EVALUATING THE GASTROINTESTINAL MICROBIOME OF ANGUS STEERS DIVERGENT IN FEEDLOT FEED EFFICIENCY FROM WEANING TO SLAUGHTER

by

CHRISTINA BREANNE WELCH

(Under the Direction of Todd R. Callaway)

ABSTRACT

The objective of this research was to determine the variation in the gastrointestinal microbial population of commercial Angus steers divergent in feedlot feed efficiency and the effect age, changes in diet, fasting, and gastrointestinal location have on it. In order to test these objectives, three experiments were conducted. The first study focused on the fecal microbiota of steers at weaning, yearling, and slaughter and found that several bacterial families consistently had divergent abundances despite changes in steers' age and diet, highlighting the importance of bacterial families in determining the host feed efficiency as far back as weaning. The second study compared the ruminal microbiota pre- and post-slaughter and found that pre-slaughter fasting increased alpha-diversity and indicated some degree of dysbiosis. The third study compared the ruminal, cecal, and fecal microbiomes at slaughter of steers and found many links between steers' feed efficiency and the bacterial population in their hindguts.

INDEX WORDS: Microbiome, Gastrointestinal, Residual Feed Intake, Angus Steer, Feed Efficiency

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DEDICATION

First and foremost, I would like to dedicate this work to my grandmother, Anna “Granny Billie” Welch. Growing up she was always my role model for she not only pursued higher education late in life but did so while raising a family as a widow. By example, she taught me to be strong by always getting back up no matter how many times life knocked her down. She always valued education and instilled in me her love of learning. I will forever be grateful for the support she gave me throughout my life, and for always encouraging me to chase my dreams no matter where they might lead. I am pleased to say I am an alumnus from the same university and this shared accomplishment is one of the things I treasure most.

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CHAPTER 1

INTRODUCTION

In beef production systems, feed costs account for the largest percentage of total input costs (Herd et al., 2003; Schnepf, 2012). In addition, ruminants spend approximately 75% of their dietary energy on unproductive purposes (e.g. maintenance and production of methane), which diminishes the profit of beef producers (Kelly et al., 2010). In order to increase the profitability of beef operations, research has been focused on increasing feed efficiency in cattle. One metric to measure feed efficiency is residual feed intake (RFI). RFI is calculated by subtracting the actual feed intake of an animal from its predicted feed intake, with predicted feed intake being based on metabolic body size (Koch et al., 1963). Low-RFI cattle eat less than expected making them more efficient. More efficient ruminants utilize the nutrients found in feed better, resulting in a reduction in feed cost and a decrease in deleterious end products involved in converting feed to meat, milk, and fiber (Nkrumah et al., 2006; Thornton, 2010).

One downside to feed efficiency measurements is that there is a certain degree of fluctuation in them within a group of animals. According to Herd and Arthur (2009), diet and digestibility can explain 19% of the variation occurring in feed efficiency; however, 81% of the variation remains to be explained. One contributing factor to feed efficiency variation among cattle may be their gastrointestinal microbial population. The microbes within the gastrointestinal tract of ruminants are responsible for producing 85% of the energy available to the host in the form of volatile fatty acids produced from the

fermentation of feedstuffs (Oh et al., 1972). Therefore, if the microbes composing the host's microbiota are fermenting nutrients more efficiently, this translates to an increase in the overall efficiency of the host.

Due to the impact the gastrointestinal microbial consortium has on the host, recent research has investigated the link existing between feed efficiency in ruminants and their microbial consortium. Studies have found that more efficient cattle have a decrease in bacterial diversity in their rumens, implying that their microbiota are composed of fewer bacterial species and possessing fewer, more specialized metabolic pathways (Shabat et al., 2016). Microbiome specialization provides the bacterial population an advantage of extracting the most nutrients from the feed, eliminating nonproductive waste, and providing the most energy to the host. One disadvantage of most of the research on this topic, however, is that they have focused primarily on the microbial population within the rumen and ignored the hindgut microbial population.

In order to better understand the impact the gastrointestinal microbiota has on the feed efficiency of the host animal, and how this underappreciated microbiota affects food production, we performed a series of experiments investigating this relationship. The objective of the present research was to determine differences in the gastrointestinal microbial population of commercial Angus steers that were divergent in feedlot feed efficiency. Additionally, we investigated if those potential differences could be detected at earlier ages (e.g. on their weaning day), and if they would be maintained regardless of age and changes in diet. Furthermore, we examined the effects of fasting and gastrointestinal location on the host's microbiota. The first experiment evaluated the fecal microbiome of steers from weaning to slaughter in terms of their feedlot feed efficiency, to determine if

consistencies in their bacterial populations were present as far back as weaning, despite aging and dietary changes. The second experiment studied the impact of pre-slaughter fasting on the ruminal microbiota of steers, to determine if the lack of nutrients causes dysbiosis, allowing pathogens to thrive and more easily enter the food chain. The third experiment described the microbial populations of the rumen, cecum, and feces in steers, at slaughter, in terms of RFI classification, to determine if differences in microbial population occurred between gastrointestinal locations and RFI status. We hypothesized there would be observable differences in gastrointestinal microbial populations of commercial Angus steers divergent in feedlot feed efficiency transcending chronological age, dietary fluctuations, fasting, and gastrointestinal location.

CHAPTER 2

LITERATURE REVIEW

Digestive Anatomy of the Ruminant

The ruminant animal has a unique set of evolutionary advantages over monogastric animals allowing them to digest and utilize plant biomass that would otherwise go to waste (Hungate, 1966). This evolutionary advantage comes from a four-chambered "stomach" which degrades feedstuffs before they get to the acid stomach (Church, 1988; Wallace, 2008). The reticulorumen is comprised of both the reticulum and the rumen and is the site of pre-gastric fermentation and much of the end product absorption (Flint, 1997; Mizrahi, 2012, 2013). The first two chambers of the ruminant's four-chambered stomach are home to a wide variety of microorganisms (bacteria, protozoa, fungi, and archaea) that degrade plant materials and produce products the host animal can utilize for both maintenance and growth (Flint, 1997; Mackie, 2002; Mizrahi, 2012, 2013). Brulc et al. (2009) performed a metagenomic study and discovered the coding sequences found in the rumen of Angus-Simmental cross steers were approximately 95% bacteria, 0.6-4% archaea, and 1.5% eukarya. The rumen accounts for an estimated 80% of the total volume of the gastrointestinal tract of the ruminant and is the site of more than 85% of the volatile fatty acid production in the gastrointestinal tract (GIT) with 10-13% of the energy being produced in the hindgut of lambs and the rest being produced in the rumen (Oh et al., 1972). In addition, approximately 50% of the protein ruminants utilize comes from their GIT microbial fermentation in the form of Microbial Crude Protein (Russell and Hespell, 1981;

Bergman, 1990; Yeoman and White, 2014). The large fermentation vat that is the rumen provides the ruminant herbivores with the ability to consume and degrade large quantities of plant materials (Mizrahi, 2013) that monogastric animals cannot.

The relationship amongst the GIT microbes can be commensal, mutualistic, competitive, and/or predatory with the same microbes interacting in both beneficial and harmful ways with other microbes, as well as the host animal (Hungate, 1968; Russell, 1988). Many bacteria produce bacteriocins (an antimicrobial peptide) which target specific bacteria within a similar niche and are toxic to them providing the bacteriocin-producing bacteria with a competitive advantage in a highly-competitive environment (Laukova, 1993; Jack et al., 1995; Morovský et al., 2001; Whitford et al., 2001; Cookson et al., 2004; Xavier and Russell, 2009; Mizrahi, 2013). However, these microbes can coexist and benefit from each other by utilizing the end products cross fed from another microbe occupying a different niche (Bryant et al., 1967; Iannotti et al., 1973), while still providing energy and nutrients for the host (Dehority, 1991; Mackie, 2002; Mizrahi, 2013). The microbe-microbe interactions enhance the ability of the microbes to maximally degrade feedstuffs and to provide energy and protein to the host animal.

Degradation of Feedstuffs

The ruminant microbial population has the ability to break down plant biomass polymers that are indigestible by non-ruminant mammals that lack significant microbial fermentative capacity in their digestive system. The microbial population converts the energy conserved in the plants consumed by the host into Volatile Fatty Acids (VFA) which are utilized by the host for energy that becomes meat, milk, or fiber that can be utilized by humans (Shabat et al., 2016). Because of their ability to convert human

indigestible products into food, humans began domesticating ruminants in the Neolithic era and animal agriculture has been a vital part of civilization ever since (Ajmone-Marsan et al., 2010). Today ruminants are estimated to consume 30% of the plants grown in agriculture and are grown on as much as 30% of the land on earth (Thornton, 2010).

One step involved in the breakdown of feedstuffs consumed by ruminants is rumination, or the act of regurgitating their feed to remasticate it and make the feed particles smaller, so that the rumen microbes can break them down more easily (Hoover and Miller, 1991). Feed particle size determines the length of time that an animal has to spend ruminating in order to thoroughly break down all the feed (Welch and Smith, 1969; Welch, 1986); therefore, a ruminant fed primarily forage will ruminate more than a ruminant fed a primarily grain-based diet. Rumination is beneficial to the animal because the partially digested feed mixes with saliva, which acts as a buffer in the rumen to help prevent a decrease in ruminal pH from fermentation end products (Counotte et al., 1979). A decrease in the pH can be detrimental to both ruminal microbes as well as the host animal. Because of this intrinsic buffering system, the rumen tends to maintain a pH between 5.5 to 7.0 but can fluctuate depending on diet and frequency of meals (Dehority and Tirabasso, 2001; Welkie et al., 2010).

Feeds are degraded in the reticulorumen through anaerobic fermentation by the native microbial consortium (Mackie, 2002; Mizrahi, 2012, 2013). The microbes of the GIT of the ruminant and host share in a mutualistic relationship that benefits both members of the relationship. The rumen provides feed and a warm, dark environment ideal for the growth of the microbes and in turn, the microbes break down the feed consumed by the ruminant and provide the animal with nutrients, mainly volatile fatty acids and microbial

crude protein, which are used to meet the animals growth and maintenance requirements (Archer et al., 1999; Mackie, 2002; Mizrahi, 2013). The rumen consortium is comprised of bacteria, archaea, protozoa, and fungi all fulfilling their individual roles in the breakdown of feedstuffs (Flint, 1997; Hobson and Stewart, 2012). The abundance of the domains in the rumen vary greatly depending on a wide array of factors and allow the rumen of the host to reach its peak functionality (Mizrahi, 2013).

After the feedstuffs have been degraded by the microbial population within the rumen, digesta exits the reticulorumen through a small opening and size sorter called the reticulo-omasal orifice. Feed particles <5 mm in size pass into the omasum which absorbs water. The omasum serves as the site of water and inorganic mineral absorption (Mizrahi, 2013) before the digesta enters the acidic environment of the abomasum. After digesta leaves the omasum, the abomasum serves as the gastric or "true" stomach of the ruminant which is similar to the stomach of monogastrics. One unique difference between the abomasum and a monogastic's stomach is the abomasum secretes lysozyme, an enzyme that degrades bacterial cell walls (Mizrahi, 2013). This enzyme ensures that bacteria which have been washed out of the rumen are thoroughly lysed and degraded so that they may be later absorbed and utilized by the ruminant as the digesta (including degraded bacteria) continue to travel throughout the GIT into the small intestine (Kay, 1969; Jolles et al., 1984; Jollès et al., 1989). Microbial crude protein is an important source of protein for the ruminant, as it provides an estimated 50-80% of the total dietary protein the animal absorbs through its GIT (Storm and Ørskov, 1983).

Volatile Fatty Acids

Volatile fatty acids (VFA), also known as short chain fatty acids (SCFA), serve as the primary energy source for ruminants and cattle receive approximately 80% of their energy from these end products of plant polymer fermentation in the rumen (Bergman, 1990; Weimer et al., 2009). Most ruminal VFA are in the form of acetate, propionate, and butyrate with the remainder coming from the less abundant, isobutyrate, valerate, and isovalerate. VFA are the main fermentative end products of complex carbohydrates from plants including starch, cellulose, hemicellulose, fiber, and sugars (Leng, 1970; Dziuk, 1984; Mizrahi, 2013; Shabat et al., 2016).

VFA are not only produced in the rumen, but also throughout the entire digestive tract in mammals. The rate of VFA production in the GIT of ruminants depends greatly on the location of production because the flow rate through the gastrointestinal tract limits the time that the resident bacterial populations have to degrade and ferment digesta. Elsden et al. (1946) discovered ruminants had the greatest VFA production in their rumen, and approximately 88% of the ruminal VFA were absorbed in the rumen by the ruminal epithelial cells with only 12% of the VFA passing through to the omasum (Sutherland et al., 1963). Elsden et al. (1946) found VFA concentration was negligible from the omasum through small intestine, but there were more VFA produced in the ruminant cecum and colon as passage rate decreased, allowing intestinal bacteria a further opportunity to extract energy from the digesta. Mammals consume a variety of diets which impacts gut VFA concentrations which are highest in herbivores, lower in omnivores, and the lowest in carnivores (Bergman, 1990). Ruminant diet also impact the amount and types of VFA produced by providing different substrates, providing competitive

advantages/disadvantages to the bacteria present (i.e. cellulolytic vs. amylolytic) based upon diet composition and the passage rate of the feed from the rumen (Hungate, 1968; Schwartz and Gilchrist, 1975; Salyers, 1979; Russell, 1988; Wallace, 1988). During the degradation process and fermentation of feedstuffs, methane and carbon dioxide are also produced as a fermentative end product that represents a loss of carbon and energy to the animal and is a significant sustainability issue (Johnson and Johnson, 1995). Blaxter (1962) demonstrated that when the ruminant increases the amount of feed they consume, the percentage of energy from the diet wasted in the form of methane is decreased because the amount of total fermentable energy available through the diet is increased (Johnson et. al., 1993).

The concentration of three primary VFA in the rumen are usually found in a proportion of 65:20:15 (acetate: propionate: butyrate ratio, respectively) (Wolin, 1960, 1981; Wolin and Miller, 1983). VFA are mainly absorbed in the reticulorumen and play a key role in many of the biochemical energetic processes in the animal, including gluconeogenesis from propionate, and acetate and butyrate taking part in fatty acid synthesis (Russell and Wilson, 1996). Acetate is the most abundant VFA found in the GIT of all mammals (Bergman, 1990), and the amount of acetate present in the rumen of an animal remains relatively constant regardless of diet (Russell, 1988). Propionate and butyrate are produced in relatively large quantities as well, but their concentrations can vary greatly depending on host diet (Bergman, 1990; Hungate, 1966). The acetate-to-propionate ratio of VFA is an important snapshot in terms of energy production for ruminants, but is also important to carcass quality because acetate is linked with subcutaneous fat in the host animal, whereas, propionate increases intramuscular fat in the

host (Smith and Crouse, 1984). This leads beef producers to a desire to reduce the acetate-to-propionate ratio in the rumen in order to increase the quality grade of the meat, but dairy producers who are paid based upon milkfat percentage prefer more acetate because it leads to milk fat.

Generally when ruminants are fed a high concentrate (starch) diet, the proportion of acetate to propionate in their rumen decreases compared to that from a diet higher in forages (Blaxter, 1962), resulting in a decrease in methane production and an increase in the amount of energy extracted from the diet (Wolin, 1960). Diet composition does impact the amount of propionate produced in the rumen; however, the underlying mechanism is unknown because both amylolytic and cellulolytic bacteria produce propionate (Hungate, 2013). Amylolytic bacteria produce a large amount of propionate directly and indirectly (mediated by lactilytic species such as *Megasphaera elsdenii* and *Selenomonas ruminantium*), and cellulolytic bacteria produce succinate which is a precursor of propionate (Li et al., 2014; Macy et al., 1978).

High grain diets are associated with a reduced ruminal pH as well as lower A:P ratios (Russell, 1998). The lower pH results in more lactate and propionate production using up reducing equivalents, so the methanogens can no longer use the reducing equivalents inside the rumen from methane production (Van Nevel et al., 1970; Van Kessel and Russell, 1996; Russell, 1998). Russell (1988) compared rumen fluid from non-lactating dairy cows either fed 100% forage or 90% concentrate, and found that rumen fluid from 90% concentrate diet fed cows had a higher total VFA production, as well as a lower acetate-to-propionate ratio resulting from increased propionate production.

Propionate is important to the ruminant animal because it is the glucogenic VFA (Bergman et al., 1966; Bergman, 1982, 1983). Glucose metabolism produces energy and acetyl units in muscle tissue which are utilized during intramuscular fat (marbling) deposition (Smith and Crouse, 1984). However, ruminal propionate production from starch is accompanied by lactate and carbon dioxide (Pennington and Sutherland, 1956), and overproduction of lactate can lead to a drop in pH and acute or chronic ruminal acidosis (Hernández et al., 2014). The production of lactate in the tissue of the animal occurs more frequently in sheep with as much as 50% of propionate produced in the rumen being utilized (Judson et al., 1968; Bergman, 1975; Weeks and Webster, 1975; Bergman, 1983); whereas, only a 3-15% loss of propionate to lactate production is reported in calves (Weigand et al., 1972) and cows (Cook et al., 1969).

Butyrate is the VFA most closely associated with healthy GIT tissue and gut integrity (Kvidera et al., 2017; Mani et al., 2013; Plöger et al., 2012). Ruminal butyrate is converted in epithelial tissues to the ketone β-hydroxybutyrate which provides energy to the epithelium (Guan et al., 2008). Butyrate has been linked to an increase in tight junction proteins making it a potential tool to treat inflammatory bowel disease in humans (Plöger et al., 2012). In humans, the presence of butyrate not only increases the growth of epithelial cells within the lower gastrointestinal (GI), but also inhibits the growth of malignant cells (Kim et al., 1982; Fleming and Arce, 1986). Butyrate production has been shown to increase in cattle's rumen when they are fed high concentrate diets (Leedle et al., 1995; Carberry et al., 2012). Protozoal populations also increase butyrate concentrations in the rumen as an end product of protozoal fermentation which means protozoa are beneficial to the health of the host (Carberry et al., 2012).

Cost of Feed

Unlike monogastric animals, ruminant producers must first feed the gastrointestinal microbial population, and in turn the microbes feed the host. This intermediate ecosystem ensures that ruminants are less efficient than monogastrics in converting feed to meat and milk, and the need to improve sustainability ensures there is a pressing need to increased feed efficiency. Animals that have poor feed efficiencies have increased environmental impacts and cost of production (Nkrumah et al., 2006; Hegarty et al., 2007). In animal production systems, feed costs account for the majority of the cost of production (Barnard, 1969; Miller et al., 2002; Schnepf, 2012), and can account for up to 80% of the total production costs (Arthur et al., 2004). As much as 75% of the dietary energy of the animal is used for non-productive purposes (e.g., maintenance, microbial growth, methane), so reducing the non-productive use of energy is critical to improving producer profitability and reducing the environmental impact of beef production (Kelly et al., 2010a).

