microcentrifuge tube.

Serum samples: On the day of analysis, 0.1 mL of aqueous formic acid 1% and 0.1 mL erythromycin 50 ng/ml were added to 0.1 mL of serum sample and 0.3 mL of aqueous phosphoric acid 4% in a 1.0 mL microcentrifuge tube.

Quality control samples: Quality controls were prepared by spiking negative control serum samples at the following concentrations: 6, 60 and 120 ng/mL. On the day of analysis, 0.1 mL of aqueous formic acid 1% and 0.1 mL erythromycin 50 ng/ml were added to 0.1 mL of quality control serum sample and 0.3 mL of aqueous phosphoric acid 4% in a 1.0 mL microcentrifuge tube. The QCs are mixed for 10 seconds using a vortex mixer.

Upon preparation, all tubes were mixed for 10 seconds using a vortex mixer. All tubes were centrifuged at 13,000 g for 5 minutes, and the supernatants were cleaned-up by solid phase extraction (SPE).

Clean-up by SPE: The serum was cleaned-up using Oasis PRIME MCX Elution plate (2 mg) from Waters Co. (Milford, MA). The SPE Elution plate was stacked on top of a spacer and a 2 mL collection plate to collect the waste. The acidified negative control, serum samples, calibration standards were loaded on the SPE plate and pushed through the plate using a Positive Pressure-96 plate Processor, (Waters Co., Milford, MA). The plate was washed with 0.3 mL of aqueous ammonium formate 100 mM containing 2% formic acid followed by 0.3 mL of methanol. Gamithromycin was eluted in a clean collection plate with 50 uL of ammonium hydroxide 5% in methanol. 50 uL of aqueous formic acid 5% was added to each well before analysis.

Validation parameters: The response (analyte over internal standard) was plotted against the concentration (ng/mL). Linear regression with a weighing factor of 1/x was the best fit selected for the calibration of gamithromycin. The coefficient of correlation was at least > 0.99, and the response was linear between 2.5 to 250 ng/mL. The lower limit of quantification was 2.5 ng/mL. Intra-day precisions at 6, 60 and 120 ng/mL were 1.9%, 1.4%, 6.9%, respectively. The intra-day accuracies were at 109.7%, 105.9% and 102.4% respectively (n=3). The inter-day precisions at 6, 60 and 120 ng/mL were 6.1, 6.9%, 6.0%, and the inter-day accuracies at 6, 60 and 120 ng/mL were 107.1%, 111.4% and 105.5% (n=6), respectively.

Analysis of gamithromycin in ileum biopsies

Negative control: Negative control bovine intestine tissue obtained from the veterinary hospital was prepared using the same method as the samples.

Biopsy sample: Biopsies were weighted (10 mg), transferred into a 15 mL conical tube and homogenized in 2 mL of aqueous ammonium formate 100 mM with 2% formic acid. The homogenate is spun down for 30 min at 4 °C and 4,500g. To 1.0 mL of sample homogenate was added 0.1 mL of erythromycin 200 ng/mL in aqueous formic acid 2% and 0.1 mL of aqueous formic acid 2%. After mixing for 10 seconds, the sample was cleaned-up by solid phase extraction. The total dilution factor was 40. Biopsies containing a high concentration of gamithromycin were diluted 5 times with mobile phase and re-analyzed.

Quality control samples: Quality controls were prepared by spiking negative control serum homogenates at the following concentrations: 6, 15 and 90 ng/mL (corresponding to 1.2, 3.0 and 18 ng/g of biopsy). On the day of analysis, to 1.0 mL of quality control homogenate was added 0.1 mL of erythromycin 200 ng/mL in aqueous formic acid 2% and 0.1 mL of aqueous formic acid 2%. After mixing for 10 seconds, the sample was cleaned-up by solid phase extraction. Following SPE, the concentrations in the HPLC vial were 30, 75 and 450 ng/mL.

Clean-up by SPE: The biopsies extracts were cleaned by using Oasis HLB (3cc, 60 mg) cartridges from Waters Co (Milford MA). After conditioning the cartridge successively with 2 mL of methanol and 2 mL of water, the sample was loaded. The cartridge was then washed with water containing 5% methanol and allowed to dry for 1 minute. Gamithromycin was eluted with 2 mL of methanol and collected in a glass tube. The

eluate was dried with a vacuum concentrator at 40°C, and the residue was reconstituted in 0.2 mL of aqueous formic acid 0.1%.

Matrix-matched calibration standards: Calibration standards working solutions were prepared freshly in aqueous formic acid 1% at the following concentrations: 50, 100, 250, 500, 1000, 2500 and 5,000 ng/mL. On the day of analysis, 10 mL of gamithromycin working standard, 10 mL of erythromycin at 1,000 ng/mL were added to 180 mL of cleaned negative control biopsy extract. The concentrations of the calibration standards in the cleaned extract were as follows: 5; 10; 25; 50; 100, 250 and 500 ng/mL.

Validation parameters: The response (analyte over internal standard) was plotted against the concentration (ng/mL). Linear regression with a weighing factor of 1/x was the best fit selected for the calibration of gamithromycin. The coefficient of correlation was at least > 0.99, and the response was linear between 5 to 100 ng/mL in the clean extract. The lower limit of quantification was 0.2 ng/g. Intra-day precisions at 0.24, 60 and 3.6 ng/g were 5.8%, 3.5%, 2.5%, respectively, and the intra-day accuracies were at 121.6%, 114.9% and 109.3%, respectively (n=3). The inter-day precisions at 0.24, 60 and 3.6 ng/g were 16.6%, 15.5%, 10.9%, and the inter-day accuracies at 0.24, 60 and 3.6 ng/g were 87.6%, 88.6% and 91.3% (n=6), respectively.

Analysis of gamithromycin in ileum lumen

Negative control: Negative control bovine feces was obtained from the veterinary hospital and was prepared using the same method as the samples.

Ileum lumen: Samples were taken out of the -80°C freezer; let thaw on the bench for about 20-30 minutes. 1 g of sample was weighted in a 15 mL conical centrifuge tube and

9 mL of ammonium formate 100 mM containing 2% formic acid (pH 4.0) was added. The samples were homogenized. The tubes were centrifuged for 30 minutes at 4 °C and 4,500 g. The supernatant was transferred into a clean tube.

Quality control samples: Quality controls were prepared by spiking negative control feces at the following concentrations: 150, 450 and 900 ng/g (final concentration of 37.5, 112.5, 225 ng/mL in the HPLC vial). On the day of analysis, to 0.5 mL of quality control homogenate was added 0.1 mL of erythromycin 200 ng/mL in aqueous formic acid 2% and 0.1 mL of aqueous formic acid 2%. After mixing for 10 seconds, the sample was cleaned by solid phase extraction.

Clean-up by SPE: The lumen homogenates were cleaned by using Oasis MCX (3cc, 60 mg) cartridges from (Waters Co.,Milford, MA). After conditioning the cartridge successively with 2 mL of methanol and 2 mL of water, the sample was loaded with the liquid being pushed through the cartridge using a vacuum manifold. The cartridge was then washed successively with 2 mL of aqueous ammonium formate 100 mM containing 2% formic acid and 2 mL of methanol. Gamithromycin was eluted with 2 mL of ammonium hydroxide 5% in methanol. The organic solvent was evaporated with a vacuum concentrator, and the residue was reconstituted in 0.2 mL of aqueous formic acid 0.1%.

Matrix-matched calibration standards: Calibration standards working solutions were prepared freshly in aqueous formic acid 1% at the following concentrations: 100, 250, 500, 1000, 2500, 5000 ng/mL. On the day of analysis, 10 uL of gamithromycin working standard, 10 uL of erythromycin at 1,000 ng/mL were added to 180 uL of cleaned

negative control lumen extract. The concentrations of the calibration standards were the following in the cleaned extract: 10; 25; 50; 100, 250, 500 ng/mL.

Validation parameters: The response (analyte over internal standard) was plotted against the concentration (ng/mL). Linear regression with a weighing factor of 1/x was the best fit selected for the calibration of gamithromycin. The coefficient of correlation was at least > 0.99, and the response was linear between 10 to 500 ng/mL in the clean extract. The lower limit of quantification was 40 ng/g. Intra-day precisions at 150, 450 and 900 ng/g were 7.8%, 7.6%, 6.1%, respectively, and the intra-day accuracies were at 114.0%, 106.7% and 108.4%, respectively (n=3). The inter-day precisions at 150, 450 and 900 ng/g were 8.0%, 6.1%, 8.0%, and the inter-day accuracies were 116.5%, 108.9% and 102.6% (n=6), respectively.

Pharmacological Results

The unbound plasma gamithromycin concentration along with the lumen contents and epimural mucosal surface of the ileum were measured and are presented in Tables 6 and 7. Table 6 summarizes the results associated with the control group. The concentration of gamithromycin in plasma, fluid, and tissue are represented in parts per billion (ppb). Areas marked as “N/A” indicate that samples were not taken during those time points. As expected, antimicrobial analysis revealed no detection (zero ppb) for the samples associated with the plasma and lumen contents. Interestingly, it is important to note that although low concentrations are reported, antibiotic was detected in the biopsy samples of the control animals. It is speculated that this could be related to contaminated analysis technique or small shifts in retention time caused by air bubbles, column wear or

blockages, or changes in the pumping pressure which may have led to read outputs containing curve spikes even though no antibiotics are present.

CONTROL	Concentration of Gamithromycin in ppb					
ID 30	Day 0	Day 3	Day 8	Day 15	Day 35	Day 70
Serum	0	0	0	0	0	0
Lumen Contents	0	0	0	0	0	0
Mucosal Biopsy	N/A	13	1,357	7	----*	N/A

* Sample was unavailable for analysis

CONTROL	Concentration of Gamithromycin in ppb					
ID 27	Day 0	Day 3	Day 8	Day 15	Day 35	Day 70
Serum	0	0	0	0	0	0
Lumen Contents	0	0	0	0	0	0
Mucosal Biopsy	N/A	11	66	6	7	N/A

Table 6: Concentration of gamithromycin (ppb) in the control samples collected during the designated time points.