Residual Feed Intake

The ruminant animal's energetic efficiency must be increased, so if two cattle grow at the same rate but one consumes less feed, then it will have the lowest cost of production and lessening the deleterious byproducts of ruminal fermentation (i.e., methane emissions) (Bradford, 1999; Thornton, 2010). Although the biological mechanisms controlling an animal's feed efficiency have yet to be discovered, some of the factors influencing it are physiological (Herd and Arthur, 2009), genetic (Herd and Bishop, 2000), and behavioral (Lancaster et al., 2009).

Residual feed intake (RFI) is one of the most commonly used methods of calculating feed efficiency as it includes factors such as the metabolic body size and growth

of the animal, is moderately heritable (Koch et al., 1963; Archer et al., 1999; Crews, 2005; Arthur and Herd, 2008; Herd and Arthur, 2009), and can be selected for in a breeding program. Each animal's RFI is unique and can vary between animals within the same herd and the same breed (Herd and Arthur, 2009; Shabat et al., 2016). It is a valuable tool when comparing feed efficiency because it is independent from growth and body size of the animal, which eliminates some biases (Archer et al., 1999; Moore et al., 2009). The RFI is established using the dry matter intake (DMI) and the metabolic weight of the animal (Nkrumah et al., 2006), and is calculated by subtracting the actual feed consumption from what expected intake based on metabolic body weight and level of production (Herd and Arthur, 2009; Shabat et al., 2016). Each animal is assigned a value to their efficiency that is either positive or negative based on whether their consumption is above or below the expected intake. Negative RFI values indicate the animal is more efficient and eats less feed than do animals with a positive RFI value (Archer et al., 1999; Crews, 2005; Arthur and Herd, 2008; Herd and Arthur, 2009; Moore et al., 2009).

More feed efficient cattle possess other positive traits for the animal, the producer, the consumer, and the environment. More efficient cattle have lower subcutaneous fat adiposity (Richardson et al., 1998), resulting in less energy being used for back fat, and instead more energy devoted to adding muscle. Low-RFI cattle have lower heat and methane emissions compared to high-RFI cattle (Nkrumah et al., 2006; Hegarty et al., 2007) which is beneficial to the producer by reducing the feed energy used for non-growth functions and is also better for the environment by reducing methane released into the atmosphere. More efficient cattle have increased dry matter and crude protein digestibility (Nkrumah et al., 2006) compared to inefficient cattle since they extract more energy and

nutrients from their feed. However, diet can have a huge impact on feed efficiency. Kelly et al. (2010b) found heifers maintained their RFI when they were fed a consistent diet and continued to maintain their RFI status throughout their life when their diet remained the same. However, Durunna et al. (2011) found the RFI of steers changed dramatically following a diet change from low energy to a high energy diet. In order to better understand feed efficiency and be able to improve energetic efficiency, we must understand the factors that affect feed efficiency.

The Gastrointestinal Microbiome

Approximately 19% of the variation in feed efficiency can be explained by diet and digestibility but, the cause of 81% of the variation remains unknown (Herd and Arthur, 2009). One factor that may explain some variation is the presence and composition of the gastrointestinal microbiome. Composition of the microbial population of the gut can alter the RFI status of the ruminant because the energy and nutrients obtained by the animal are direct products of the microbial fermentation (Kim et al., 2011; Hernandez-Sanabria et al., 2012; McCann et al., 2014).

Older methods of quantifying bacterial cells present in GIT samples included counting colonies on agar plates or counting bacterial cells under a microscope. Unfortunately viable cell counts lead to an inaccurate representation of the bacterial abundance since only an estimated 10% of bacteria found in rumen samples from cattle and sheep have been cultured and identified (Bryant and Burkey, 1953a; Bryant and Burkey, 1953b; Maki and Foster, 1957; Dehority and Tirabasso, 2001; Kobayashi, 2006; Creevey et al., 2014; Weimer, 2015). This limitation to the knowledge of what bacteria were present in the rumen and the metabolic pathways they were utilizing prevented a more

detailed understanding of the host-microbe relationship. Although rumen function and microbiology have been studied for many years, the development of next-generation sequencing has opened the doors to information on microbial density, the function of the microbial community, and the effects the GIT microbiota has on the host (Dowd et al., 2008; Pitta et al., 2010; Rice et al., 2012; Xie et al., 2013; Myer et al., 2017). In order to gain a better understanding of the microbes making up the GIT consortium, gene sequencing is performed, normally by amplifying the V1-V3 region of the 16S rRNA gene (Myer et al., 2015a; Myer et al., 2015b; Myer et al., 2015c; Myer et al., 2016; Lourenco et al., 2019). The increased resolution allows researchers to determine which microbes are present in the rumen or hindgut without the need to culture the bacteria.

Microbial Diversity Indices

One way to compare the ecosystem's composition is by comparing the diversity among and between samples. "Biological diversity means the variability among living organisms from the ecological complexes of which organisms are part, and it is defined as species richness and relative species abundance in space and time" (Schloss and Handelsman, 2006; Kim et al., 2017). The first measure of diversity is alpha (α) diversity which compares the diversity within a sample (Barko et al., 2018), which is compared to beta (β) diversity which compares different samples to one another (Barko et al., 2018). When evaluating the diversity of a microbial sample, the evenness and richness of species need to be considered. Kim et al. (2017) described evenness as having an equal number of individual species present; whereas, richness is the number of different species present in a sample. As evenness and richness are increased, diversity also typically increases. Two common indices are used to quantify diversity: the Shannon-Weaver diversity index, and

the Simpson diversity index (Simpson, 1949; Schloss et al., 2009; Lemos et al., 2011; Magurran, 2013). Both indices measure the relative abundance of species in a sample using evenness and richness simultaneously; however, they both have limitations and biases. The Shannon-Weaver index considers richness more impactful than evenness, and the Simpson index values evenness higher than richness (Schloss and Handelsman, 2006; Schloss et al., 2009). With the Shannon-Weaver index, the diversity increases when more species are present and as the difference in the number of species present becomes more uniform (Lemos et al., 2011; Magurran, 2013). The Simpson index compares the likelihood of two randomly selected species both being from the same species (Simpson, 1949). It is measured between 0 and 1; with 1 indicating a less diverse sample. As with most biological indices, comparing diversity indices between samples with a different number of sequences present requires an adjustment due to sample size to avoid skewed results, since diversity increases as the number of species increases (Lemos et al., 2011).

To better understand the richness within a specific niche or community, the number of species must be counted (Kim et al., 2017). Communities with a small number of species present are conducive to this method of analysis, but practically speaking most communities have too many species present to count. A further problem is that sample size can skew the richness estimate, so samples must be adjusted in order to be compared fairly (Sanders, 1969; Hughes et al., 2001). A popular index used to infer differences in species richness within a population is Chao 1, which examines richness using a nonparametric method designed to quantify the number of species within a sample (Kim et al., 2017). Chao 1 estimates richness by calculating the expected operational taxonomic units (OTU) using the OTU present in the sample (Chao, 1984; Chao and Lee, 1992; Chao and Yang,

1993). This specific index favors rare species, so it uses singletons and doubletons making it ideal for samples mostly comprised of many species with low abundance (Hughes et al., 2001).

Bacterial Abundance

Bacteria compromise the largest percentage of microbes within the rumen making up as much as 95% of the DNA sequences (Brulc et al., 2009) and are responsible for the break down and fermentation of the majority of the plant materials in feedstuffs (Windham and Akin, 1984; Akin and Benner, 1988). Many factors can alter the concentration of specific bacteria within the ruminants GIT including diet, age of the animal, ruminal pH, the presence of antibiotics, protozoal concentrations, and the overall health of the host (Stewart et al., 1997; Bevans et al., 2005; Mosoni et al., 2011; Franzolin et al., 2012). The two main bacterial phyla found in the gastrointestinal tract of ruminants are Bacteroidetes and Firmicutes (Brulc et al., 2009; Jami and Mizrahi, 2012; Rice et al., 2012; Mizrahi, 2013; Myer et al., 2015a; Myer et al., 2015b; Myer et al., 2015c; Myer et al., 2016; Myer et al., 2017; Zhou et al., 2018). Myer et al. (2015b) found the remaining phyla in the rumen of steers differing in feed efficiency were comprised of Proteobacteria, Tenericutes, Cyanobacteria, and Spirochaetes. Both efficient and inefficient steers had a large abundance of bacteria from the orders Bacteroidales and Clostridiales and the families *Ruminococcaceae*, *Lachnospiraceae*, and *Veillonellaceae* (Myer et al., 2015b). The most numerous genera found in the rumen were from the genus *Prevotella* from the phyla Bacteroidetes (Stevenson and Weimer, 2007; Pitta et al., 2010; Myer et al., 2015b; Myer et al., 2017). Myer et al. (2015b) found that after *Prevotella*, the next most abundant

bacterial genera found in the rumen of steers regardless of efficiency status were *Dialister*, *Succiniclasticum*, *Ruminococcus*, *Butyrivibrio*, and *Mitsuokella*.

The bacterial consortium in the rumen can be broadly divided into two main groups – fibrolytic and amylolytic bacteria (Mizrahi, 2013). When a ruminant is fed a diet mainly consisting of forages, cellulolytic bacteria tend to dominate the ruminal population with the best known being *Ruminococcus albus, R. flavefaciens*, and *Fibrobacter succinogenes* (Flint and Bayer, 2008; Flint et al., 2008; Hobson and Stewart, 2012). When ruminants are fed a high concentrate diet, amylolytic bacteria such as: *Ruminobacter amylophilus*, *Streptococcus bovis*, *Succinomonas amylolytica, Butyrivibrio fibrosolvens, Selenomonas ruminantium,* and many species from the genus *Prevotella* tended to predominate (Krause et al., 2003).

Bacterial concentrations remain unchanged in sheep feed anywhere from one to six times a day (Moir and Somers, 1957; Warner, 1966a, b; Dehority and Tirabasso, 2001); however, bacterial concentrations have been shown to increase in sheep feed 24 times a day (Dehority and Tirabasso, 2001). Ruminal bacterial concentrations fluctuate in response to feeding times. When cattle are fed once a day, immediately prior to the daily meal there is a decrease to the bacteria immediately after feeding due to the consumption of feed and water diluting the rumen. The bacterial concentration in the rumen increases after mealtime reaching its highest population approximately 16 hours post feeding before steadily decreasing until the next feeding (Warner, 1966a; Leedle et al., 1982).

Archaeal Abundance

A metanalysis of several studies (Janssen and Kirs, 2008) found the three most abundant rumen methanogens in ruminants belonged to the genus *Methanobrevibacter*

(61.6%), *Methanomicrobium* (14.9%), and a group of uncultured archaea known as rumen cluster C (RCC) (15.8%). Methane is a critical deleterious end product of the ruminal fermentation that many strategies have been devised to reduce (McAllister et al., 2015; McAllister and Newbold, 2008; Wright et al., 2004). Methane is produced by organisms in the kingdom Archaea which is represented by ruminal methanogens (Mizrahi, 2013). The end product of methanogenic fermentation is methane which is the result of either the reduction of carbon dioxide with H_2 or the degradation of formate or acetate into methane and carbon dioxide (Thauer et al., 2008). Hungate et al. (1970) found the dissimilation of acetate to be a very insignificant method of methane production in the rumen, so the majority of the methane produced by the rumen is through the process of reducing carbon dioxide or formate (Mizrahi, 2013). The methane produced is eructated into the environment from the reticulorumen resulting in an estimated 12% loss of the energy from the feed (Johnson and Johnson, 1995). The presence of methanogens in the rumen is not all negative though; when grown in co-culture with the cellulolytic bacteria *R. flavefaciens* and *R. albus*, methanogens manipulate the process of fermentation resulting in a greater production of energy for the ruminant by recycling reducing equivalents (Latham and Wolin, 1977; Pavlostathis et al., 1988; Stams and Plugge, 2009).

Protozoal Abundance

Ruminal protozoa make up about 50% of the biomass of the rumen (Mizrahi, 2013). The main source of nutrients utilized by protozoa are carbohydrates in the form of starches and sugars, so they are more abundant in higher concentrate diets (Grubb and Dehority, 1975; Mizrahi, 2013). In fact, Dehority and Orpin (1997) found 40-60% concentrate diets results in the highest abundance of protozoal populations; however, diet, ruminal pH,

turnover rate, feeding frequency are all factors found to manipulate the abundance of protozoa in the rumen.

There is some debate about the importance of protozoa to the nutrition of the ruminant animal. Becker and Hsiung (1929) discovered protozoa present in the rumen of goats provided no nutritional advantages over goats without protozoa in their rumens. Conversely, Ushida et al. (1986) found protozoa may have a negative impact on the availability of both dietary and microbial protein to the ruminant animal by consuming the protein that would otherwise be available to the host animal. Although there might not be nutritional benefits to the ruminant, protozoa provide a functional advantage to the rumen itself (Williams and Coleman, 2012) by producing butyrate with provides energy to GIT tissue so the gut remains healthy and fully functional.

Carberry et al. (2012) found ruminal *Entodinium* spp. increased in abundance when beef cattle were on a low forage diet compared to a high forage diet. This is due to the fact that *Entodinium* spp. flourish on concentrate diets when there is a high presence of starch available (Dennis et al., 1983). Protozoa engulf any starch found in the rumen along with any amylolytic bacteria currently breaking down the starch, allowing them to control the overfermentation of starch in the rumen (Dennis et al., 1983). Another way protozoal abundance increase in the rumen is when animals are fed more frequent meals throughout the day (Moir and Somers, 1957; Clarke et al., 1982).

Fungal Abundance

The majority of the fungi found in the rumen are thought to be cellulolytic (Orpin and Joblin, 1997), and their role is the rumen is to initially colonize fiber and break down cellulose, hemicellulose, starch, and some protein (Dehority, 2003). Fungi are crucial to

the degradation of fiber in the rumen because they are capable of separating both the cuticle and the lignified cell wall of plants (Hobson and Stewart, 2012). They break apart the lignocellulose tissues of plants through the use of their rhizoids which penetrate and separate the plant fiber allowing other microbes in the rumen to then break down the smaller pieces (Ho et al., 1988; Akin et al., 1989). Since they play such an important role in fiber degradation, studies show diets high in forage have a higher abundance of fungi present (as much as 8% of total ruminal abundance) and a longer retention time than diets high in grain (Bauchop, 1979; Russell and Rychlik, 2001). Fungal concentrations have been shown to remain constant in both sheep and fistulated steers regardless of the frequency of feeding from one all the way to 24 times per day (Obispo and Dehority, 1992; Dehority and Tirabasso, 2001). The fungi that exists in the rumen come in two forms occupying different locations in the rumen. Zoospores having a relatively low abundance (10^3-10^4/mL of rumen fluid) can be free-living cells that float in the rumen fluid or can attach to plant particulates in the rumen along with fungal sporangia (Carberry et al., 2012).

The Hindgut Microbiota

The ruminal microbiome has been relatively well explored, but there have been few examinations of the ruminant hindgut microbiome. The ruminant hindgut is broadly similar to the hindgut of monogastric animals by absorbing nutrients across the epithelium and being home in the hind gut to a secondary microbial fermentation similar to that of the rumen (Yeoman and White, 2014). The most dominant phylum in the ruminant hindgut tend to be Firmicutes rather than Bacteroidetes as is seen in the rumen (Myer et al., 2015b; Myer et al., 2016; Myer et al., 2017). The most abundant genera of Firmicutes found in the ruminant hindgut are *Butyrivibrio* and *Ruminococcus.*

Throughout the GIT, the environmental conditions within and the function of the different intestinal compartments dictates the microbial population present in that particular section. For example, the low pH of the duodenum means bacterial populations are fairly low but increase distally with increasing pH. The jejunum absorbs both proteins and carbohydrates, so as expected, its microbial population looks a lot different than the microbial population of the rumen (Myer et al., 2017). Myer et al. (2016) found the abundance of Firmicutes in the jejunum of steers, regardless of feed efficiency, was approximately 90% of the total OTU, whereas, Bacteroidetes was only 0.4 to 1.1% with addition phyla present being Actinobacteria, Proteobacteria, and Tenericutes. Unlike the rumen, the most abundant genus in the jejunum was *Ruminococcus* followed by *Butyrivibrio*, *Lactobacillus*, *Bulleidia*, *Mogiobacterium*, *Mitsuokella*, and *Pripionibacterium* all having an abundance greater than 1% (Myer et al., 2016). Perea et al. (2017) found that in lambs the ileal microbiota was most similar to the jejunum when comparing samples throughout the gastrointestinal tract and found that both locations showed variation in alpha-diversity between efficient and inefficient lambs. The microbial populations of the ileum and jejunum clustered together along with the duodenum (de Oliveira et al., 2013) and the most dominant small intestinal bacteria were *Clostridiaceae* and *Ruminococcaceae*. Furthermore, *Prevotellaceae* was the most dominant family from the phylum *Bacteroidetes* in the small intestine (de Oliveira et al., 2013).

Due to similarities in function and proximity, Myer et al. (2017) found cecal and colonic bacterial populations from steers to be very similar. Since these are the locations of hindgut degradation of cellulose and starch that has escaped ruminal degradation and intestinal digestion, the microbial composition of both of them is similar to the rumen

(Myer et al., 2017). In both the cecum and colon, Spirochaetes, Tenericutes, and Actinobacteria are some of the most numerous phyla (Myer et al., 2015a; Myer et al., 2015c) with the addition of Fibrobacteres and Proteobacteria in the colon (Myer et al., 2015c). Many similarities exist between the two hindgut regions of the GIT at the genus level, with *Prevotella*, *Turcibacter*, *Coprococcus*, *Ruminococcus*, *Dorea*, *Blautia*, and *Oscillospira* being among the most abundant genera in both regions with the addition of *Clostridium* being in the most numerous in the cecum, and *Parabacteroides* being in the most numerous in the colon (Myer et al., 2015a; Myer et al., 2015c; Myer et al., 2017).

Development of the Microbiota

When ruminants are born, their rumens are non-functional and are smaller in volume; however, the microbial population starts to develop shortly after birth (Fonty et al., 1987; Mizrahi, 2013). Because the rumen is not functioning, when young ruminants consume milk from their dam the suckling reflex induces the engagement of the esophageal groove (Mizrahi, 2013) which provides a passage for the milk past the rumen and ultimately past the fermentation taking place there (Titchen and Newhook, 1975; Wise et al., 1984). One of the first ways a young ruminant's microbiota develops is from coming into contact with their mother or other ruminants (Bryant et al., 1958; Dehority, 2003). One of the first bacteria to inhabit the rumen is *Escherichia coli* which was found in the rumen of calves as early as 1 day of age but then began to decrease until leveling out approximately 6-8 weeks old (Minato et al., 1992). A study performed by Song et al. (2018) corroborated other studies by finding many pathogenic bacteria including *Escherichia-Shigella* and *Salmonella* were more prevalent in the hindgut of calves who were 1-wk old which could result in digestive upset of calves. Fonty et al. (1987) and Bryant et al. (1958)

found similar patterns by showing facultative anaerobic bacteria decreased as the ruminant matured. Both types of eukaryotes found in the rumen are some of the first to inhabit the rumen of newborn ruminants. Protozoa are introduced through the saliva of the mother from licking or coming into contact with feed (Dehority, 2003) and quickly begin to colonize the rumen in the first few days after birth (Bryant et al., 1958; Bryant and Small, 1960; Eadie and Hobson, 1962). Fungi enter the rumen via the same route as protozoa, as researchers have found anaerobic fungi present in the saliva of mature ruminants (Trinci et al., 1988). Bryant et al. (1958) found that contact with the mother and other mature ruminants is also critical for the introduction of cellulolytic microbes in the rumen as evident by lambs kept in isolation not having cellulolytic species in their rumen consortium. The rumen consortium is mostly comprised of strictly anaerobic species a few days after birth, and cellulolytic and methanogenic bacteria and anaerobic fungi began to colonize the rumen at around 1 week of age with the aerobic and facultative anaerobic populations decreasing in abundance as the animal matures (Fonty et al., 1987). On the other hand, other types of bacteria such as amylolytic bacteria, lactate utilizers, sulfate reducing bacteria, and xylan and pectin fermenting bacteria which normally start in really low concentrations, quickly increase in abundance and reach their constant abundance and level off at 3 days of age (Mizrahi, 2013).

Dietary Effect on the Microbial Consortium

One of the main environmental factors affecting the ruminant's microbial population is diet (Tajima et al., 2001; De Menezes et al., 2011; Carberry et al., 2012). Specifically, one of the most extreme changes in the ruminant's microbiota occurs during the transition from a high forage to a high concentrate diet, and this transition is where

many dietary illnesses occur in feedlot cattle (Nagaraia and Chengappa, 1998; USDA/APHIS, 1995). Because the fermentation must rapidly shift from the degradation of cellulose and hemicellulose to degrading starch and soluble sugars, the rumen microbial population must undergo a profound shift (Fernando et al., 2010; McAllister, 2000; Carberry et al., 2012). The bacteria present in the rumen are a direct consequence of the nutrients provided to them and which bacteria have the most competitive advantage and can utilize the feedstuffs the best. One example of this is in sheep feed a high roughage diet, cellulolytic bacterial abundances comprised 68.5, 54.6, and 23.5% of the total bacterial population when fed one, 6, or 24 times a day, respectively (Dehority and Tirabasso, 2001). Fibrolytic fungal populations varied in sheep with feeding frequency as well, which made up 82.4, 80.4, and 92.9% of total fungal populations when fed one, 6, and 24 times a day, respectively (Dehority and Tirabasso, 2001).

RFI's Effect on the Microbial Consortium

In recent years, many studies have suggested a link between the bacteria that make up the ruminal consortium and the feed efficiency of the host animal (Guan et al., 2008). Hernandez-Sanabria et al. (2012) stated the microbiota of the rumen must play a part in the host animal's feed efficiency because 10% of RFI can be attributed to the ruminant's digestion (Herd and Arthur, 2009). The rumen microbial fermentation controls the efficiency of feedstuff conversion to VFA and Microbial Crude Protein which in turn impacts animal growth efficiency and feed intake (Hernandez-Sanabria et al., 2012). Changes in diet and the microbiota do not always impact the animal's RFI; Hernandez-Sanabria et al. (2012) found when steers were switched from a low-energy to a high-energy diet some of the steers retained their feed efficiency, but their microbial populations

changed. However, Guan et al. (2008) compared the ruminal microbiomes of 18 steers with different RFI statuses in the post feedlot period to determine any links between RFI and microbiome composition. Low-RFI steers' (higher efficiency) microbiotas were more closely similar in composition than were microbiotas of the high-RFI steers (91% vs 71% similarity, respectively). The rumen fluid from Low-RFI, Medium-RFI, and High-RFI steers were analyzed further, and the ruminal bacteria showed similar grouping effects as the original group of Low- and High-RFI steers (Guan et al., 2008). They concluded the more efficient animals' microbial populations were likely to use the same metabolic pathways contributing to their increased feed efficiency. However, the less efficient steers' microbiotas were less related to each other, leading to the assumption they utilized a larger variety (broader diversity) of metabolic pathways being expressed resulting in more nutrients being wasted instead of being utilized by the animal.

The Firmicutes-to-Bacteroidetes ratio is thought to play an important role in energy harvesting of animals. Increased levels of Firmicutes is correlated with fat deposition in ruminants, pigs, humans, and mice (Ley et al., 2005; Turnbaugh et al., 2006; Guo et al., 2008; Turnbaugh and Gordon, 2009; Waldram et al., 2009; Zhang et al., 2009; Myer et al., 2015b), suggesting that Firmicutes plays a role in extracting more energy from their digesta (Ley et al., 2006). Myer et al. (2015b) demonstrated higher levels of Firmicutes were correlated with increased average daily gain (ADG) and an increase in fat levels in steers. In a study with pigs that were classified as either obese or lean, the obese pigs had lower levels of the phyla Bacteroidetes than the lean pigs (Guo et al., 2008). In humans that had undergone gastric bypass surgery, Firmicutes populations were decreased in individuals undergoing weight loss (Zhang et al., 2009). Individuals colonized by a microbiota

containing more Firmicutes had a more efficient microbial population allowing them to harvest more energy in their diets making them predisposed to obesity (Turnbaugh and Gordon, 2009).

The microbiome can also change the feed efficiency of the host via a change in alpha- and/or beta-diversity. Efficient cattle were shown to have lower species diversity in the rumen compared to inefficient animals (Zhou et al., 2009). Other researchers found no differences in α- or β-diversity in the rumen of steers (Myer et al., 2015b), bulls (McCann et al., 2014), or lambs (Perea et al., 2017) in terms of feed efficiency. Shabat et al. (2016) however, utilized low- and high-efficiency dairy cows and found that more efficient dairy cows had lower microbial richness in the rumen. They also noted a lower diversity in the efficient cows' ruminal microbiomes and further found that the most efficient cows' ruminal microbiomes utilized fewer microbial metabolic pathways leading to fewer metabolites that were more utilizable by the host, which provided the host with more energy than inefficient cows.

Ruminal methanogen populations have been correlated with the efficiency status of cattle (Nkrumah et al., 2006; Zhou et al., 2009; Hernandez-Sanabria et al., 2012; Mizrahi, 2012; Jami et al., 2014; Kittelmann et al., 2014; Shi et al., 2014; Wallace et al., 2015). A study performed by Nkrumah et al. (2006) found energetically inefficient (high-RFI) beef cattle produced more methane compared to more energetically efficient beef cattle. Shabat et al. (2016) found that more efficient dairy cows produced less ruminal methane than did inefficient cows, which is logical given that ruminal methane represents a loss of up to 12% of the digestible energy in the diet (Johnson and Johnson, 1995; Myer et al., 2017).

Feed efficiency is not generally correlated with the "core microbiome" that have a large overall abundance, but rather is correlated with a few individual taxa that impact plant fiber degradation, GIT health, and ruminal dysbiosis (McCann et al., 2014; Myer et al., 2015b; Perea et al., 2017). Increased ruminal populations of the genera *Butyrivibrio* and *Succinivibrio*, family *Ruminococcaceae*, and order Bacteroidales have all been correlated with increased feed efficiency of cattle; whereas, decreased ruminal populations of genera *Lactobacillus*, *Robinsoniella*, *Selenomonas*, and *Succiniclasticum* have been correlated with increased feed efficiency in cattle (Hernandez-Sanabria et al., 2012; McCann et al., 2014; Myer et al., 2015b). A similar pattern was found by Perea et al. (2017) in lambs that differed in feed efficiency, with increased populations of the family *Ruminococcaceae,* which contains many cellulolytic species, in the rumen and feces of efficient lambs suggesting the importance of forage degrading bacteria in the rumen as well as the hindgut. There was a reduction in succinate-producing bacteria, *Succinivibrio* and *Alloprevotella* spp., in the rumen and colon in more efficient compared to inefficient lambs. *Prevotella* was in greater abundance in heifers who were less efficient (Carberry et al., 2012). These differences are likely due to animals differing in efficiency possessing differences in their ruminal microbial fermentation pathways affecting the fermentative end products of feedstuffs. In the case of *Prevotella* spp., starch, proteins, peptides, hemicellulose, and pectin are utilized by the bacteria to create acetate, succinate, and propionate for the host (Carberry et al., 2012).

Perea et al. (2017) demonstrated improved performance in lambs with an elevated abundance of acetate-producing bacteria (i.e., *Bifidobacteriaceae*) in the rumen, small intestine, and lower GIT, possibly because an increase in *Bifidobacteriaeae* is closely

related to gut health (Mitsuoka, 1990). Conversely, an increase in *Proteobacteria* in the hindgut of less efficient lambs (Perea et al., 2017) was indicative of a dysbiosis as this group is more abundant in humans suffering from inflammatory bowel syndrome (Mukhopadhya et al., 2012), cats with diarrhea (Suchodolski et al., 2015), and obese individuals (Zhao, 2013). A member of the *Proteobacteria* phylum, *E. coli*, possesses an endotoxin that increases subcutaneous fat deposition (Cani et al., 2008). *Christensenellaceae* has been repeatedly linked to a healthy well-functioning GIT compared to individuals with inflammatory bowel syndrome (Goodrich et al., 2014), more abundant in pigs that displayed lower *E. coli* shedding (Jenkins et al., 2015), and negatively correlated with loose stools (Tigchelaar et al., 2016). In addition to being linked to overall gut health, *Christensenella minuta* has been linked to decreased adipose tissue deposition in germ-free mice inoculated with a human fecal sample inoculated with *C. minuta* (Goodrich et al., 2014) compared to germ-free mice.

Zhou et al. (2018) used crossbred steers to examine changes in ruminal microbiota and RFI status when ruminal contents from efficient steers was exchanged with inefficient. Efficient steers that received ruminal contents from inefficient steers had increased abundance of *Lactobacillus* which is linked with acute rumen acidosis (Hernández et al., 2014). Previous findings show *Coriobacteriaceae* and *Coprococcus* populations were higher in jejunal contents of steers with low feed intake and a large amount of gain (Myer et al., 2016). Zhou et al. (2018) found similar results in low-RFI steers after receiving the contents from high-RFI steers where both *Coriobacteriaeae* and *Coprococcus* were more abundant. *Coriobacteriaceae* and *Coprococcus* have previously been linked to increased volatile fatty acid production in the rumen (Song et al., 2018). These findings suggest the

microbial consortium may alter its mechanisms of fermentation to try to regain the host's original efficiency by having a predisposition to specific bacteria associated with feed efficiency.

RFI's Effect on Energy Availability

Studies have demonstrated that low-RFI steers had more digestible and metabolizable energy available to the tissues (Nkrumah et al., 2006) suggesting the efficiency of steers can be explained as result of increased microbial fermentation increasing energy supplied to the host animal (Guan et al., 2008). There are many different ways in which the additional VFA production could occur from interactions between bacteria, protozoa, fungi, and the host animal (Kamra, 2005). Guan et al. (2008) found low-RFI steers had higher ruminal concentrations of butyrate and valerate than did high-RFI steers. They also reported total VFA and acetate concentrations were numerically higher, supporting the concept that more efficient animals captured more dietary energy via their rumens as a result of increased microbial fermentation or a shift in pathways utilized by the microbial population. Shabat et al. (2016) found more efficient dairy cows had a higher production of propionate, butyrate, valerate, and isovalerate along with an overall 10% increase in VFA production. They also reported a decrease in the acetate-to-propionate ratio in efficient cows compared to inefficient cows, suggesting increased energy retention by the ruminant (Russell, 1998).

Carberry et al. (2012) fed heifers both low- and high-forage diets and noted a correlation between microbial fermentation end products (e.g. acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate) and the high-forage diet. More propionate and valerate was found in the rumen of heifers fed a high-forage diet. Conversely, there

was more butyrate and total VFA concentration as well as an increase in the acetate: propionate ratio in the rumen of low forage-fed heifers. *R. albus* was found to be 1.7 times more abundant in the more efficient heifers while they were fed the high-forage diet (Carberry et al., 2012) which is thought to be caused by the cellulolytic activity of *R. albus* as a fibrolytic bacterial species. Carberry et al. (2012) also found *R. albus* did not differ in abundance based on RFI categorization when the host was fed a low-forage diet because the higher amount of starch was more slowly utilized by cellulolytic and utilitarian species (Mackie et al., 1978; Orskov, 1982). This increase disappeared when animals were switched to a higher energy diet where it was found that *Prevotella* was negatively correlated with both isobutyrate and isovalerate concentrations.

Conclusions

The ruminant's gastrointestinal microbial population provides it a competitive advantage of utilizing plant materials that are indigestible by other animals lacking a fermentative forestomach. The ruminal and gastrointestinal fermentation provides both energy and protein to the host animal via a mutualistic relationship. However, this relationship is not perfectly efficient in converting feed to energy and protein used to synthesize meat or milk. In order to maximize production profitability for producers, feed efficiency must be increased to allow cattle to gain more weight on less feed and ultimately increase producer profitability. Increasing the feed efficiency of the ruminant can be achieved by manipulating the gastrointestinal microbial ecosystem. To achieve this goal, we must understand the key microbes and the relationships they have with each other and with the host animal in order to optimize metabolic pathways utilized to lead to more efficient nutrient utilization.

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CHAPTER 3

EVALUATION OF THE FECAL MICROBIOME OF ANGUS STEERS WITH DIVERGENT FEED EFFICIENCIES ACROSS THE LIFESPAN FROM WEANING TO SLAUGHTER

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ABSTRACT

Many studies have examined the link between specific bacteria of the gastrointestinal tract and cattle feed efficiency. However, cattle undergo dietary changes during their productive life which can cause fluctuations in their microbial consortium. The objective of the present study was to assess changes in the fecal microbiome of beef steers genetically selected to be divergent in feedlot feed efficiency, to determine whether differences in the fecal microbiome could be detected as early as weaning, and continued throughout rearing of steers regardless of dietary changes. Fifteen commercial Angus steers divergent in feed efficiency based on their feedlot-finishing performance were selected and divided into two groups according to their residual feed intake (**RFI**): efficient steers (low-RFI; n=7) and inefficient steers (high-RFI; n=8). Fecal samples were collected at weaning, yearling, and slaughter. DNA extraction and 16S rRNA gene sequencing were performed to ascertain their fecal microbial consortium. One-way ANOVA with feedlot feed efficiency classification as a fixed effect, and repeated-measures ANOVA for each efficiency group were performed. Overall, microbial evenness and diversity were greater at weaning compared to yearling and slaughter for both efficiency groups ($P < 0.001$). Feedlot RFI linearly decreased as both Shannon diversity and Ruminococcaceae abundance increased (R^2 = 65.6% and 60.7%, respectively). Abundances of *Ruminococcaceae*, *Rikenellaceae*, and *Christensenellaceae* were higher at weaning versus yearling and slaughter ($P < 0.001$); moreover, these families were consistently more abundant in the feces of the efficient steers (for most of the timepoints evaluated; $P \leq 0.05$), compared to the inefficient steers. Conversely, abundances of *Bifidobacteriaceae* and *Lactobacillaceae* were numerically higher in the feces of the inefficient steers throughout

their lives. Total volatile fatty acid concentrations increased at slaughter compared to weaning and yearling for both efficiency groups ($P < 0.001$). The acetate:propionate ratio decreased linearly ($P < 0.001$) throughout the life of the steers regardless of their efficiency, reflective of dietary changes. Our results indicate that despite fluctuations due to animal age and dietary changes, specific bacterial families may be correlated with feed efficiency of steers. Furthermore, such differences may be identifiable at earlier stages of cattle's production cycle, potentially as early as weaning.

Keywords: Beef cattle, *Bifidobacteriaceae*, *Christensenellaceae*, Microbiome, Residual Feed Intake, *Rikenellaceae*, *Ruminococcaceae*

Introduction

In beef production systems, feed represents the largest single cost and accounts for an estimated 60-75% of the total cost of production (Herd et al., 2003). In order to increase the profitability of beef operations, producers seek to improve the efficiency by which cattle convert ingested feed into body weight gain (Nkrumah et al., 2006; Arthur and Herd, 2008). One method used to identify feed efficiency of cattle involves calculating their residual feed intake (**RFI**), which is a measure that utilizes the difference in an animals' expected feed intake based on their metabolic body weight and their actual feed intake. Values of RFI express how efficient animals are while producing a certain level of body weight gain. If an animal eats less than expected for that level of gain (low-RFI), it is considered more efficient (Koch et al., 1963; Arthur and Herd, 2008). Therefore, low-RFI animals are more efficient than animals that have high RFI values.

An estimated 19% of the variation in RFI can be attributed to diet and digestibility of feed (Herd and Arthur, 2009). Variation in RFI can also be linked to the microbial population within the gastrointestinal tract (**GIT**) of cattle, because these microbes are responsible for 70% of the energy and 50% of the protein the ruminant animal uses (Russell and Hespell, 1981; Bergman, 1990). Many studies have found a direct link between feed efficiency and the microbial population in cattle's GIT (McCann et al., 2014; Shabat et al., 2016; Myer et al., 2017); however, most of the studies concerning the GIT of cattle have focused on the ruminal microbial population.

The present study was designed to evaluate the composition of the fecal microbial population of beef cattle that differed greatly in feed efficiency (as assessed by RFI). In most practical beef production situations, obtaining a fecal sample from the animals is

substantially more feasible than obtaining a ruminal sample. Therefore, fecal samples were collected from beef steers at three different times during their production cycle: at weaning, yearling, and immediately post-slaughter. The fecal microbiome of the most efficient (low-RFI) and least efficient (high-RFI) steers were evaluated and compared at each stage. We hypothesized that fecal microbiomes would consistently differ at different points in the lifecycle of steers based on their feed efficiency evaluated during the feedlot-finishing phase.

Materials and Methods

Animals, diets, and steer selection

The animals utilized in this study were cared for using the guidelines approved by the University of Georgia's Animal Care and Use Committee (AUP #A2012 11-006-R1). The steers used in the present study are from the fifth generation of a genetic selection program involving Angus cattle being selected for residual average daily gain and intramuscular fat (marbling). All animals were calved and raised at the Northwest Georgia Research and Education Center, located in Calhoun, GA (34° 30 N, 84° 57 W) where they were reared on a pasture-based system until approximately 10 months of age. The steers (n=63) were then transported to a commercial feedlot located in Brasstown, NC (35° 10 N, 83° 23 W) where they were backgrounded prior to starting the feedlot trial. They were fed a 3-week transition diet prior to being switched to a finishing diet, which was fed for 110 days. The finishing diet provided 14.51% crude protein, 2.10 Mcal/kg NE_m, 1.43 Mcal/kg NE_g, 0.70% Ca, and 0.45% P. Further composition information on both the transition and finishing diets can be found in Supplementary Table 1.

During the feedlot period, the feed intake of steers was individually measured using a GrowSafe System (GrowSafe Systems Ltd., Calgary, Canada). Intake data was then used to calculate their individual feed conversion rates, which were expressed as RFI. Upon conclusion of the feedlot period, steers were rank-ordered based on their feed efficiencies (RFI) and the 12 most efficient (lowest RFI values), along with the 12 least efficient (highest RFI values) were transported to the University of Georgia Meat Science Technology Center, a federally inspected meat plant located in Athens, GA (33° 57 N, 83° 22 W). The steers were housed on site overnight where they were fasted but given *ad libitum* access to water prior to humane slaughter the next morning. In order to avoid marginal differences in RFI and create an even greater biological distinction between the two groups of steers, and due to sample availability at the previous collection timepoints, further selection of the samples was performed. This resulted in a total of 15 steers being used in this study: 7 were classified as low-RFI (efficient steers), and 8 were classified as high-RFI (inefficient steers).