Table 7 summarizes the antimicrobial concentration in the animals that were treated with gamithromycin. Samples collected at day 0 were all determined to be negative, as expected. Samples at day 3 indicating time point associated with the terminal half-life, day 8 samples reflecting the time point before the gamithromycin is still expected to be above 1ug/ml (MIC90) in PELF, day 15 samples representing the one week time point from when the concentration remains above the MIC90 in BAL cells and lung tissue, day 35 samples for the established meat withdrawal time, and day 70 representing one and half times the established meat withdrawal were all determined.

A drop in concentration from day 3 to day 8 and a spike back on day 15 was noted. This short-term biphasic absorption effect is characteristic of macrolides and plays an important role on inflammatory modulation (Matzneller et al. 2013). A significant higher drug concentration was seen in the mucosal biopsy samples for all time points for all

animals, especially if compared to plasma. This finding is in agreement with the pharmacological characteristics of gamithromycin. As a basic compound with a high volume of distribution (24.9 ± 2.99 L/kg) this macrolide promptly leaves the central compartment and moves into the deep peripheral compartments. This distribution characteristic is responsible for the efficacy to target tissues as an unbound free drug performing its antimicrobial activity very effectively.

	Concentration of Gamithromycin in ppb					
ID 31	Day 0	Day 3	Day 8	Day 15	Day 35	Day 70
Serum	0	103.2	11.5	20.7	5	0
Lumen Contents	0	544	475	47	21	0
Mucosal Biopsy	N/A	28,366	2,096	865	69	N/A

	Concentration of Gamithromycin in ppb					
ID 29	Day 0	Day 3	Day 8	Day 15	Day 35	Day 70
Serum	0	51.7	10.2	19.6	0	0
Lumen Contents	0	1018	179	48	0	0
Mucosal Biopsy	N/A	37,369	9,423	2,606	141	N/A

	Concentration of Gamithromycin in ppb					
ID 22	Day 0	Day 3	Day 8	Day 15	Day 35	Day 70
Serum	0	75.2	28.9	9.5	0	0
Lumen Contents	0	723	127	50	0	N/A
Mucosal Biopsy	N/A	79,274	9,754	----*	122	N/A

* Sample was unavailable for analysis

	Concentration of Gamithromycin in ppb					
ID 18	Day 0	Day 3	Day 8	Day 15	Day 35	Day 70
Serum	0	57.5	7.1	18.2	0	0
Lumen Contents	0	633	168	50	0	0
Mucosal Biopsy	N/A	33,352	10,630	1,669	----*	N/A

* Sample was unavailable for analysis

Table 7: Concentration of gamithromycin (ppb) in the samples collected from the treatment group during the designated time points.

Microbiome Results

For this study, the microbiome profile was determined at the same time points used for the antibiotic analysis and was conducted for the lumen contents and mucosal biopsy of the ileum. After rigorous quality sequence curation, a total of over 1.7 million sequences were identified within the Bacteria and Archaea domains and utilized for final microbiota analyses. The average reads per sample was approximately 48.000.

As discussed previously, it is imperative to note gamithromycin targets, in bovine medicine, infections associated with *Mannheimia haemolytica*, *Pasteurella multocida* and *Histophilus somni*, all pathogenic bacteria belonging to the phylum *Proteobacteria*. Initially, for comparison purposes, only the bacteria belonging to the phylum level were studied. Figure 16 shows the top five most abundant bacterial phyla present on the mucosal surface of the ileum. The control group (no treatment given) microbiome consisted of *Firmicutes* (55%) and *Bacteroidetes* (21%) followed by smaller percentages of *Fusobacteria* (3%), *Proteobacteria* (6%), and *Euryarchaeota* (2.3%). In contrast, the microbiome present in the treated group consisted of *Firmicutes* (61%) and *Bacteroidetes* (22%) followed by smaller percentages of *Fusobacteria* (<0.5%), *Proteobacteria* (3.8%), and *Euryarchaeota* (2.5%).

Figure 17 shows the top five most abundant bacterial phylum present in the lumen contents of the ileum. The control group (no treatment given) microbiome consisted of *Firmicutes* (61.4%) and *Bacteroidetes* (25.6%) followed by smaller percentages of *Fusobacteria* (2%), *Proteobacteria* (1.5%), and *Euryarchaeota* (1.9%). In contrast, the microbiome present in the treated group consisted of *Firmicutes* (61.7%) and *Bacteroidetes* (27.6%) followed by smaller percentages of *Fusobacteria* (1%), *Proteobacteria* (1.9%), and *Euryarchaeota* (2.3%).

At the phylum level, when compared between the cattle and over the days the study was conducted, individual variability appeared greater in the mucosal biopsy microbiota than luminal populations (Fig 18 & 19). The proportion of different phyla in samples was generally similar for each animal; however, it appears to be rather distinct among the cattle.

In figure 19, the variability appears to be less evident not only individually but also amongst the study days.