Fecal collection and storage

The first set of samples used in this study was collected several months before the steers were slaughtered, on their weaning day (approximately 9 months of age). Fecal contents were aseptically collected via fecal grab (approximately 50 g) along with production and growth data using a separate palpation sleeve for each sample which was placed in 50 mL conical tubes and stored on wet ice until they could be transferred to a -20°C freezer in Athens, GA. After the steers were backgrounded, transported to the feedlot, and adapted to their finishing diets, the second set of fecal samples were collected, which corresponded to the yearling phase (approximately 13 months of age). These samples were

collected and prepared as described above. The final set of samples was collected from their rectum upon evisceration of the carcasses on the slaughter day (approximately 18 months of age). The samples from the kill floor were immediately placed in a -20℃ freezer for storage.

DNA extraction and sequencing

DNA was extracted from all samples using a hybrid DNA extraction protocol utilizing both mechanical and enzymatic methods as previously described by Rothrock et al. (2014). This procedure uses 0.33 g of fecal material placed into 2 mL Lysing Matrix E tubes (MP Biomedicals LLC, Irvine, CA, USA) which are homogenized using a FastPrep 24 Instrument (MP Biomedical LLC, Irvine, CA, USA) to mechanically break open the cells. InhibitEX Tablets (QIAGEN, Venlo, Netherlands) were used as the enzymatic means of increasing DNA yields. An automated robotic workstation (QIAcube; QIAGEN, Venlo, Netherlands) was used for elution and purification of DNA from the samples. DNA concentration and purity were determined spectrophotometrically using the Synergy H4 Hybrid Multi-Mode Microplate Reader along with the Take3 Micro-Volume Plate (BioTek Instruments Inc; Winooski, VT, USA). The samples required a minimum requirement of 20 µL of volume and a concentration of 10 ng/µL of DNA in order to proceed to sequencing. Samples that failed to meet these minimum requirements were processed through a new cycle of DNA extraction. Once all samples were adequate in both volume and DNA concentration, they were stored at 4℃ overnight.

After overnight storage, the samples were transported to the Georgia Genomics and Bioinformatics Core (https://dna.uga.edu) for library preparation and 16S rRNA gene sequencing. The library preparation included PCR replications using the forward primer:

S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and reverse primer: S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013). 16S rRNA gene sequencing was performed using the Illumina MiSeq with v3 2 x 300 base pairs kit (Illumina Inc., San Diego, CA, USA).

Sequencing data

After sequencing was performed, the data was demultiplexed and converted into FASTQ files. Pair-end reads were set and merged using BBMerge Paired Read Merger v37.64, and files were analyzed using QIIME pipeline v1.9.1 (Caporaso et al., 2010). The files were then filtered based on quality and merged into one single file that was then converted into the FASTA format. Sequences were grouped together at 97% similarity into operational taxonomic units (OTU) using the Greengenes database (gg_13_8_otus) with singletons being excluded from the analysis. Sequence depth was set at 17,542 sequences per sample for further analysis.

Volatile fatty acid analysis

Analysis of volatile fatty acids (**VFA**) was performed according to the procedure described in Lourenco et al. (In Review). Briefly, 1 g of fecal material was acquired from each sample, diluted with 3 mL of distilled water, and placed into 15 mL conical tubes. The tubes were vortexed for 30 seconds to produce a homogeneous sample and 1.5 mL of the mixture was transferred to microcentrifuge tubes. The tubes were centrifuged at 10,000 x *g* for 10 minutes. One mL of the supernatant was transferred into a new microcentrifuge tube and mixed with 0.2 mL of metaphosphoric acid solution (25% w/v). The samples were vortexed for 30 seconds and stored at -20℃ overnight. The next morning samples were thawed and centrifuged at 10,000 x *g* for 10 minutes**.** The supernatant was removed and

transferred into polypropylene tubes combined with ethyl acetate in a 2:1 ratio of ethyl acetate to supernatant. Tubes were vortexed for 10 seconds to thoroughly mix them and allowed to settle for 5 minutes for optimum separation. Then 600 µL of the top layer was transferred into screw-thread vials. VFA analysis was performed using a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan) with a flame ionization detector and a capillary column (Zebron ZB-FFAP; 30 m x 0.32 mm x 0.25 µm; Phenomenex Inx., Torrance, CA, USA). Sample injection volume was set to 1.0 µL, and helium was used as a carrier gas. Column temperature started at 110℃ and increased to 200℃ over the course of 6 minutes. The injector temperature was set to 250℃, and the detector temperature was set to 350℃.

Statistical analysis

Statistical analyses were performed using Minitab v19.1. Animal performance data, alpha diversity indices, and bacterial abundances were analyzed using a one-way ANOVA with feedlot RFI classification (i.e. high- or low-RFI) as a factor. In addition, repeated-measures ANOVA was carried out for each group of steers to investigate potential differences across the 3 samples collected across their lifecycle (weaning, yearling, and kill floor), and Tukey's pairwise comparisons were performed to assess further differences. Multiple correlations between RFI values and the microbial traits were evaluated, both Shannon diversity and the abundance of *Ruminococcaceae* at slaughter were found to be highly significant. Linear regression analysis was performed to investigate the relationship between Shannon diversity index at the kill floor and RFI; as well as between the abundance of *Ruminococcaceae* at the kill floor and RFI. For all statistical tests, results were considered significant at $P \leq 0.05$, and treated as trends when $0.05 < P \leq 0.10$.

Results

Animal Performance

Animal performance, in terms of body weight, was similar throughout the life cycle of the efficient and the inefficient steers (Table 1; $P \geq 0.51$). Dry matter intake during the feedlot-finishing period was lower for the Low-RFI (efficient) steers ($P < 0.001$) compared to the High-RFI (inefficient) steers. Feed conversion expressed as a feed:gain ratio was lower ($P = 0.001$) for the more efficient steers. Likewise, the efficient and inefficient steers were divergent ($P < 0.001$) in terms of feedlot RFI, with the efficient steers consuming 4.04 kg less dry matter/day when comparing to the inefficient steers (RFI = 2.02 and -2.02 for the inefficient and efficient steers, respectively).

Diversity Indices

Alpha diversity indices for the efficient and inefficient steers at weaning, yearling, and slaughter were examined (Table 2). In the inefficient steers, Chao 1, an indicator of microbial richness, was higher ($P = 0.02$) in the feces at weaning compared to the feces collected at slaughter. In the efficient steers microbial richness at slaughter and yearling numerically decreased compared to weaning, but this was not significant ($P = 0.19$). In both the efficient and inefficient steers, the species within the feces at weaning were more similar in evenness ($P < 0.001$) than at yearling and slaughter. Similarly, the Shannon diversity index of fecal samples was higher ($P < 0.001$) at weaning than at the later timepoints, regardless of feedlot efficiency status of the steers. Regression analysis revealed that as the Shannon diversity index increased, RFI decreased (Figure 1; $R^2 = 65.6\%$). The Shannon diversity index did not differ between efficient and inefficient steers

at weaning and yearling (Figure 2; $P > 0.18$); however, at slaughter, fecal microbial diversity was greater ($P = 0.004$) in the efficient steers compared to inefficient steers.

Bacterial Relative Abundance

The relative abundances of the bacterial families S24-7, *Bifidobacteriaceae*, and *Lactobacillaceae* were greater ($P \leq 0.05$) in the inefficient steers at weaning compared to the efficient steers (Figure 3). At both yearling and slaughter, abundance of the families *Ruminococcaceae*, *Rikenellaceae*, and *Christensenellaceae* were higher ($P < 0.05$) in feces of efficient steers than in feces of the inefficient steers.

As the population of *Ruminococcaceae* increased in the feces collected at slaughter, the RFI of the host animal was lower (Figure 4; $R^2 = 60.7\%$). *Ruminococcaceae* abundance was higher ($P < 0.001$) at weaning than at both yearling and slaughter in both feed efficiency groups (Figure 5). At weaning, there were no differences ($P = 0.46$) in *Ruminococcaceae* abundance between efficient and inefficient steers. At yearling and slaughter, *Ruminococcaceae* abundance was higher ($P = 0.01$) in efficient steers compared to inefficient steers.

The fecal abundance of *Rikenellaceae* was higher at weaning than at yearling and slaughter for both groups of steers (Figure 6; $P < 0.001$). Fecal abundance of *Rikenellaceae* tended to be higher ($P = 0.10$) at weaning in the efficient steers than in the inefficient steers. At yearling and slaughter, the most efficient animals had greater fecal abundances of *Rikenellaceae* compared to the least efficient animals ($P \leq 0.05$). For both groups of steers, the abundance of *Christensenellaceae* was greater (Figure 7; $P < 0.001$) in feces collected at weaning than in the feces collected at yearling and slaughter. Moreover, abundance of

Christensenellaceae tended to be higher at weaning ($P = 0.08$) in efficient steers; and was higher ($P \leq 0.05$) at both yearling and slaughter in the efficient steers.

Fecal *Bifidobacteriaceae* abundance was higher ($P = 0.011$) at slaughter compared to weaning in the inefficient steers (Figure 8). Conversely, abundances of *Bifidobacteriaceae* remained relatively consistent throughout the life of efficient steers ($P = 0.142$), although a numerical increase was observed. At weaning, there were more species from the family *Bifidobacteriaceae* present in the feces of inefficient steers versus those in the feces of efficient steers ($P = 0.02$). *Bifidobacteriaceae* abundance was on average the same in the feces of the steers at the yearling stage regardless of their feed efficiency status ($P = 0.14$). Inefficient steers tended to have greater abundances of *Bifidobacteriaceae* in the feces at slaughter compared to efficient steers ($P = 0.06$).

In the most efficient steers, the abundance of *Lactobacillaceae* was higher at the yearling stage than it was at weaning (Figure 9; $P < 0.01$). There was a tendency for abundance of *Lactobacillaceae* to vary widely during the life of the inefficient steers ($P = 0.082$). Moreover, inefficient steers had a larger abundance of *Lactobacillaceae* at weaning than did efficient steers ($P = 0.05$); however, at yearling and slaughter, the abundance of this family was similar between efficient and inefficient steers ($P \geq 0.13$).

Volatile fatty acid concentrations

Fecal acetate concentrations were greater (Table 3; $P = 0.002$) at slaughter compared to weaning and yearling stages in the inefficient steers, whereas, it was only greater ($P = 0.019$) at slaughter compared to the yearling stage of the efficient steers. More propionate and butyrate were present in the feces at slaughter compared to the feces at weaning and yearling ($P \leq 0.003$) in both efficient and inefficient steers. Valerate

concentrations in the feces at slaughter were higher ($P < 0.001$) in the efficient animals compared to at the weaning and yearling stages; however, fecal valerate was only higher ($P = 0.049$) at slaughter compared to the yearling stage in the inefficient steers. Total VFA concentrations were increased ($P < 0.001$) in all steers, regardless of feedlot feed efficiency, in the fecal samples collected at slaughter compared to either the weaning or yearling stages. Regardless of feedlot feed efficiency status, the ratio of acetate to propionate decreased throughout the life of the steers, with the highest in the feces at weaning, intermediate in the feces at the yearling stage, and lowest in the feces at slaughter ($P < 0.001$).

Discussion

Animal Performance

As expected based on previous generations' performance within this commercial Angus herd (Detweiler et al., 2019), the steers were consistent in terms of growth throughout their lives. However, steers differed in the amount of feed consumed and in their conversion of feed into body weight. The efficient steers were able to eat on average 3.9 kg feed less per day (of dietary dry matter) while gaining approximately the same amount of weight as the inefficient steers. This translated into the efficient steers needing 5.3 kg less feed than their inefficient counterparts to gain 1 kg of body weight, given that their feed:gain ratios were 10.54 and 15.84, respectively. Similarly, the calculated RFI values were very distinct between the 2 groups of steers, with the lowest RFI values observed in the efficient steers. Feed conversion differences are important for producers because feed is the most expensive input cost of animal production systems, therefore

having cattle that can gain the same amount of weight while consuming less feed can have a significant impact on feeder profit margins (Herd et al., 2003; Schnepf, 2012).

Diversity Indices

Research has broadly demonstrated that bacterial richness and diversity are decreased in more efficient ruminants (Zhou et al., 2009; Shabat et al., 2016); however, those assessments are normally made relative to the ruminal microbial populations and have not been extensively evaluated the intestinal microbiome (Durso et al., 2017). Shabat et al. (2016) hypothesized that ruminal microbial populations with less richness and diversity had fewer, but more specific metabolic pathways present, leading to a more limited metabolite pool and less production of non-metabolizable products in the rumen. However, in the intestinal environment, Welch et al. (In Review) found that bacterial richness and diversity were greater in both cecal contents and the feces of steers with higher feed efficiency compared to low efficiency steers. Other researchers have also shown that microbial diversity measures differed between the rumen and the feces of cattle (de Oliveira et al., 2013; Myer et al., 2017). The present study found bacterial diversity to be greater in the feces of the most efficient steers which somewhat contradicts the hypothesis proposed by Shabat et al. (2016); however, our findings conform to biological theory in regard to the intestinal environment. Nutrient availability differs widely throughout the gastrointestinal tract of cattle, digesta that reaches the large intestine contains less-digestible nutrients, which are essentially "leftovers" that escaped ruminal microbial degradation and small intestinal digestion. Therefore, increased bacterial diversity in the hindgut will in turn result in a greater array of microbial enzymes to degrade the intestinal digesta, allowing the more efficient steers to capture more leftover nutrients that would

have been unutilized in the GIT and convert them into a metabolic end product that is utilized by the animal for growth.

Beyond gastrointestinal geography there are many other contributing factors to microbial consortium composition variation including animal age and diet (Durso et al., 2012; Liu et al., 2017; Myer et al., 2017). Thus, the changes in alpha diversity within the present study are also related to both changes in age of the steers and diet fed during each growth phase. Overall, we found that steers had the greatest microbial richness, evenness, and diversity in their feces at weaning, when they were younger and were reared on their dams in a pasture-based production system that included highly digestible nutrients from their dam's milk and less digestible forages. During the backgrounding process leading to the yearling stage, starch concentrations in the ration increase, resulting in a selective pressure on the complex microbial ecosystem of the ruminant gastrointestinal tract. Since ruminant amylase activity is lower than that found in monogastric animals, starch that escapes ruminal degradation can also avoid mammalian digestion and reach the cecum and colon of cattle and impact bacterial populations in this portion of the gut (Diez-Gonzalez et al., 1998). The feedlot ration contains an even larger concentration of starch and the selective pressure of this ration decreased the microbial evenness and diversity of all steers regardless of feed efficiency status and decreased bacterial richness (Chao1) in the inefficient steers. It has been hypothesized that as grain levels in diets increased to the point of incurring ruminal acidosis, that the environmental pressures would select for reduced bacterial diversity and for a microbial population largely of Lactic Acid Bacteria (Russell and Hino, 1985; Maounek and Barton, 1987; Nagaraja and Titgemeyer, 2007).

The present results tend to support this hypothesis as to a cause and type of change in the microbial consortium diversity in the feces of cattle.

Bacterial Relative Abundances

Diet alters not only the alpha-diversity of the gastrointestinal microbiome as a whole, but also causes fluctuations in the individual bacterial populations within the gastrointestinal tract throughout the life of a steer (Tajima et al., 2001; Carberry et al., 2012). Since the steers utilized in this study changed from a pasture to a feedlot-based system, the variation in bacterial populations at each timepoint can easily be explained by diet. However, the more novel results were found that despite fluctuations in abundance throughout the steers' lives, certain bacterial families were consistently higher in one group of steers based on their efficiency status, regardless of the diet.

Ruminococcaceae is a family comprised of primarily cellulolytic and hemicellulolytic bacterial species that produce acetate, formate, and hydrogen as fermentation end products (Helaszek and White, 1991; Biddle et al., 2013). *Ruminococcaceae* is able to degrade many substrates that other bacterial families cannot (Brulc et al., 2009). Biddle et al. (2013) reported that *Ruminococcaceae* possess many genes which allow them to bind to cellulose, hemicellulose, and xylan giving them a competitive advantage relative to degradation of plant materials (Ding et al., 2001; Rakotoarivonina et al., 2005). In the present study, regardless of feed efficiency status, abundances of *Ruminococcaceae* were greatest at weaning, while animals were on a forage-based diet, which contains substantially more fiber than feedlot diets. *Ruminococcaceae*, along with *Lachnospiraceae*, have a higher abundance of and more diverse carbohydrate-active genes present, specifically glycoside hydrolases and

carbohydrate-binding modules compared to *Clostridiaceae (*Biddle et al.,2013). Genetic diversity of CAZymes provides *Ruminococcaceae* an advantage when it comes to nutrient uptake and utilization of diverse polysaccharides (Wang et al., 2019). The negative relationship between RFI and *Ruminococcaceae* found in the present study at slaughter corroborate with our previous findings (Welch et. al., In Review) that have shown as this bacterial family increased, RFI decreases resulting in the steer becoming more efficient by utilizing a broader nutrient spectrum, allowing the animal to absorb more energy from the diet. Therefore, we suggest in the present study that the greater abundance of *Ruminococcaceae* in the most efficient steers allowed them to extract greater amounts of energy from the digesta reaching their hindguts, resulting in greater metabolizable energy levels compared to inefficient steers.

Rikenellaceae is a currently discovered family consisting of bacteria found within the gastrointestinal tract and fecal material from animals and humans (Graf, 2014), and was present in the colon of steers regardless of their efficiency status (Myer et al., 2015b). Bacteria within *Rikenellaceae* can utilize mucin as a source of carbohydrates and energy which provides them a competitive advantage over other bacteria (Bomar et al., 2011). *Rikenellaceae* produce acetate, succinate, and propionate as fermentative end products (Abe et al., 2012), all of which can be utilized by the host animal. Moreover, *Rikenellaceae* was found to be more prevalent as a member of the core microbiome of heifers fed a forage-based diet, compared to a forage-grain mixed or all grain diet (Petri et al., 2013), which agrees with present results, given that the greatest abundances of *Rikenellaceae* were observed at weaning when the steers were consuming primarily forages. The abundance of *Rikenellaceae* was consistently higher in the feces of the most efficient steers at all

timepoints evaluated indicating that the efficient steers possibly produced more mucin in their hindguts resulting in a greater population of *Rikenellaceae*. Furthermore, because the abundance of this bacterial family was consistently higher in the feces of the most efficient steers, this family is a candidate marker of cattle feed efficiency, even at early stages of growth.