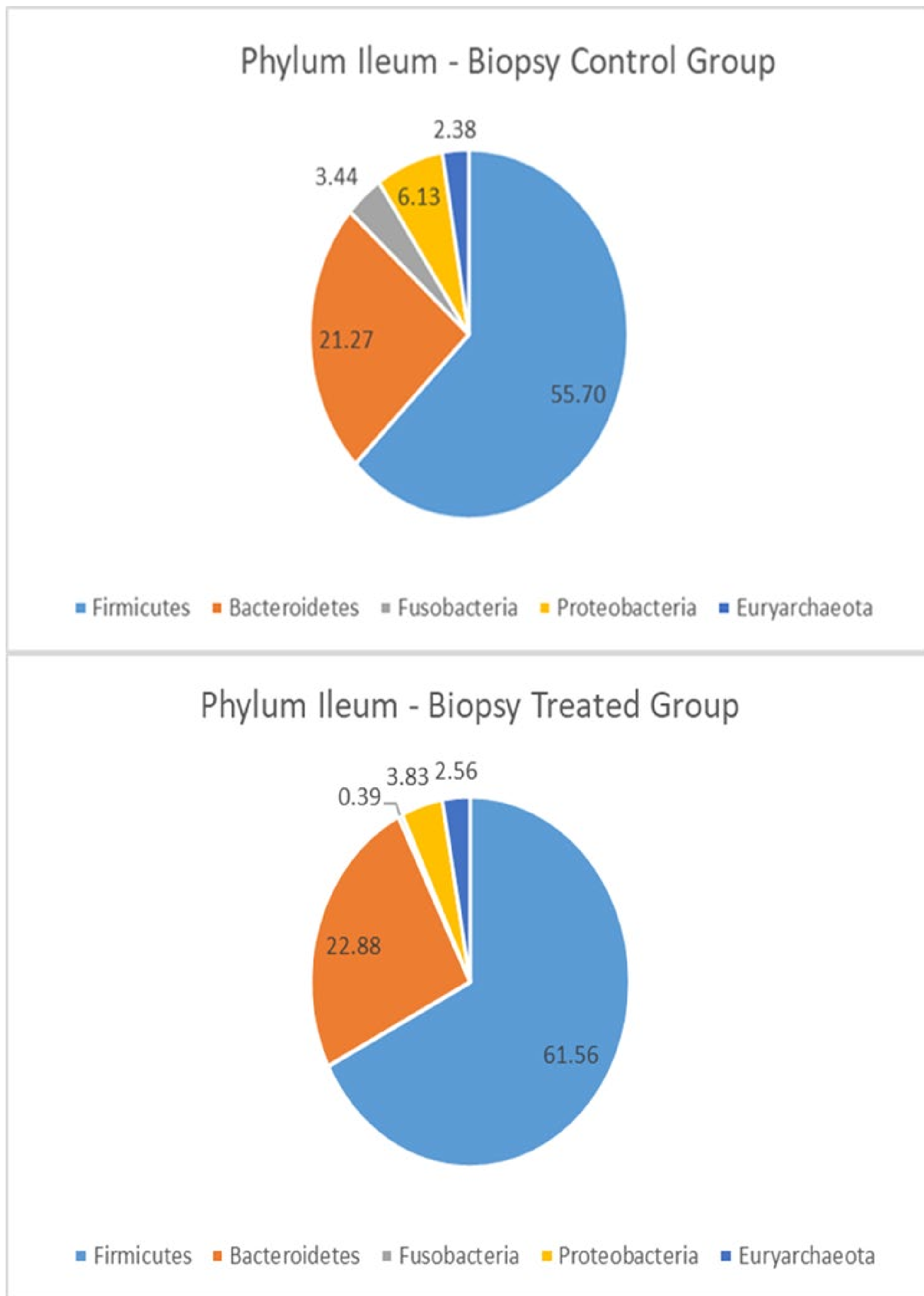


Figure 16. Composition, at the phylum level, of the top five most abundant bacterial populations present in mucosal surface of the ileum of the control group and treated group.

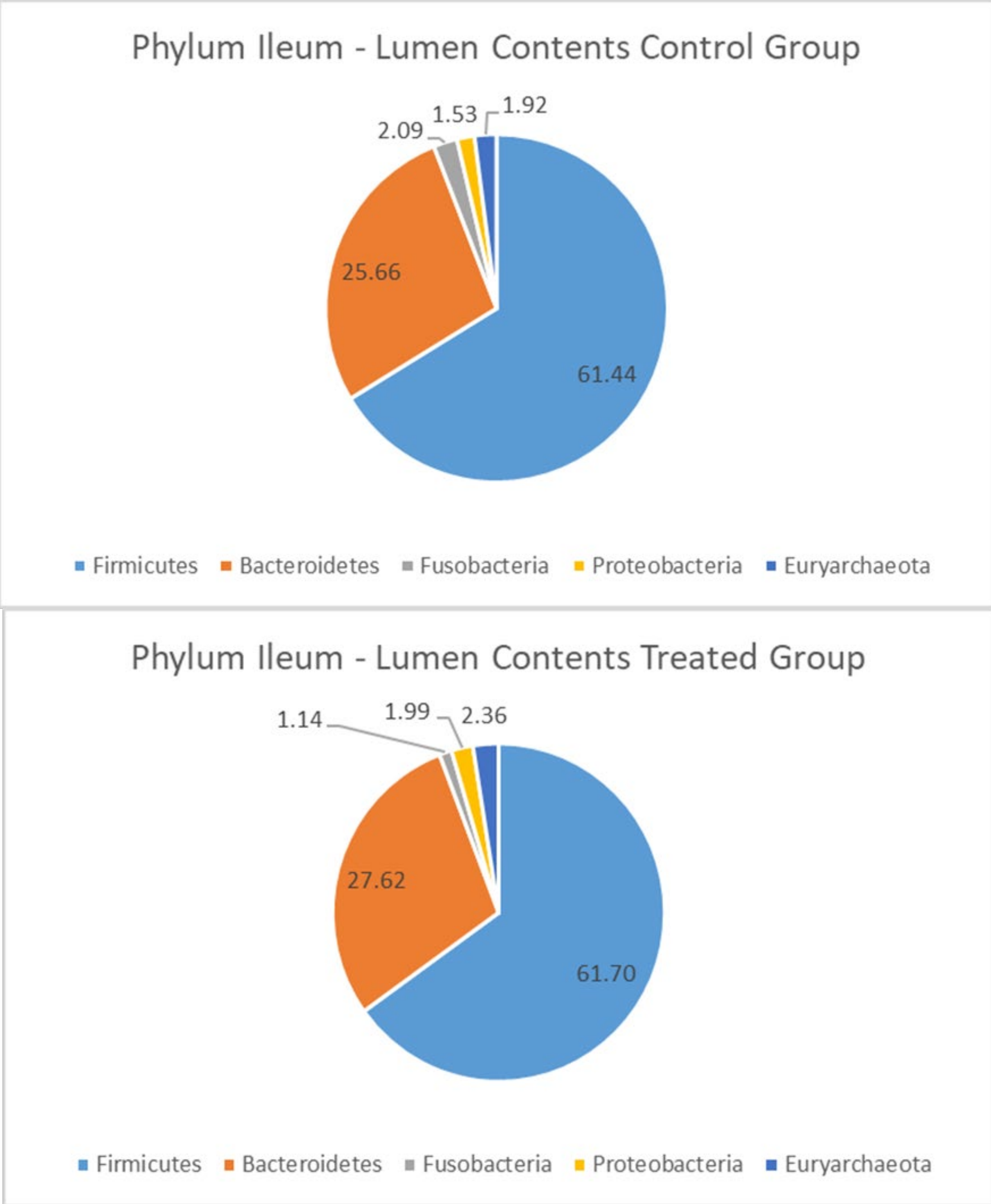


Figure 17. Composition, at the phylum level, of the top five most abundant bacterial populations present in lumen contents of the ileum of the control group and treated group.