Christensenellaceae has been associated with a healthy digestive system in humans, a reduction in adipose tissue, and a lower Body Mass Index (Goodrich et al., 2014). The family *Christensenellaceae* consists of species which produce α-arabinosidase, β-galactosidase, and β-glucosidase (Perea et al., 2017), which are components of plant fibers, thus it is logical that the highest fecal abundances were found at weaning when the steers were grazing forages. The decrease in abundance of *Christensenellaceae* seen in the feces at yearling and slaughter can be attributed to a decrease in the overall health of the digestive system during the feedlot finishing period (USDA/APHIS, 1995; Hernández et al., 2014; Steele et al., 2016), where the diet is much more concentrate-based and has been linked to a reduction in ruminal and intestinal health (Russell and Rychlik, 2001; Plaizier et al., 2012; Kvidera et al., 2017; Li et al., 2019). Additionally, *Christensenellaceae* produce butyrate (Morotomi et al., 2012) which is used by epithelial tissue for energy (Russell, 2002; Guan et al., 2008a). Increased butyrate production by *Christensenellaceae* may reduce the incidence of leaky gut which may be responsible for leakage of lipopolysaccharide (LPS) and resultant inflammation in less efficient steers (Li et al., 2012; Mani et al., 2013; Zhang et al., 2016; Kvidera et al., 2017).

Bifidobacteriaceae is comprised of a fructo- and galacto-oligosaccharide-fermenting bacteria to produce acetate (Perea et al., 2017), and is often associated with a

healthy gastrointestinal tract and is often used as a probiotic component (Gomes and Malcata, 1999; Ross et al., 2010; Maldonado-Gómez et al., 2016), and have been found in the rumen, small intestine, and hindgut of ruminants (Uyeno et al., 2010; Xia et al., 2015; Perea et al., 2017). *Bifidobacteriaceae* were negatively correlated with *Christensenellaceae* populations in human fecal samples (Goodrich et al., 2014) which was replicated in the present study, with *Christensenellaceae* abundance being highest at weaning, and *Bifidobacteriaceae* being highest at slaughter. In addition, *Christensenellaceae* abundance was higher in the most efficient steers, whereas *Bifidobacteriaceae* abundance was higher in the inefficient steers. *Bifidobacteriaceae* was most abundant in the cecum of steers that had a high rate of gain and high feed intake but was not found in steers with high gain and low feed intake (i.e., more efficient; Myer et al. 2015a). Our present results support this conclusion, because *Bifidobacteriaceae* were lowest in the more efficient steers. Similar to *Bifidobacteriaceae*, *Lactobacillaceae* populations tended to be greater in the feces of the less efficient steers. *Lactobacillaceae* are carbohydrate-utilizing bacteria and are classified as Lactic Acid Bacteria because they produce copious amounts of lactate, acetate, and CO_2 (Hammes and Vogel, 1995; Hammes and Hertel, 2009). *Lactobacillaceae* proliferate in carbohydrate-rich environments such as acidified foods, and human and animal oral and gastrointestinal microbiotas (Hammes and Hertel, 2009; Felis and Pot, 2014). In cattle, (Li and Guan, 2017) found *Lactobacillaceae* to be correlated with four different metabolic pathways (e.g., methane metabolism, and valine, leucine, and isoleucine degradation), coupled with the high level of lactic acid production, these pathways represent a significant loss of energy and amino acids. For instance, up to 12% of the gross dietary energy can be lost in the form of methane (Hales

et al., 2020). Therefore, increased *Lactobacillaceae* populations may have shifted intestinal fermentation pathway expression, contributing to the reduced efficiency of steers.

Volatile fatty acid production

Many studies have found low-RFI (more efficient) steers to have an increase in energy converted in the rumen (Nkrumah et al., 2006; Guan et al., 2008b), suggesting that host efficiency may be directly related to additional VFA produced by microbial fermentation of the rumen. Welch et al. (In Review) found a numerical increase in VFA production in the rumen of efficient steers compared to inefficient steers; however, fecal samples were reversed with VFA concentrations being highest in the less efficient steers. The present study observed similar patterns, with the inefficient steers having more total fecal VFA than did efficient ones. Moreover, the lower numerical concentrations of important VFA such as acetate, propionate, and butyrate observed in the feces of the efficient steers suggests that the VFA values quantified in the fecal material are strong indicators of increased lower gut VFA absorption rather than production. This result suggests a need to investigate the linkage between the microbiome of the lower gut and gut epithelial integrity and health, potentially explaining differences in feed efficiency.

Diet greatly impacts VFA concentration (Russell, 1988; Hungate, 2013), and it is unsurprising that fecal VFA concentrations varied greatly throughout the steers lifetimes due to the different diets consumed at each stage of growth. Furthermore, most individual VFA (and total VFA) fecal concentrations were highest at slaughter, suggesting that the hindgut microbial activity increased with age, resulting in more VFA production. The acetate-to-propionate ratio in the rumen is directly related to energy availability to the host animal, because propionate is glucogenic (Bergman et al., 1966; Bergman, 1982, 1983).

In the present study the A:P decreased throughout the life of the steers, regardless of their efficiency status, reflecting the dietary composition as a key factor in these changes. The A:P ratio is decreased in ruminants fed a high concentrate diet (reflective of an increase in propionate production) compared to cattle fed a high forage diet (Blaxter, 1962; Diez-Gonzalez et al., 1998; Hales et al., 2020). Although our measurements were made in the fecal material, the acetate-to-propionate ratio was highest when the steers consumed pasture and was lower when the steers were fed a feedlot ration. The acetate-to-propionate ratio shift in the hindgut fermentation may have a large economic impact as propionate production is linked to the development of intramuscular fat (marbling) which results in improved carcass quality and a higher price per carcass (Smith and Crouse, 1984).

Conclusions

Collectively, our results demonstrate that bacterial populations as a whole fluctuate throughout the life of steers, some specific bacterial families can be consistently found at differential abundances in steers with different feed efficiencies. Surprisingly, for some bacterial families this holds true throughout the production continuum of beef steers, even when major diet changes occur. Abundances of *Ruminococcaceae*, *Rikenellaceae*, and *Christensenellaceae* were numerically greater in the feces of the efficient steers from weaning until slaughter. Conversely, *Bifidobacteriaceae* and *Lactobacillaceae* were more abundant in the feces of inefficient steers at multiple stages of their lives, suggesting a potential negative impact on feed efficiency. Moreover, microbial diversity in the hindgut was strongly correlated with feedlot RFI, and it was consistently highest in the most efficient steers. Collectively, our results illustrate that the ruminants' intestinal microbiota

can significantly impact feed efficiency, and this microbiome divergence can be detected as early as weaning.

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Table 3.1. Performance of the efficient and inefficient steers (n = 7 efficient and n = 8 inefficient) at different points in the beef production continuum**.**

Item	Inefficient	Efficient	SEM	*P*-value
Birthweight, kg	38.4	37.2	1.3	0.52
Weaning (9 mo) weight, kg	300	298	10.5	0.90
Yearling weight (13 mo), kg	501	487	14.4	0.51
Feedlot final body weight (18 mo), kg	599	584	15.6	0.51
Feedlot dry matter intake, kg/d	13.6	9.7	0.87	<0.001
Feedlot feed:gain ratio, kg	15.84	10.54	1.32	0.001
Feedlot Residual Feed Intake (RFI), kg/d	2.02	-2.02	0.82	<0.001

Table 3.2. Alpha-diversity indices calculated for efficient and inefficient steers at different stages of production: weaning, yearling, and slaughter.

	Inefficient Steers				
Index	**Weaning**	**Yearling**	**Slaughter**	**SEM**	***P*-value**
Chao1	4360.6[a]	3956.9[ab]	3384.7[b]	206	0.02
Species evenness	0.788[a]	0.687[b]	0.658[b]	0.009	<0.001
Shannon diversity	8.73[a]	7.42[b]	6.99[b]	0.14	<0.001
	Efficient Steers				
	Weaning	**Yearling**	**Slaughter**	**SEM**	***P*-value**
Chao1	4424.8	3621.4	3834.8	303	0.19
Species evenness	0.804[a]	0.710[b]	0.724[b]	0.006	<0.001
Shannon diversity	8.95[a]	7.61[b]	7.81[b]	0.11	<0.001

[ab] Values not sharing a common superscript within each row significantly differ ($P \leq 0.05$).

Table 3.3. Volatile fatty acid (VFA) concentration (m*M*) in the feces of efficient and inefficient steers at different stages of production: weaning, yearling, and slaughter.

	Inefficient Steers				
	Weaning	**Yearling**	**Slaughter**	**SEM**	***P*-value***
Acetate	39.9[b]	29.6[b]	55.9[a]	4.08	0.002
Propionate	6.2[b]	6.4[b]	16.0[a]	1.05	<0.001
Butyrate	2.5[b]	4.0[b]	7.6[a]	0.87	0.003
Valerate	0.70[ab]	0.40[b]	1.09[a]	0.18	0.049
Total VFA	51.0[b]	40.4[b]	82.6[a]	5.37	<0.001
Acetate:Propionate	6.5[a]	4.6[b]	3.6[c]	0.23	<0.001
	Efficient Steers				
	Weaning	**Yearling**	**Slaughter**	**SEM**	***P*-value***
Acetate	33.0[ab]	30.6[b]	43.8[a]	2.95	0.019
Propionate	6.0[b]	5.9[b]	14.1[a]	0.82	<0.001
Butyrate	1.8[b]	3.4[b]	6.5[a]	0.61	<0.001
Valerate	0.5[b]	0.3[b]	1.4[a]	0.10	<0.001
Total VFA	42.7[b]	40.2[b]	68.7[a]	3.54	<0.001
Acetate:Propionate	6.3[a]	5.2[b]	3.2[c]	0.28	<0.001

[*] *P*-value for the repeated measures ANOVA using collection time as a factor.
[abc] Values not sharing a common superscript within each row significantly differ according to Tukey's pairwise comparison ($P \leq 0.05$).

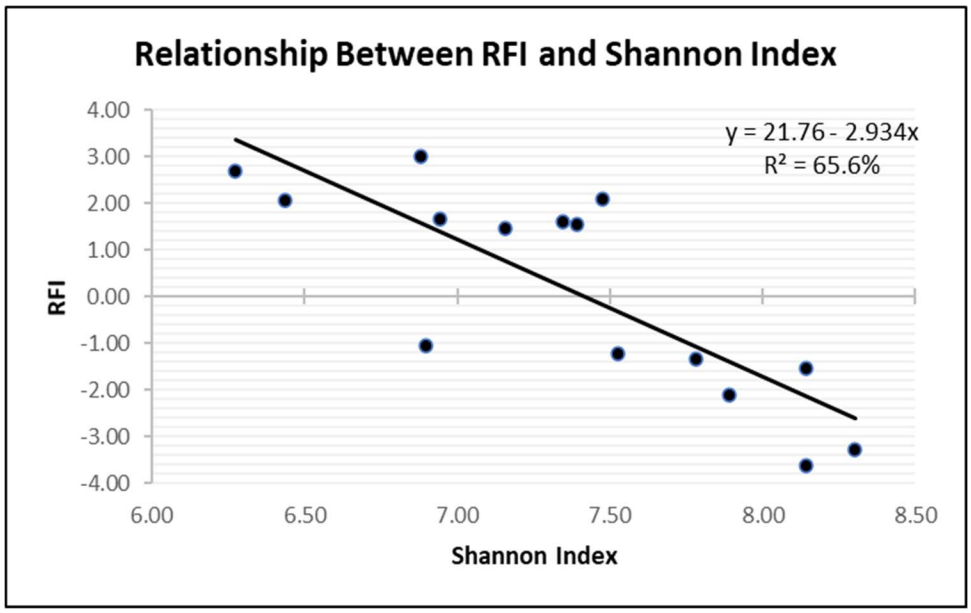

Figure 3.1. Linear regression expressing the relationship between residual feed intake (RFI) and Shannon diversity in the feces of Angus steers (n=15) observed at slaughter.

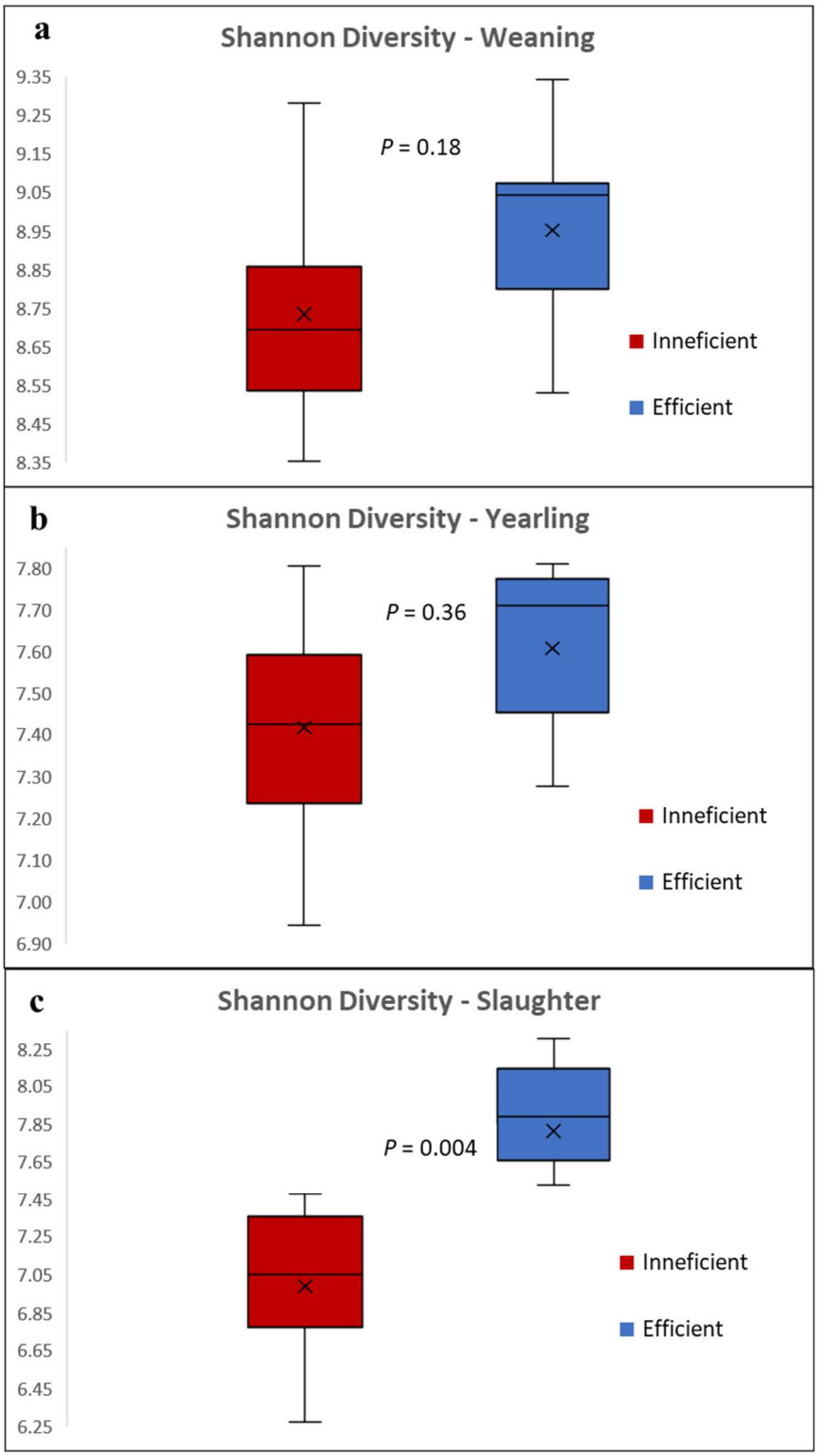

Figure. 3.2. Shannon diversity index at weaning (**a**), yearling (**b**), and slaughter (**c**) of steers (n=15) differing in feed efficiency. **P*-value indicates the significance of contrast between efficient and inefficient steers.

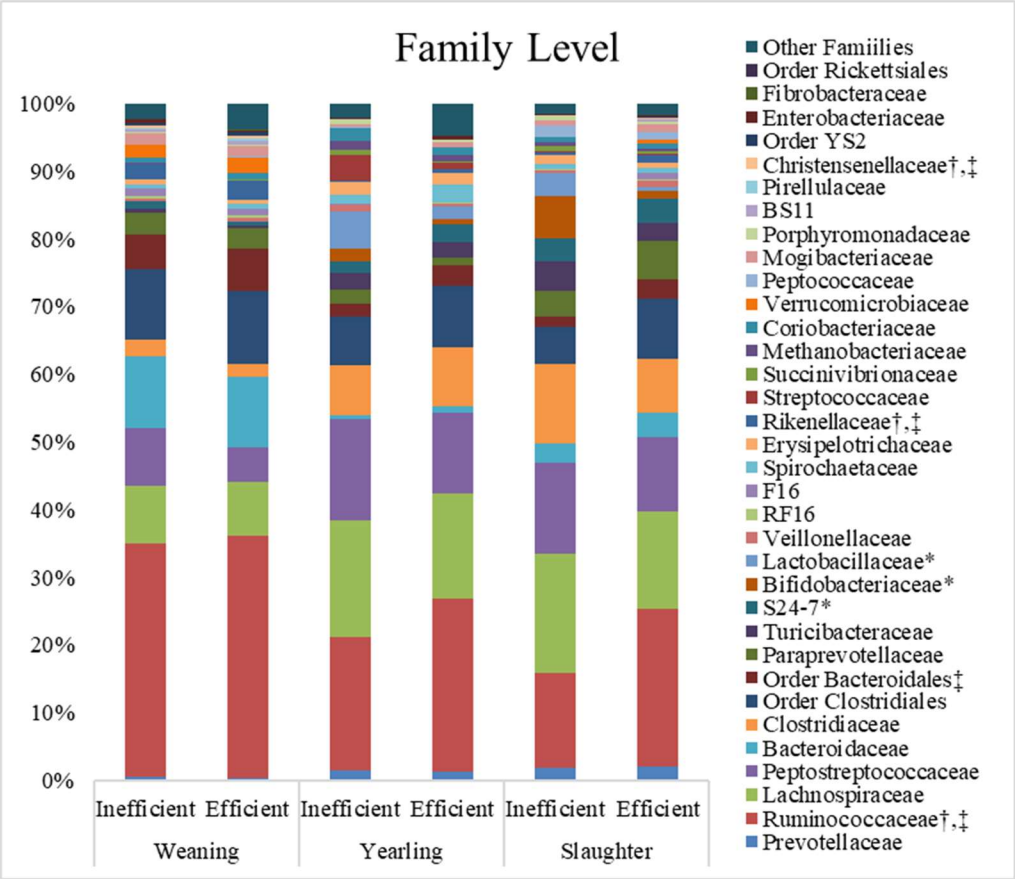

Figure 3.3. Relative bacterial abundance at the family level found in the feces of efficient and inefficient steers at weaning, yearling, and slaughter. Differences (P< 0.05) between the two groups of steers are denoted by * at weaning, † at yearling, and ‡ at slaughter ($P \leq 0.05$).