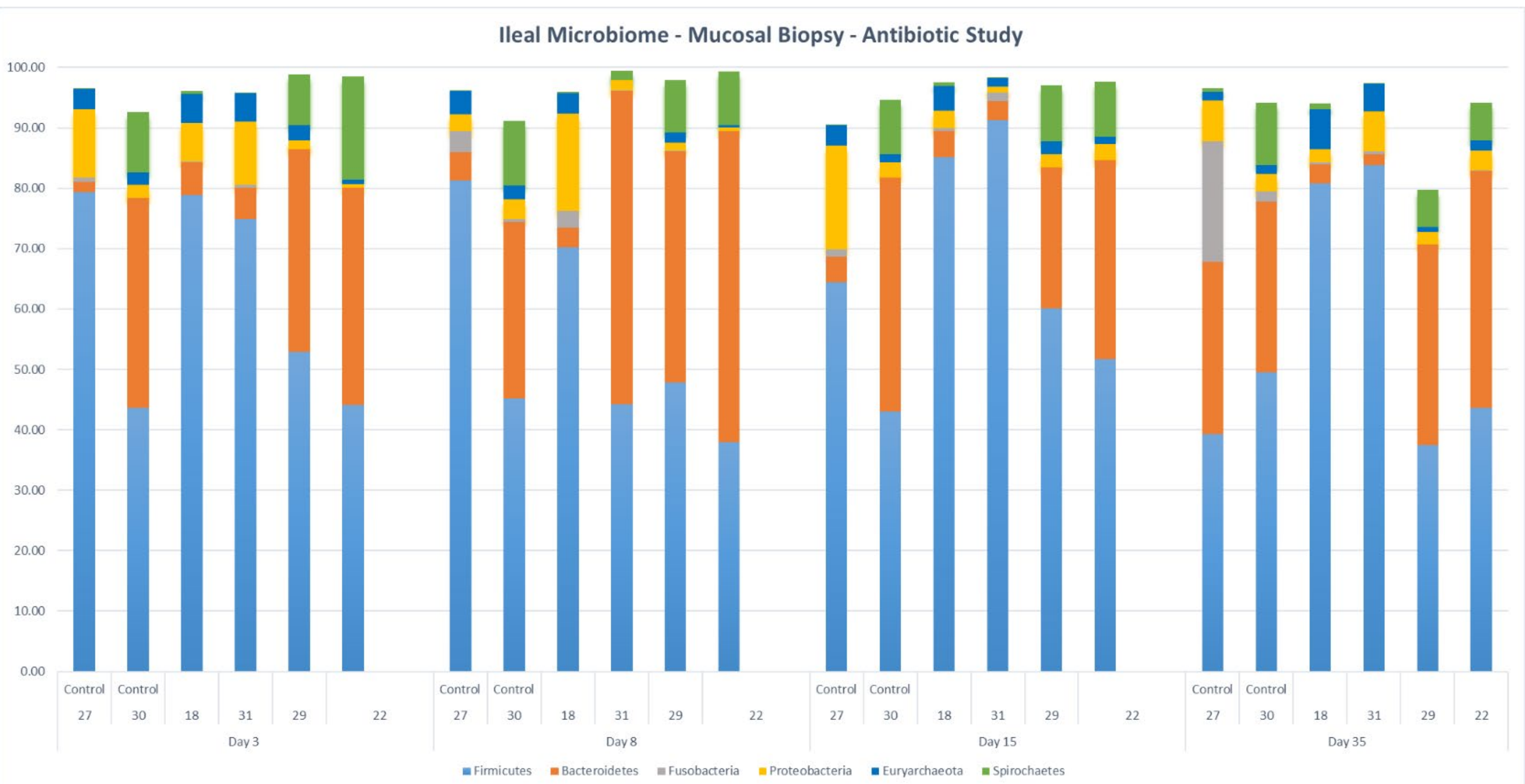


Figure 18. Top 6 phylum-level composition and abundance of microbiota associated with the mucosal biopsy of each animal per day. Note the first two animals are the control followed by the treated individuals

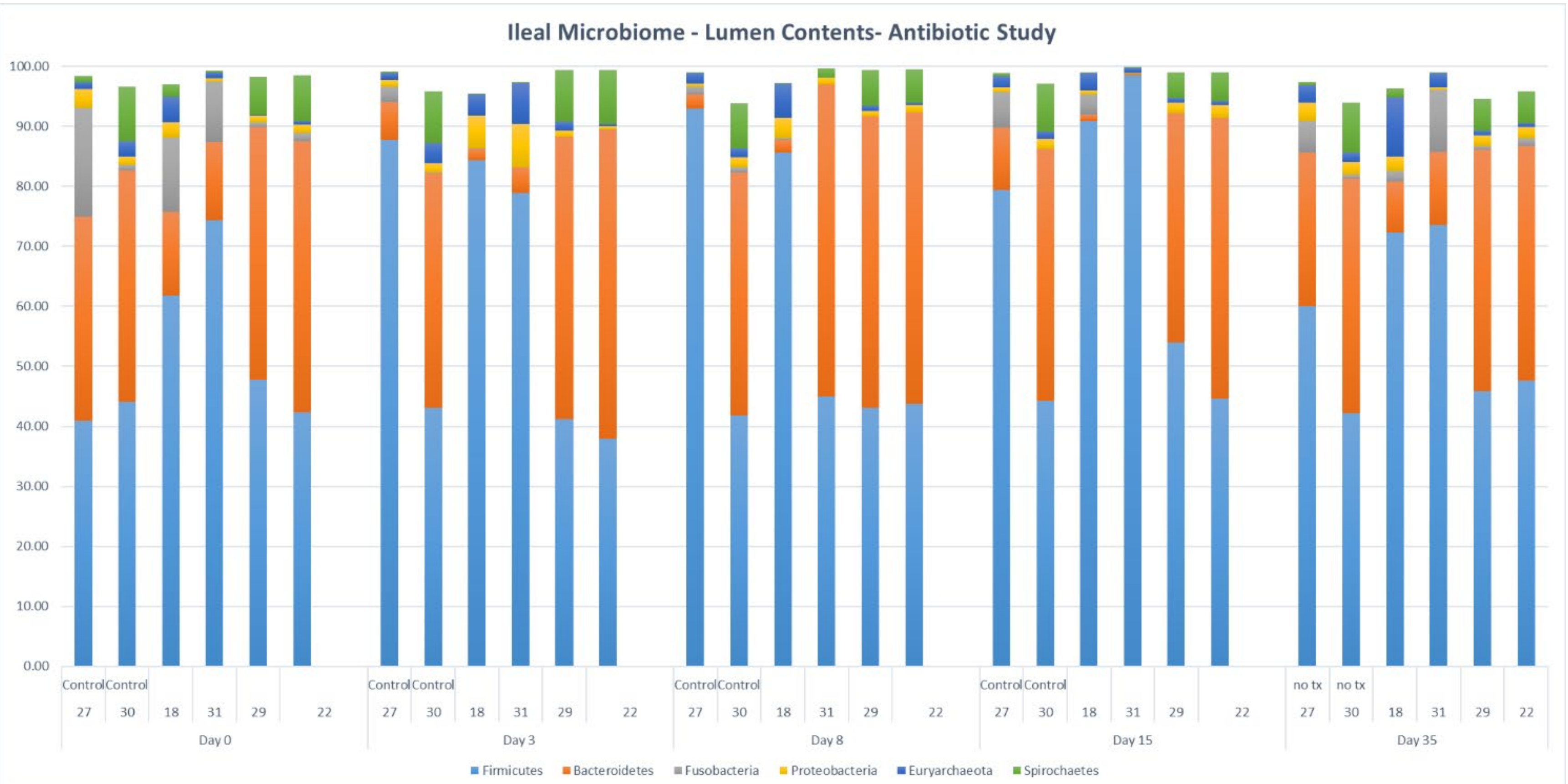


Figure 19. Top 6 phylum-level composition and abundance of microbiota associated with the luminal surface for each animal/per day. Note the first two animals are the control followed by the treated individuals.