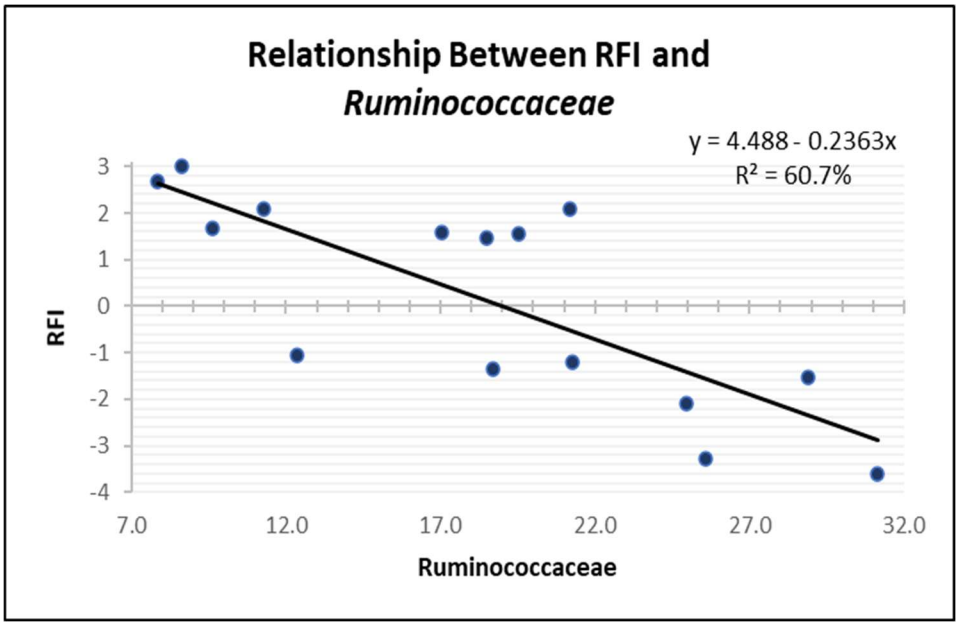

Figure 3.4. Linear regression expressing the relationship between RFI and abundance of *Ruminococcaceae* in the feces of Angus steers (n=15) at slaughter.

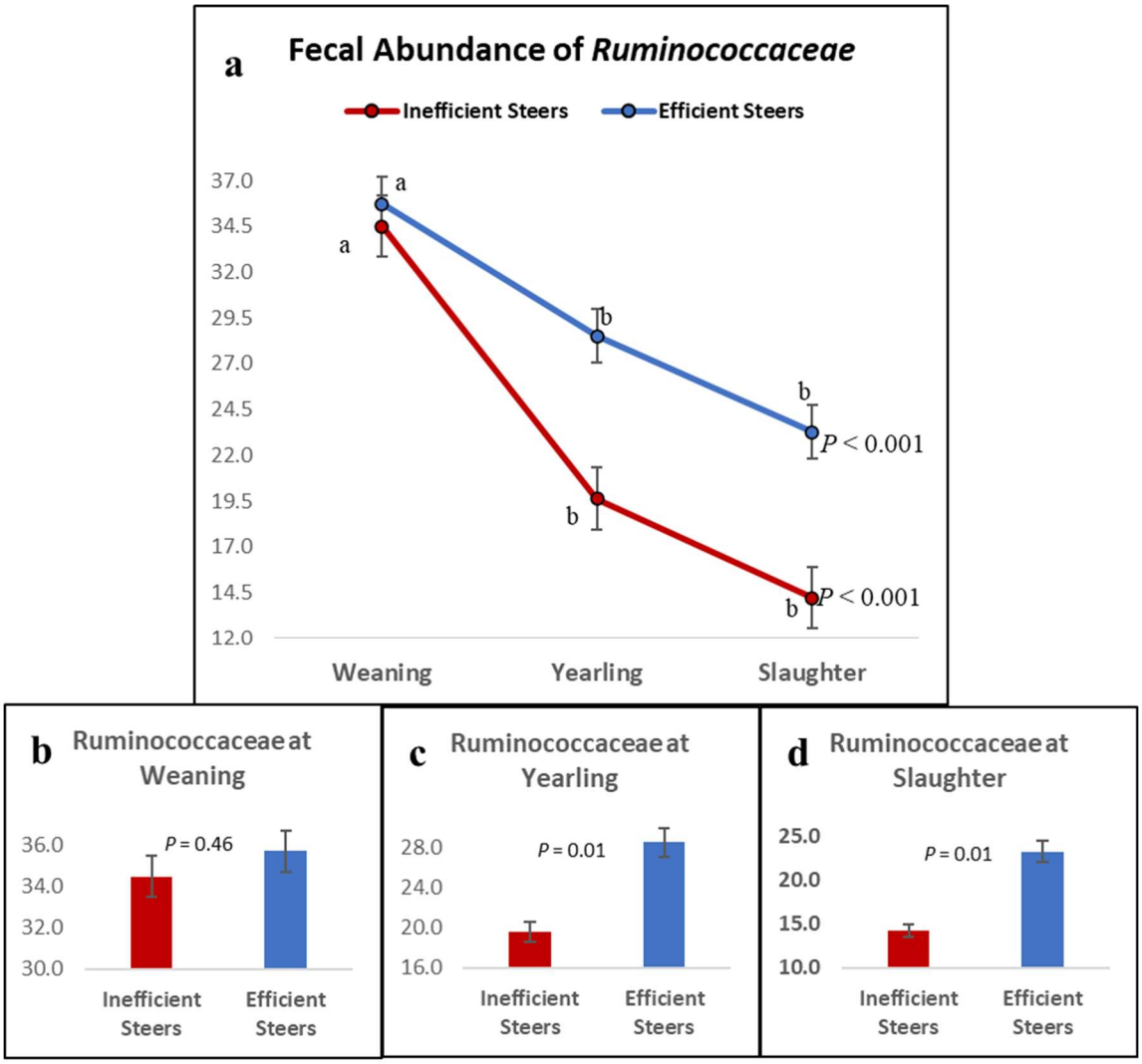

Figure 3.5. Fecal abundance of *Ruminococcaceae* throughout the beef production continuum (**a**), at weaning (**b**), at yearling (**c**), and at slaughter (**d**) of inefficient (n=8) and efficient (n=7) steers. *P*-values indicate differences (P<0.05) between efficiency groups. Error bars indicate standard error. In panel (**a**) [a,b] indicate a significant difference for the different timepoints ($P \leq 0.05$).

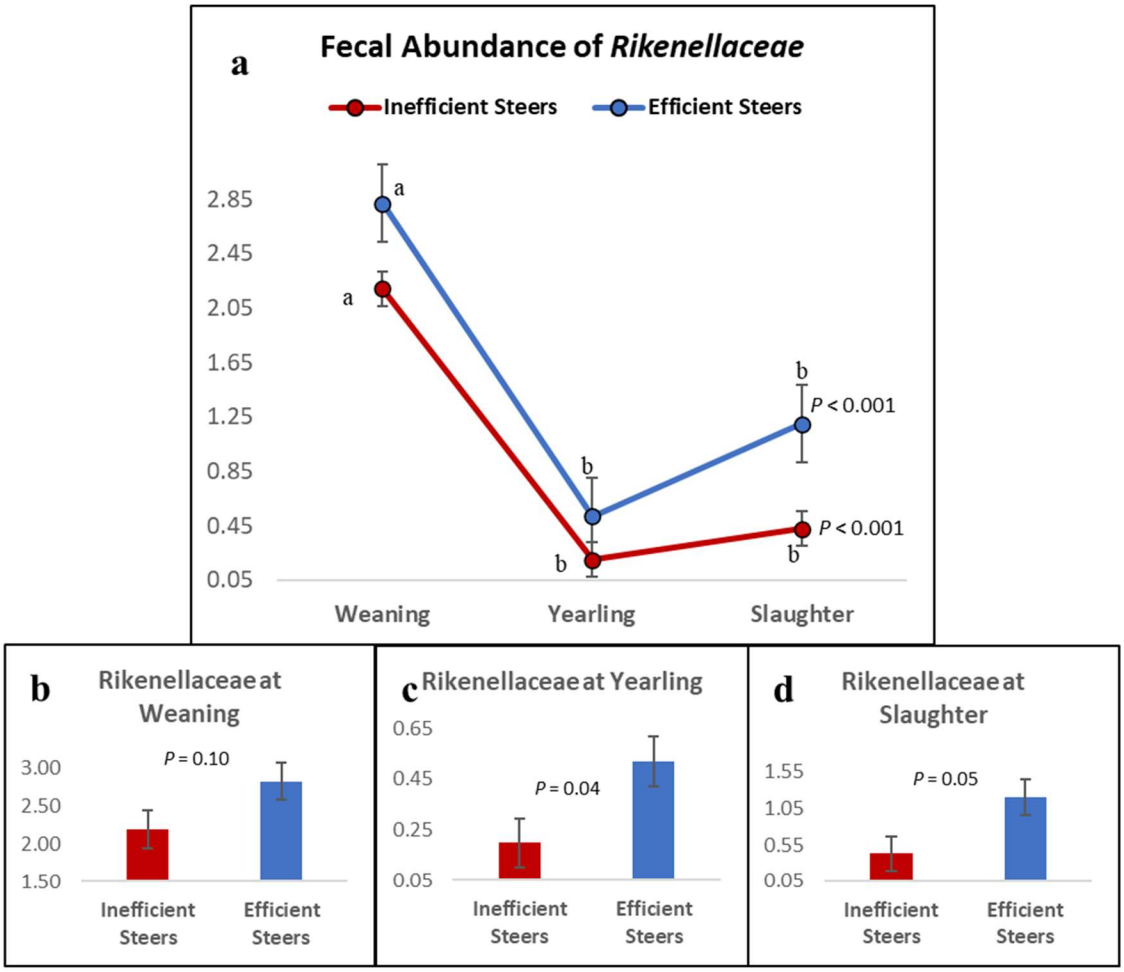

Figure 3.6. Fecal abundance of *Rikenellaceae* throughout the production continuum (**a**), at weaning (**b**), at yearling (**c**), and at floor (**d**) of inefficient (n=8) and efficient (n=7) steers. *P*-values indicate the significance of the difference between efficiency groups. Error bars indicate standard error. In panel (**a**) [a,b] indicate a significant difference for the different timepoints ($P \leq 0.05$).

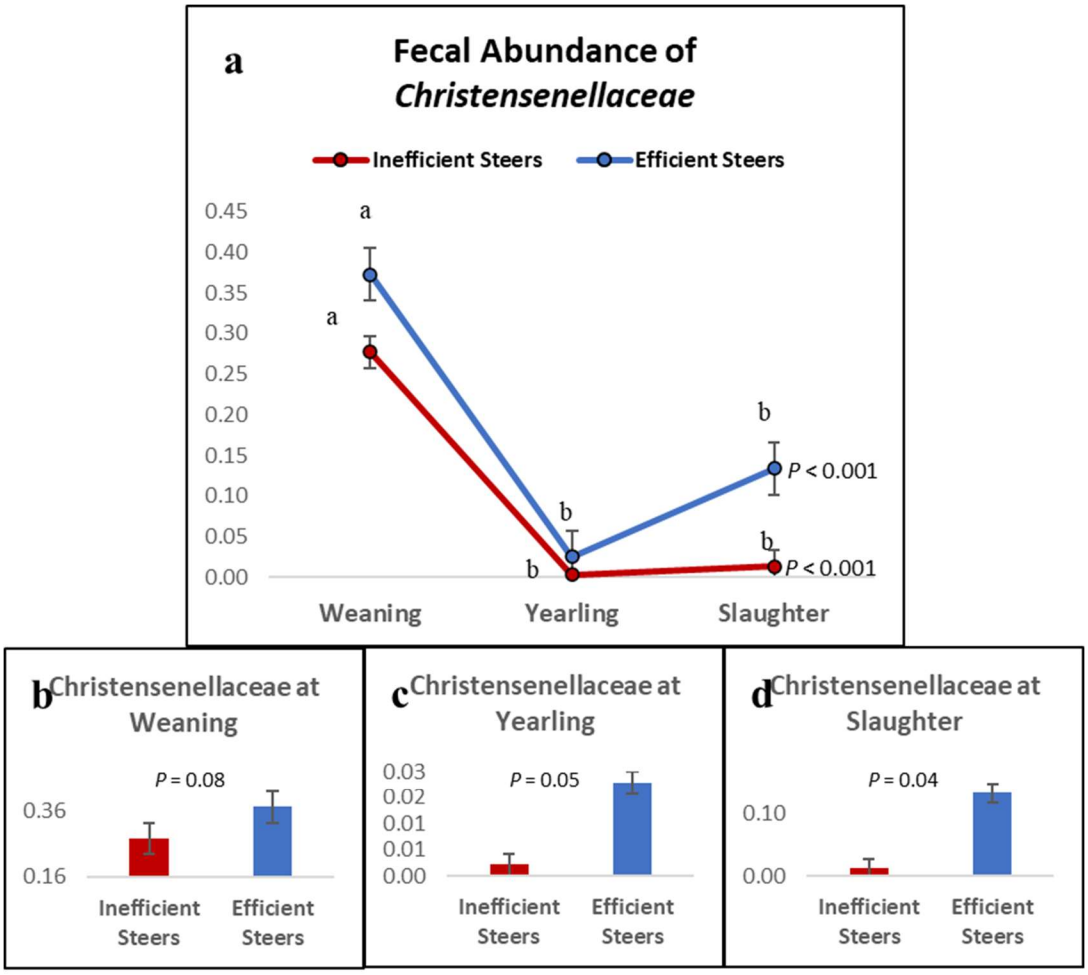

Figure 3.7. Fecal abundance of *Christensenellaceae* throughout the production continuum (**a**), at weaning (**b**), at yearling (**c**), and at slaughter (**d**) of inefficient (n=8) and efficient (n=7) steers. *P*-values indicate the significance of the difference between efficiency groups. Error bars indicate standard error. In panel (**a**) [a,b] indicate a significant difference for the different timepoints ($P \leq 0.05$).

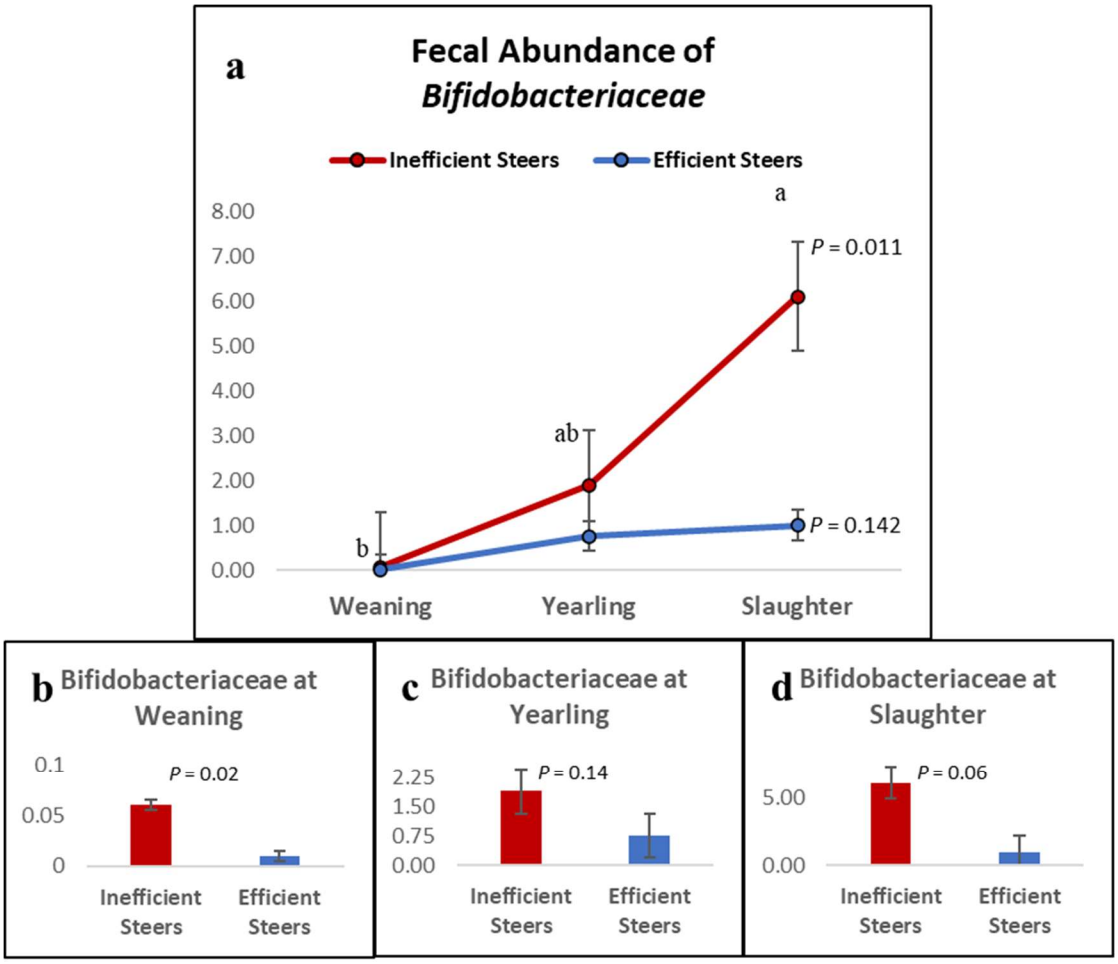

Figure 3.8. Fecal abundance of *Bifidobacteriaceae* throughout the beef production continuum (**a**), at weaning (**b**), at yearling (**c**), and at slaughter (**d**) of inefficient (n=8) and efficient (n=7) steers. *P*-values indicate the significance of the difference between efficiency groups. Error bars indicate standard error. In panel (**a**) [a,b]

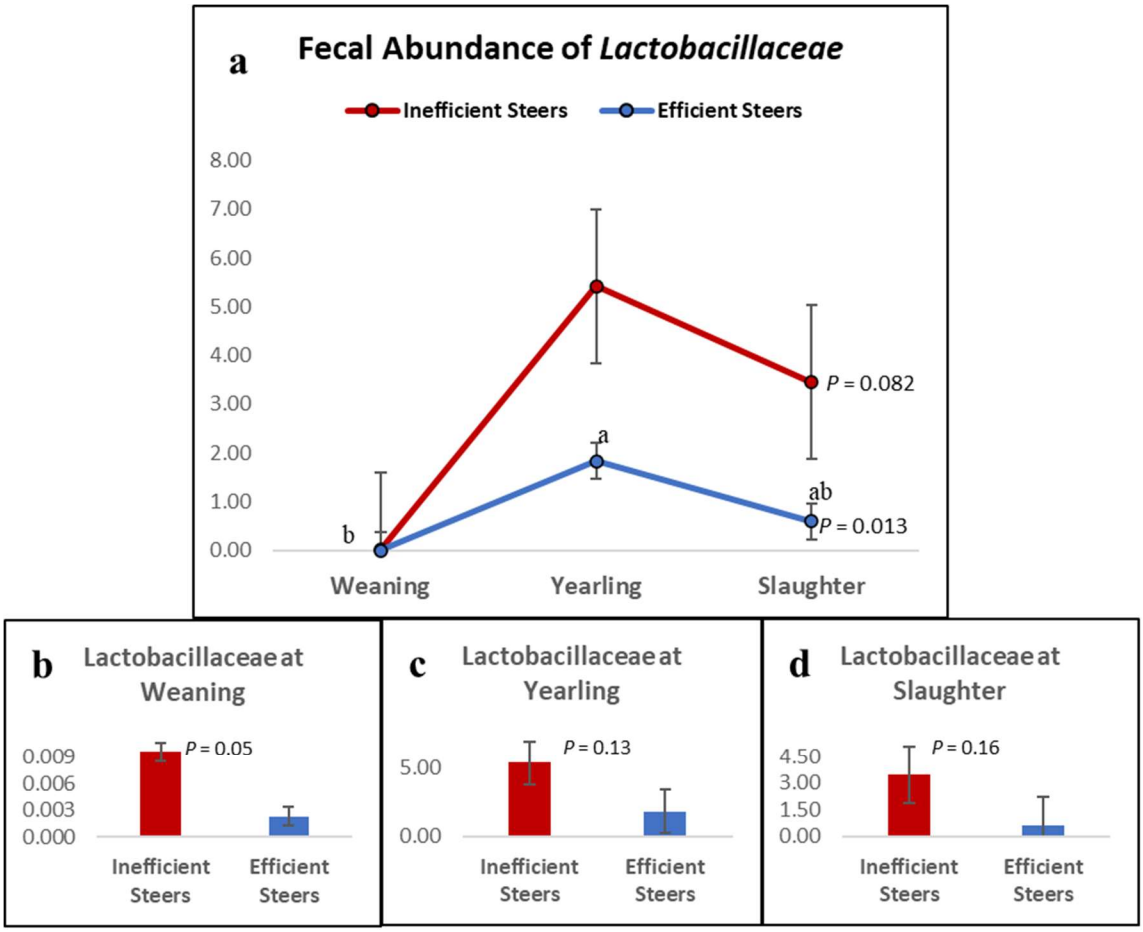

Figure 3.9. Fecal abundance of *Lactobacillaceae* throughout the beef production continuum (**a**), at weaning (**b**), at yearling (**c**), and at slaughter (**d**) of inefficient (n=8) and efficient (n=7) steers. *P*-values indicate the significance of the difference between efficiency groups. Error bars indicate standard error. In panel (**a**) ^{a,b} indicate a significant difference for the different timepoints ($P \leq 0.05$).

CHAPTER 4

THE IMPACT OF PRE-SLAUGHTER FASTING ON THE RUMINAL MICROBIAL POPULATION OF COMMERCIAL ANGUS STEERS[1]

[1]Welch, C. B., J. M. Lourenco, T. R. Krause, D. S. Seidel, T. D. Pringle, T. R. Callaway. To be submitted to *Journal of Animal Science.*

Abstract

Foodborne illnesses are a nationwide concern resulting in an estimated 9.4 million illnesses every year and $77 million in total costs incurred. In order to decrease outbreaks associated with these pathogens, they must be prevented from entering the food chain. One opportunity these pathogens have of entering the food chain is by increasing their populations in the gastrointestinal tract of cattle following pre-slaughter fasting. The objective of this study was to determine the impact of pre-slaughter fasting on the ruminal microbial consortium to determine if fluctuations indicate that dysbiosis occurred. Fifteen commercial Angus steers (n=15) divergent in residual feed intake (RFI) were utilized during this study. Rumen samples were collected prior to pre-slaughter fasting and post-slaughter. DNA extraction and 16S rRNA gene sequencing were performed to determine the ruminal microbiota. Paired t-tests were performed for all alpha-diversity, volatile fatty acid, and bacterial analyses. Bacterial richness (Chao 1), evenness, and the Shannon diversity index were all increased after fasting regardless of feed efficiency ($P \leq 0.04$). The two predominant families *Prevotellaceae* and *Ruminococcaceae* were decreased ($P \leq 0.029$) as a result of fasting, whereas, the remaining minor families increased ($P < 0.001$) in relative abundance. This was further highlighted at the genus-level, where *Blautia* and *Methanosphaera* increased ($P \leq 0.003$); and the well-known pathogens *Campylobacter* and *Treponema* tended to increase ($P \leq 0.075$) following fasting. These findings, combined with the trend for butyrate production to decrease ($P = 0.068$) as a result of pre-slaughter fasting, corroborates the idea that fasting causes deleterious effects to the rumen. As a result of fasting, nutrient depletion can result in dysbiosis which can allow opportunistic pathogens to fill the void in the ruminal niche left by the decreased core microbiome.

Key words: microbiome, rumen, steer, fasting, dysbiosis, slaughter, *Blautia*, *Methanosphaera*, *Campylobacter*, *Treponema*

Introduction

Foodborne illnesses are a concern both nationwide and globally. The Center for Disease Control (CDC) reported that 31 pathogens known to cause foodborne illness were responsible for 9.4 million illnesses, 55,961 hospitalizations, and 1,351 deaths in the United States each year (Scallan et al., 2011). With such high incidence of food-related illnesses, there is a major economic impact associated with not only medical costs, but also through industry cost associated with recalls. There is an estimated $77 million in cost incurred from direct or indirect costs annually in the United States (Scharff, 2012).

One of the ways pathogens enter the food chain is during the slaughter process, with contamination from nicked gastrointestinal tracts or from hides (Bosilevac et al., 2004, 2005; Koomaraie et al., 2005). Cattle are natural reservoirs of *Escherichia coli* O157: H7, a strain of *E. coli* causing hemorrhagic colitis in humans (Callaway et al., 2003). Many studies have found that when cattle are fasted prior to slaughter, the growth of many foodborne pathogens including *Salmonella* and *E. coli* within the rumen and feces of cattle are increased (Brownlie and Grau, 1967; Rasmussen et al., 1993; Pointon et al., 2012). As ruminants fast, their gastrointestinal tract (GIT) microbiome uses up the nutrients available. This may cause their core microbial population to decrease leading to dysbiosis, allowing opportunistic pathogens to colonize their GIT. The objective of this study was to evaluate the ruminal bacterial abundance fluctuations occurring as a result of pre-slaughter fasting, to determine if this fluctuation can result in ruminal dysbiosis in cattle.

Materials and Methods

Animals selection and handling

The steers involved in this research were handled using the guidelines approved by the University of Georgia's Animal Care and Use Committee (AUP #A2012 11-006-R1). They were reared on a pasture-based system for their first year at the Northwest Georgia Research and Education Center, located in Calhoun, GA (34° 30 N, 84° 57 W). After being weaned and backgrounded, a total of 63 steers were transported to a commercial feedlot located in Brasstown, NC (35° 10 N, 83° 23 W). The animals were then transitioned to a feedlot-finishing ration using a step-up approach for 3 weeks. After that, the steers were fed a finishing diet for 110 days. The finishing diet provided 14.51% crude protein, 2.10 Mcal/kg NEm, 1.43 Mcal/kg NEg, 0.70% Ca, and 0.45% P.

During the feedlot-finishing period, the steers were fed using a GrowSafe System (GrowSafe Systems Ltd., Calgary, Canada) which measured the individual feed intake of each steer. This data, along with animal performance information, allowed calculation of the residual feed intake (RFI) for those steers. The steers most divergent in terms of RFI status (n=17) were utilized during this study. Of this group, 8 were considered less-efficient (high-RFI) and 9 were assigned to the more-efficient group (low-RFI). These animals were transported to the University of Georgia Meat Science Technology Center, a federally inspected meat plant located in Athens, GA (33° 57 N, 83° 22 W). The steers were housed on site overnight with *ad libitum* access to water before humane slaughter the next morning.

Rumen content collection and storage

Upon arrival at the slaughter facility, rumen fluid was individually collected from all steers following the procedures described in Lourenco et al. (2019). Briefly, this

procedure uses esophageal tubing and a perforated metal probe, which is attached to an electric vacuum pump for collection of the ruminal contents. Approximately 350 mL of ruminal contents were collected from each animal. Immediately after, a subsample of 45 mL was transferred to a sterile conical tube, and the tubes were stored in a -20℃ freezer. In the following morning, after slaughter and evisceration of the carcass, the rumen was cut into using a flame-sterilized scalpel. Approximately 45 mL of contents were aseptically removed from the rumen and placed into sterile conical tubes, and the tubes were immediately taken to a -20℃ freezer for long term storage.

DNA extraction and sequencing

Bacterial DNA extraction was performed on the ruminal samples using a hybrid protocol described by Rothrock et al. (2014) utilizing mechanical and enzymatic means of extraction to optimize results. Briefly, this procedure used either 330 µL of rumen fluid (pre-lairage) or 0.33 g of rumen contents (post-lairage) placed in 2 mL Lysing Matrix E tubes (MP Biomedicals LLC, Irvine, CA, USA). The tubes were homogenized using a FastPrep 24 Instrument (MP Biomedical LLC, Irvine, CA, USA) in order to mechanically disrupt the cells. Enzymatic extraction occurred with the use of InhibitEX Tablets (QIAGEN, Venlo, Netherlands). DNA from each sample was eluted and purified using an automated robotic workstation (QIAcube; QIAGEN, Venlo, Netherlands). After extraction was completed, the concentration and purity of the DNA within each sample was measured spectrophotometrically using the Synergy H4 Hybrid Multi-Mode Microplate Reader along with the Take3 Micro-Volume Plate (BioTek Instruments Inc; Winooski, VT, USA). In order to have sequencing performed, each sample had to meet a minimum requirement of 20 µL of volume present with a DNA concentration of at least 10 ng/µL. Samples failing

to meet these minimum requirements were discarded and a new DNA extraction cycle was performed. Prior to sequencing, samples were stored at 4°C overnight.

16S rRNA gene sequencing was performed at the Georgia Genomics and Bioinformatics Core (https://dna.uga.edu). The library was prepared using PCR replications with the forward primer: S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and the reverse primer: S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013). An Illumina MiSeq v3 2 x 300 base pairs kit (Illumina Inc., San Diego, CA, USA) was utilized for the 16S rRNA gene sequencing.

At the completion of sequencing, the data was demultiplexed and converted to FASTQ files. BBMerge Paired Read Merger v37.64 was used to set and merge pair-end reads. The files were then analyzed using QIIME pipeline v1.9.1 (Caporaso et al., 2010). The data files were quality filtered and merged into a single file and converted to the FASTA format. Sequences were grouped into operational taxonomic units (OTU) at 97% similarity using the Greengenes database (gg_13_8_otus). Singletons were excluded from the analysis. The sequence depth was set at 17,542 sequences per sample for further analysis.

Volatile fatty acid analysis

For the rumen fluid samples collected when the steers arrived at the slaughter facility, the samples were thawed and vortexed for 30 seconds to produce homogenized samples. Then, 1.5 mL of each sample was pipetted into a centrifuge tube. For the rumen contents collected on the kill floor, the tubes were thawed, and 1 g of rumen contents were diluted with 3 mL of distilled water and placed into 15 mL conical tubes. The conical tubes

were vortexed for 30 seconds to homogenize the samples, and 1.5 mL of the mixture from each tube was transferred into new centrifuge tubes. The tubes were centrifuged at 10,000 x *g* for 10 minutes. For each sample, 1 mL supernatant was then transferred to a new centrifuge tube and combined with 200 µL of a metaphosphoric acid solution (25% w/v). Each sample was vortexed for 30 seconds to ensure proper mixture and then stored at -20°C overnight. The following morning, samples were thawed and centrifuged at 10,000 x *g* for 10 minutes**.** The supernatant was then placed into polypropylene tubes with ethyl acetate in a 2:1 ratio of ethyl acetate to supernatant. The samples were vortexed for 10 seconds and allowed to settle for 5 minutes to optimize separation. Next, 600 µL of the top layer was transferred into screw-thread vials for analysis of the volatile fatty acid (VFA) concentrations. A Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan) with a flame ionization detector and a capillary column (Zebron ZB-FFAP; 30 m x 0.32 mm x 0.25 µm; Phenomenex Inx., Torrance, CA, USA) was used for VFA analysis. This equipment utilized helium as the carrier gas. Sample injection volume was 10 µL. The column temperature started at 110°C and gradually increased to 200°C. The injector and detector temperatures were set to 250°C and 350°C, respectively.

Statistical Analysis

Statistical analysis was performed using Minitab v.19.1. Differences in the alpha diversity indexes and bacterial relative abundances upon arrival at the slaughter facility and at the kill floor were calculated using paired t-tests. Results were considered significant at $P \leq 0.05$ and treated as trends when $0.05 < P \leq 0.10$.

Results and Discussion

Pre-slaughter fasting majorly impacted the overall alpha-diversities of the ruminal environment (Table 1). For the high-RFI steers, we observed an increase in bacterial richness (Chao 1; $P = 0.001$), evenness ($P = 0.040$), and diversity (Shannon index; $P = 0.001$) in the rumen samples taken from lairage to the ones collected at slaughter. Similarly, bacterial richness (Chao 1), evenness, and diversity (Shannon index) were higher ($P \leq 0.03$) in the rumen of low-RFI steers after slaughter, compared to lairage. Since the impact of pre-slaughter fasting displayed similar patterns in bacterial populations regardless of feed efficiency status, we combined the steers from both efficiency groups (n=15) for further analyses. These results highlighted the immense effect fasting had on the bacteria within the rumen. Normally with a constant flow of nutrients into the rumen, the bacteria best suited to the niche determined by the diet develop a competitive advantage, allowing them to overcome other bacteria. This claim has been shown in may studies finding that the bacteria inside the rumen fluctuates as a consequence of diet (Carberry et al., 2012; Petri et al., 2013; Thoetkiattikul et al., 2013). However, when the selection pressure applied to bacteria from the diet is removed, the competitive advantage provided with it is also removed. In the present study, we saw the effect of diet selection pressure removal in the rumen due to fasting, which resulted in the amount of bacterial species (richness) being increased, and how each species was more evenly distributed (evenness), ultimately increasing diversity in the rumen post-fasting.

Figure 1 shows bacterial relative abundance at the family-level of the rumen environment of steers before and after they fasted overnight. There were fluctuations in the number of bacteria present at the family level. In order to make more sense of the data,

Figure 2 shows all the bacterial families combined, except for the two most abundant families (*Prevotellaceae* and *Ruminococcaceae*). Before fasting occurred, the two most abundant families comprised 53.87% of the rumen abundance, leaving 46.13% of the relative abundance to be made up of more than thirty minor families. However, after fasting occurred, the abundance of *Prevotellaceae* decreased from 28.18% to 21.36% ($P = 0.029$), and *Ruminococcaceae* abundance decreased from 25.69% to 17.03% ($P = 0.002$) for a combined 38.39% of the rumen environment. Consequently, the bacterial abundance of the minor families was increased from 46.13% to 61.61% ($P < 0.001$) during the overnight pre-slaughter fasting period.

At the genus level, one bacterium that increased ($P = 0.003$) in abundance after fasting was *Blautia* (Figure 3a). Zeineldin et al. (2018) found this bacterial genus to be increased in the feces of feedlot cattle displaying hemorrhagic diarrhea, along with other pathogenic bacteria. This carbohydrate fermenting bacteria has been found throughout the GIT of young calves; however, its relative abundance was found to decrease as calves age (Dias et al., 2018). This bacteria was previously found in higher abundance in the rumen of cattle fed higher grain diet compared to forage diets (Kim et al., 2014), which may indicate that they have a chance to increase in abundance and scavenge available carbohydrates in the rumen when more numerous bacterial populations decrease.

Methanosphaera was increased ($P < 0.001$) in the rumen after fasting compared to before fasting (Figure 3b). Methanogens are generally thought to waste nutrients in the rumen by converting them into methane. However, *Methanosphaera* was found to be negatively correlated to methane emissions in sheep (Kittelmann et al., 2014). This particular methanogen has been found in greater abundance in inefficient steers compared

to efficient steers based on RFI (Zhou et al., 2009). Although *Methanosphaera* may not be producing methane, it may still be negatively affecting the host by wasting energy and contributing to host inefficiency.

Campylobacter is responsible for 9% of the foodborne illnesses in the United States and 15% of the hospitalizations occurring as a result of a foodborne pathogen (Scallan et al., 2011). It along with Non-typhoidal *Salmonella* are the main causes of foodborne illnesses in the United States, England and Wales, and Australia. It is estimated that the total economic impact of each case of *Campylobacter* is $1,846 including all direct and indirect costs (Scharff, 2012). This bacterial genus was found to be trending toward an increase ($P = 0.075$) in the rumen of steers after fasting compared to pre-fasting (Figure 3c). This indicates that this opportunistic pathogen can increase in abundance during pre-slaughter fasting, which increases its chances of entering the food chain and cause detrimental economic impacts as a result of foodborne illnesses.

The genus *Treponema* trended toward an increase ($P = 0.086$) in abundance after pre-slaughter fasting compared to before fasting (Figure 3d). Tajima et al. (2001) found that the concentration of *Treponema bryantii* in the rumen decreased when the diet was changed from forage to grain. This bacterial group has been shown to interact with cellulolytic bacteria in the rumen (Kudo et al., 1987); however, this genus also has many pathogenic bacteria. Many studies have found multiple species of *Treponema* in digital dermatitis in dairy cows (Trott et al., 2003; Döpfer et al., 2012). In addition, it has been shown to be closely associated with ovine digital dermatitis bacteria (Sullivan et al., 2015b). Sullivan et al. (2015a) found the gastrointestinal tract to be a reservoir for

pathogenic *Treponema* species in both beef cattle and sheep. Therefore, the *Treponema* could potentially act as an opportunistic pathogen.

Further corroborating our claims that fasting decreased beneficial bacteria and increased pathogenic species is the potential amount of energy being produced before and after fasting (Table 2). We found acetate, propionate, isobutyrate, isovalerate, valerate, and total VFA production to remain consistent ($P > 0.29$) before and after fasting. Conversely, butyrate production was trending toward a significant decrease ($P = 0.07$) from lairage to slaughter. This data suggests that although there was not a constant flow of nutrients, the bacteria present were scavenging nutrients and continuing to produce energy for the host animal. These findings are not in alignment with some with previous researchers, which found VFA concentration in the rumen to decrease with prolonged fasting (Stewart et al., 1958; Bryant, 1964). In the present study, the only VFA trending toward a decrease after fasting was butyrate. This VFA is known as the "healthy gut" VFA because once produced in the rumen, the ruminal epithelium utilizes it as an energy source to keep the gut healthy and functioning properly (Guan et al., 2008). The decrease shown in butyrate could signify that the minor families which increased in abundance after fasting are not as beneficial to the host animal as the major families found in higher abundance prior to pre-slaughter fasting.

Collectively, our results indicate that as steers were fasted prior to slaughter, their ruminal microbial consortium experienced major fluctuations. The total number of bacteria present increased, along with the bacterial species becoming more evenly distributed, resulting in an increase in the overall diversity of the microbiota. This shift was partially explained by the two most abundance bacterial families (*Prevotellaceae* and

Ruminococcaceae), which decreased after the period of fasting and created niches for other opportunistic bacteria. Furthermore, the minor families found to be less numerous before fasting increased in abundance after fasting. Finally, many bacterial genera found to have deleterious effects in cattle and humans, were found to increase after fasting, including *Campylobacter* and *Treponema*. This suggests that when the selection pressure applied to the ruminal bacteria by the diet is removed, the microbial consortium becomes unbalanced. The dysbiosis that results gives pathogens an opportunity to thrive in the rumen and ultimately enter the human food chain.