Discussion

The enteric microbiome of the bovine small intestine remains largely uncharacterized. Previous studies have focused on the rumen and fecal microflora while usually ignoring in vivo sampling of the duodenal, ileal, and jejunal microflora.

Chapters 3, 4, and 6 of this dissertation indicated that the commensal bacterial populations between the luminal contents and epimural surface may differ significantly with respect to diversity and abundance. As described, the methods utilized are unique and innovative, as previous work published in the literature has largely centered on samples collected post mortem from the luminal contents of the gastrointestinal tract without examination of bacterial populations located in the epimural tissue surface. Studies that looked into the impact of antibiotics on pre-weaning calves' microbiota have focused mainly on fecal microbiota because of the difficult and/or cost of sampling the rest of the GIT. *Escherichia coli* is usually high in the hindgut, but its population tends to increase with higher incidences of diarrhea associated with the ileum (Constable 2004). There is an incline to the family *Enterobacteriaceae* to have more potential pathogenic bacteria which is a primary initiator of diarrhea in calves (Constable 2004). Yousif and colleagues determined that low concentrations of antibiotics had a differential effect at different levels in the gut microbiota of pre-weaning calves. The use of antibiotic combinations in milk replacer appeared to cause significant reduction in *Escherichia coli*; however, it is wise to remember that *Escherichia* is a commensal genus that plays an important role in the establishment of the gastrointestinal microbiota (Yousif et al. 2018). The use of antibiotics as growth promoters and to improve feed efficiency is a common practice. In beef cattle, Reti et al. (2013), analyzed and examined the bacterial communities associated with the mucosa and within the digesta throughout the intestinal

tract after the use of growth promoters. The results reported that the maintenance of weight gain in the presence of respiratory disease in calves had no significant differences in the composition of the primary bacterial populations following the mandated withdrawal period.

The use of antimicrobials in food-producing animals is coming under greater scrutiny with a continued emphasis on judicious use of antibiotics in both human and veterinary medicine. Many studies reported the host physiologic processes, mucosal immunity, and inflammatory responses are influenced by the health of the normal enteric microbiome. The impact of antimicrobial therapy on the gastrointestinal commensal microbiota at the mucosal surface or within the lumen is unknown (De Rodas et al. 2018; Firkins and Yu 2015; Jami and Mizrahi 2012b, 2012a; Jami, White, and Mizrahi 2014).

As mentioned previously, many significant microbial metabolic and pathologic processes take place in the ileum. Thus, appropriate balance of the ileal microbiome is thought to be important to the health of the individual in both the healthy and diseased states. The proposed study is designed to address the breaches in knowledge regarding the bovine ileal microbiome and the effects of systemic antimicrobial administration thereon.

In agreement with the literature, the pharmacological results were consistent with the macrolides characteristic with respect to its high volume biphasic distribution and low protein binding which allows basic lipophilic drugs to quickly be absorbed and leave the central circulation and gain access to the extracellular fluid and target organs providing higher and long lasting concentrations (Giguère et al. 2011).

Gamithromycin concentrated in tissues were effectively compared to plasma for the samples during the study period. The average of gamithromycin in plasma was

approximately 105 ppb compared to 53.934 ppb in the mucosal biopsies; same was true for lumen contents to plasma concentration where approximately 1.021 ppb to 105 ppb, respectively, were detected. The results also indicated, as expected, that drug clearance occurred by the expected meat withdrawal date, 35 days, and dropped below the tolerance levels set for cattle in liver (500 ppb) and muscle (150 ppb). The author finds it important to note that at 70 days, doubled the established meat withdrawal, all results indicated zero ppb detected on the samples analyzed.

It is common knowledge that drug concentrations in plasma is imperative to reach the target tissue where an infection is located; however, it is important to remember that the actual drug concentration time profile at a peripheral body site may be quite different from that of plasma and such is determined by the drug's molecular charge and size, lipid solubility, extent of plasma protein binding, and blood flow at the site of infection. It is vital to note that during the analysis technique, in order to prepare the tissue to be analyzed, homogenization occurs, and this procedure does disrupt cell membranes and produces a suspension containing both intracellular and extracellular fluid and particles. It has been reported in the literature that this necessary procedural step may result in considerable overestimation of antimicrobial concentrations in the extracellular environment (Nix et al. 1991).

With respect to the microbiome profile of the ileum between the control and treated groups, at the phylum level it does not appear to have a great impact in abundance of the top 5 bacterial phylum present. Although statistical significance is unknown, the abundance of bacteria belonging to the phylum *Proteobacteria* seems to have decreased by half in the biopsy treated group compared to control, 3.8 % versus 6.1%, respectively.

Several medically and scientifically important groups of bacteria such as *Enterobacteria*, *Vibrionaceae*, and *Pseudomonadaecea*, including the intended pathogenic bacteria gamithromycin is labeled for treatment, belong to this phylum. We speculate that this shift may lead to a potential dysbiosis of the ileum, especially in face of a healthy gut; however, this finding is not in agreement with what is known in human medicine where it is common knowledge that antibiotic use further shifts the composition of the gut microbiota toward an increased abundance of *Proteobacteria* by depressing *Bifidobacterium* populations (Frank et al. 2011; Langdon, Crook, and Dantas 2016). The same was reported in a review, providing details that the *Proteobacteria*, including *Enterobacteriacea*, is the bacterial phylum that commonly increases in the gut following antibiotic use. They further mentioned that these are pro-inflammatory and often carry antibiotic resistance genes (Langdon, Crook, and Dantas 2016).

The number and diversity of these genes commonly expands following antibiotic use. The published study speculates, in humans, the gut resistant genes among healthy adults is a reflection of antibiotic use practices in both humans and food-producing animals, further mentioning the possibility of transmission of antibiotic resistance genes through the food supply (McDonald 2017). Clearly, the results of the current research do not support such supposition.

Perhaps, since in cattle *Proteobacteria* belongs to the commensal microbiome of the GIT as reported previously (Creevey et al. 2014; Jami and Mizrahi 2012a; Snelling et al. 2019), the impact of such is not as relevant as it is in humans; however, it would be extremely relevant to analyze further into the lower taxonomic levels in order to potentially pinpoint the location of the greatest microbiota impact.

Conversely, such an impression does not appear to be true for the luminal contents. The top 5 phyla analyzed seemed to be similar in abundance between the treated and control groups. These results are expected as it is speculated that a more dynamic environment is associated with the lumen as digesta is constantly flowing. In addition, a low protein bound drug has more active drug concentrate to enter the tissues in higher concentrations rather than in locations with high protein levels such as plasma and organs filled with digesta. Aside from drug pharmacokinetics, the fact that the concentration of gamithromycin was below the lowest MIC_{50} (500 ppb) known for gamithromycin for over 90% of the time points, may support such observations.

The effects of antibiotics on microbial succession, diversity, and gene resistance is an ongoing topic, much is known; however, much still needs to be studied. The results described here add some knowledge to the literature regarding the impact of a commonly used antibiotic in cattle on the ileum microbiome. Further microbiome analysis is in order, as well as the use of a different class of antibiotic to continue enriching the literature with respect to the safety and impact of antimicrobial usage in the animal and human health.

Chapter 7

CONCLUSION

In the past two decades microbiome research has advanced significantly, especially in human medicine, mostly due to major advances in the technology, understanding of bioinformatics and decline in the cost associated with analytical methods. Microbiome research has and still provides a large amount of data contributing to the literature by understanding the nature of the microbial communities. This includes their interactions and effects, both within a host and in an external environment as part of an ecological community.

This dissertation accomplished three practical milestones: (1) The use of a unique methodology that allowed sample collection to take place while healthy cattle were undergoing ordinary husbandry. (2) The description of the luminal and epimural microbiome across 3 different locations – rumen, duodenum and ileum. (3) Lastly, the impact of a specific systemic antimicrobial on the microbiome and its pharmacological distribution were studied. Understanding the role of microbiota in physiology and disease processes is imperative, especially the important commensal bacteria and their interactions with the host and other microbes. This knowledge allows the potential for development of new diagnostic techniques and interventional strategies that can be used in several areas including agriculture, medicine and forensics (García et al. 2020).

In ruminants, the characterization of the ruminal and fecal microbiome and its impact on bovine health and production have been previously investigated; the majority of studies examined only intraluminal samples harvested post-mortem. The bovine luminal and mucosal-associated microbiota has not been characterized concomitantly, particularly in

the live animal, as this is relevant due to most metabolically-active processes occurring at the mucosal interface. The results obtained in this project were in agreement with the literature concerning the presence of a core rumen, duodenum and ileal microbiome. However, it was inspirational to find a great community diversity and richness between the epimural surface and the luminal contents. An important observation were the differences in variability and distribution of the microbiota that occurred between animals or varied between the weeks the study took place in individual animals. These results add to the understanding of “what is normal and expected” in reference to the microbiome in cattle, enabling the potential development of new strategies and research projects to leverage applications of the microbiome.

In conclusion this dissertation will aid in future endeavors towards research in the bovine microbiome. One can systematically understand the relationship between the state of the microbiome and biological processes, such as infectious and or inflammatory disease processes, within the host. Additionally the development of studies associated with the impact of different pharmaceuticals in different body systems, the influence of the microbiome and virome to the innate immune system, as the equilibrium between the immune system and immune regulatory bacteria appears to be of vital importance. Last but not least, the expansion of the knowledge associated with the impact of the microbiome involving public health and animal welfare is the future of food production and population medicine.

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