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Table 4.1. Ruminal alpha-diversity of high-RFI (n=8) and low-RFI (n=9) steers observed at lairage and kill floor (after fasting overnight).

Index	High-RFI Steers			
	Lairage	**Slaughter**	**SEM**	***P*-value**
Chao 1	2,791	3,786	177	**0.001**
Shannon Index	8.18	8.59	0.072	**0.001**
Evenness	0.768	0.786	0.007	**0.04**
	Low-RFI Steers			
	Lairage	**Slaughter**	**SEM**	***P*-value**
Chao 1	2,499	3,051	204	**0.027**
Shannon Index	7.92	8.45	0.161	**0.011**
Evenness	0.753	0.788	0.014	**0.03**

Table 4.2. Ruminal volatile fatty acid production (m*M*) of commercial Angus steers (n=15) at lairage and kill floor (after fasting overnight).

Volatile fatty acid	Lairage	Slaughter	SEM	*P*-value
Acetate	62.50	62.73	6.740	0.97
Propionate	26.13	22.50	4.520	0.43
Isobutyrate	1.29	1.27	0.081	0.88
Butyrate	14.06	10.71	1.710	0.07
Isovalerate	3.27	3.70	0.397	0.30
Valerate	1.71	1.76	0.467	0.91
Total volatile fatty acid	109.21	103.56	13.100	0.67
Acetate:Propionate	2.63	3.18	0.332	0.12

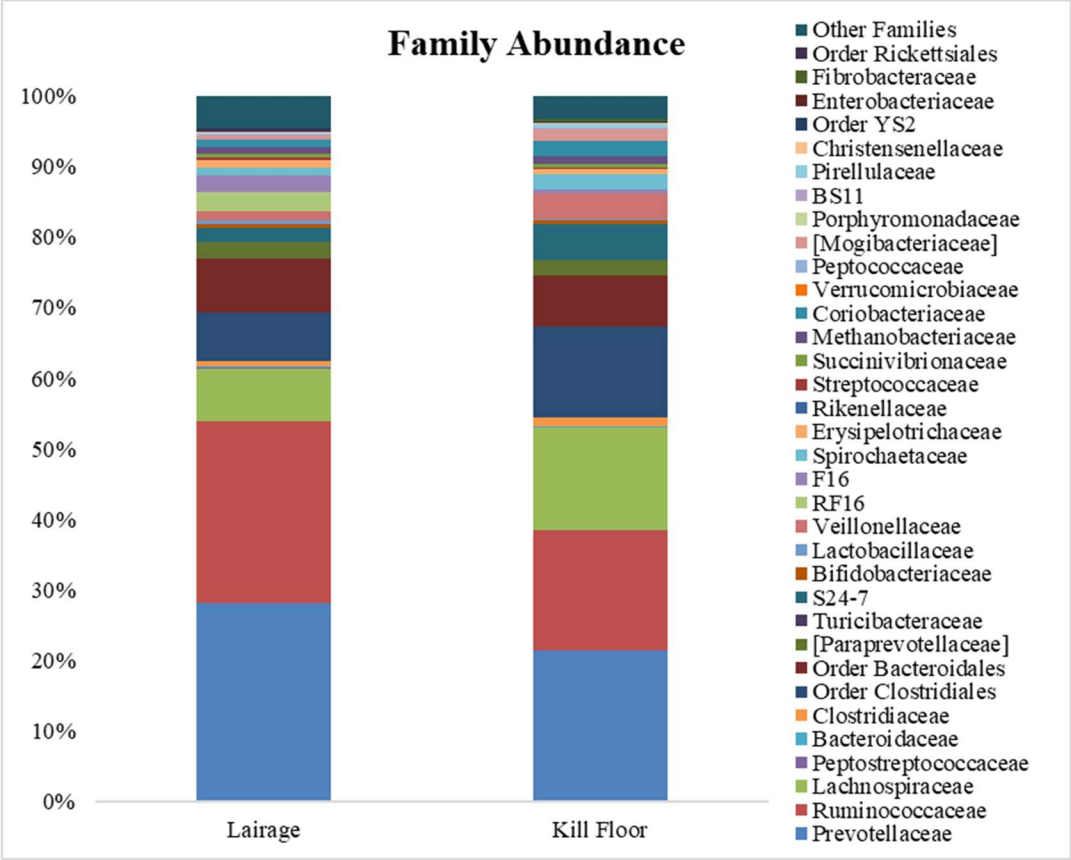

Fig. 4.1. Relative bacterial abundance in the rumen of Angus steers (n=15) observed at lairage and kill floor (after fasting overnight).

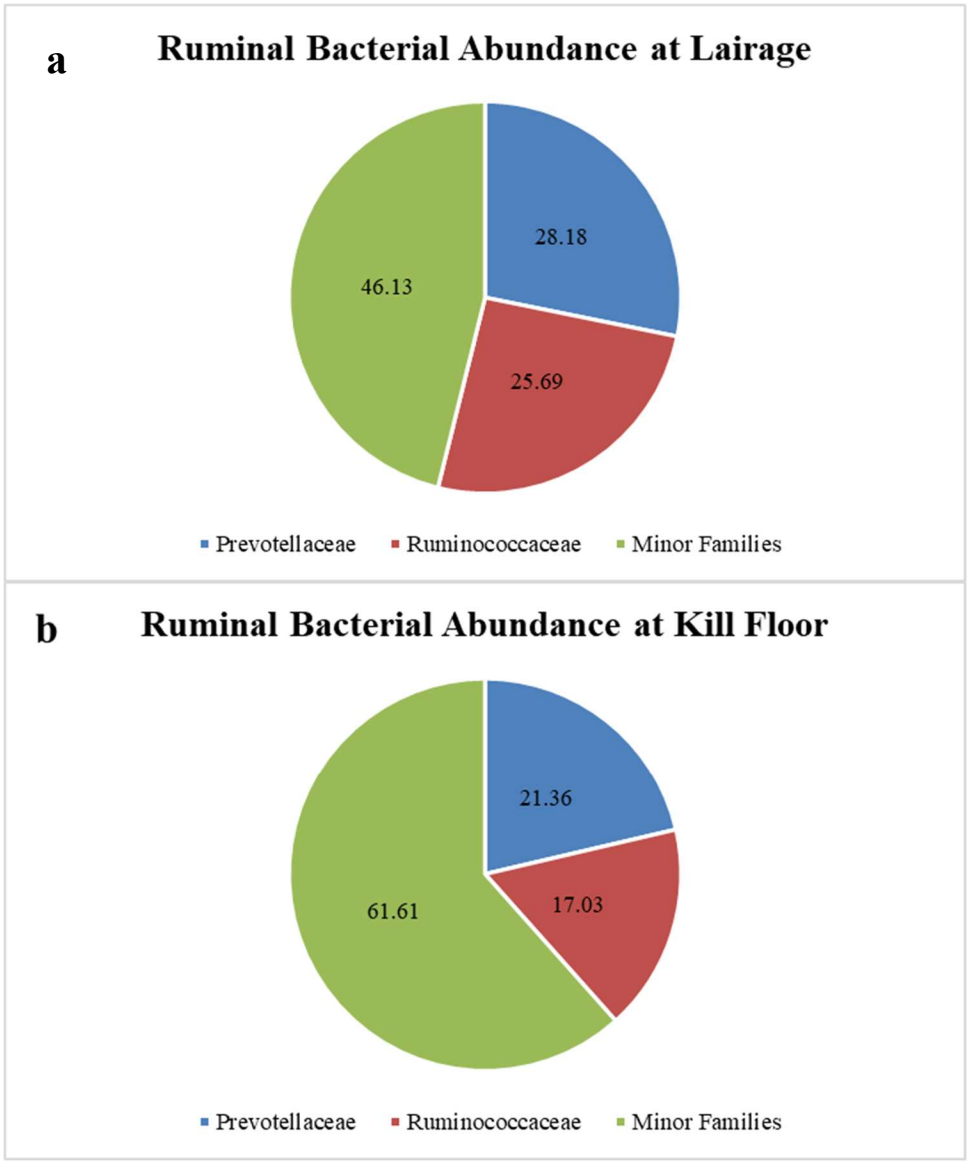

Fig. 4.2. Relative bacterial abundance in the rumen of Angus steers (n=15) observed at lairage (**a**) and kill floor (**b**) (after fasting overnight). Family abundance was different after fasting ($P \leq 0.03$).

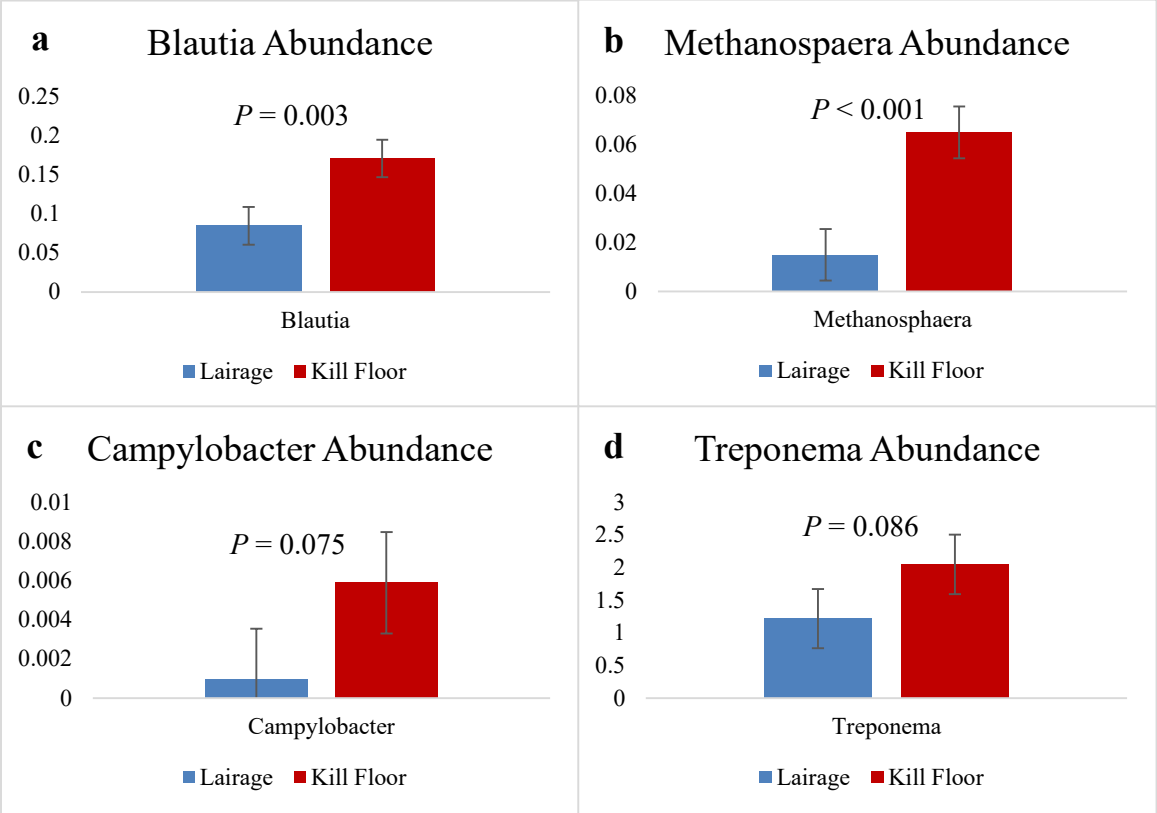

Fig. 4.3. Relative bacterial abundance of (**a**) *Blautia*, (**b**) *Methanosphaera,* (**c**) *Campylobacter*, and (**d**) *Treponema* in the rumen of Angus steers (n=15) before (lairage) and after (kill floor) pre-slaughter fasting. Error bars indicate the standard error of the mean. *P*-value indicates the difference between bacterial abundance from lairage to kill floor.

CHAPTER 5

THE IMPACT OF FEED EFFICIENCY SELECTION ON THE RUMINAL, CECAL, AND FECAL MICROBIOMES OF ANGUS STEERS FROM A COMMERCIAL FEEDLOT[1]

[1]Welch, C. B., J. M. Lourenco, D. B. Davis, T. R. Krause, M. N. Carmichael, M. J. Rothrock, T. D. Pringle, T. R. Callaway. Submitted to *Journal of Animal Science*.

Abstract

Feed costs are the greatest cost of animal production so reducing it is critical to increase producer profits. In ruminants, the microbial population within the gastrointestinal tract (GIT) are critical to nutrient digestion and absorption in both the rumen and the hindgut. The objective of this study was to determine the microbial taxonomic profile of the rumen, cecum, and feces of feedlot steers at slaughter in order to link feed efficiency and the GIT microbiota from these 3 locations. Twenty (n = 20) commercial Angus steers were selected and divided into two groups according to their residual feed intake (RFI) classification determined during the feedlot-finishing period: high-RFI (n=10) and low-RFI (n=10). After the ruminal, cecal, and fecal samples were collected at slaughter, DNA extraction and 16S rRNA gene sequencing were performed to determine their microbial composition. One-way ANOVA was performed on the microbial traits using RFI classification as the fixed effect. Overall, the ruminal microbiome was the most different in terms of taxonomic profile compared to the cecal and fecal as revealed by beta diversity analysis (P<0.001). Moreover, bacterial richness (Chao1) was greatest (P=0.01) in the rumen of the high-RFI group compared to the more efficient, low-RFI group. In contrast, bacterial richness and diversity in the intestinal environment showed that Chao1 was greater (P=0.01) in the cecum, and Shannon diversity index was greater in both the cecum and feces of low-RFI compared to high-RFI steers (P=0.01 and P<0.001, respectively). *Ruminococcaceae* was more abundant in the low-RFI group in the cecum and feces (P=0.01); and fecal *Bifidobacteriaceae* was more abundant in high-RFI steers (P=0.03). No correlations (P≥0.07) between any ruminal bacterial family and RFI were detected; however, *Ruminococcaeae*, *Mogibacteriaceae*, *Christensenellaceae,* and BS11 were

negatively correlated with RFI (P<0.05) in the cecum and feces. *Succinivibrionaceae* in the cecum was positively correlated with RFI (P=0.05), and fecal *Bifidobacteriaceae* was positively correlated with RFI (P=0.03). Results collectively indicate that in addition to the ruminal fermentation, the lower gut fermentation has a significant impact on feed efficiency and nutrient utilization in feedlot steers, therefore the intestinal microbiome should also be considered when examining the basis of ruminant feed efficiency.

Key words: 16S rRNA, feedlot cattle, gastrointestinal microbiota, residual feed intake, *Ruminococcaceae*

Introduction

Feed costs account for 60-75% of the input costs in most beef production systems (Herd et al., 2003), requiring beef cattle producers to focus attention on traits like feed efficiency, because profit margins are narrow (Schnepf, 2012). Beef cattle are often selected for breeding based upon improved feed intake and feed efficiency (Nkrumah et al., 2006; Arthur and Herd, 2008). Residual feed intake (RFI) is a measure of feed efficiency within a herd that is defined as actual feed intake minus predicted feed intake based on body size and growth (Herd et al., 2003; Nkrumah et al., 2006; Arthur and Herd, 2008), where a lower RFI value is more desirable for producers. Low-RFI cattle eat less than expected based on their body weight and growth rate and use feed more efficiently than high-RFI cattle. Additionally, selection for low-RFI cattle has led to animals that ate less feed per unit weight gain in a feedlot without negative impacts on growth or product yield, leading to the conclusion that low-RFI steers are more profitable than high-RFI steers (Herd et al., 2003).

Cattle obtain the majority of their metabolizable energy in the form of volatile fatty acids (VFA) produced by ruminal microbial fermentation (Perea et al., 2017). In the rumen of beef cattle, increased abundances of the phyla *Firmicutes* have been observed in feed efficient animals (i.e. animals with high average daily gain [ADG] and low daily feed intake) (Myer et al., 2017). In other studies, differences in the rumen microbial populations were correlated with RFI, with *Prevotella* populations in a greater abundance in the rumen of less efficient (high-RFI) cattle (McCann et al., 2014).

In recent years, it has become increasingly clear that the gastrointestinal microbial fermentation plays an important role in host body composition and physiology because the

resident microbiota determines the ability of the host to harvest energy from its diet (Ley et al., 2006; Turnbaugh and Gordon, 2009; Yeoman and White, 2014) The ratios of gastrointestinal bacterial populations in mice, particularly of the phyla *Firmicutes* and *Bacteroidetes,* have been correlated with differences in host body composition (Ley et al., 2005). Consequently, although most of the research involving beef cattle was performed in the rumen, some studies have focused on the lower gastrointestinal tract (GIT) and feces of ruminants in order to assess their impacts on feed efficiency (Myer et al., 2015; Myer et al., 2017; Perea et al., 2017).

The present study investigated the relationship between the microbiome of the rumen, cecum, and feces and host animal feed efficiency (in the form of RFI) in finishing beef cattle. We hypothesized that cattle with different RFI values would have distinct microbiota in some or all of those gastrointestinal compartments, potentially linking feed efficiency and microbiome composition throughout the GIT of feedlot beef steers.

Materials and Methods

Animals, diets, and steer selection

All animals in this study were cared for following guidelines approved by the University of Georgia's Animal Care and Use Committee (AUP #A2012 11-006-R1). Angus steers utilized in the study were calved and raised at the Northwest Georgia Research and Education Center, located in Calhoun, GA (34^{o} 30 N, 84^{o} 57 W). Steers utilized were the fifth-generation offspring of a genetic selection experiment performed using Angus cattle that were selected for both residual average daily gain (RADG) and carcass marbling (intramuscular fat). All steers (n=63) were reared in a pasture-based

system for the first year, before being transported to a commercial feedlot located in Brasstown, NC (35º 10 N, 83º 23 W), where they were fed a high-grain finishing ration for approximately 4 months. Upon arrival at the feedlot, steers were adapted to the finishing diets using a 3-week step-up approach and then were fed the same finishing diet for 110 days. The feedlot diet composition is shown in Table 1. Feed intake of all steers was measured using a GrowSafe System (GrowSafe Systems Ltd., Calgary, Canada), which allowed individual measures of feed intake and computation of feed efficiency (RFI). Following finishing, steers were rank ordered based on determined RFI over the course of the feedlot period and the 12 most efficient and the 12 least efficient steers were selected. These steers (n = 24) were transported to the University of Georgia Meat Science Technology Center, a federally inspected meat plant located in Athens, GA (33º 57 N, 83º 22 W). Steers were housed at this location overnight where they had *ad libitum* access to water but were fasted prior to humane slaughter the following morning. Following evisceration, samples from three different locations in the GIT were aseptically collected: rumen, cecum, and rectum. In order to avoid marginal differences and work only with animals that were distinctly different regarding feed efficiency, samples from four animals that had intermediate RFI values were not used in this study, resulting in a total of 20 steers utilized: 10 classified as low-RFI (-1.93) and 10 classified as high-RFI (1.97).

Collection of GIT samples, DNA extraction and sequencing

Following the removal of the viscera, the rumen, cecum and rectum were identified, and approximately 40 milliliter (mL) of contents were aseptically collected. All samples were collected in individual sterile 50-mL conical tubes and stored at -20°C until further analysis. DNA was extracted from the samples following the procedure described by

Rothrock et al. (2014), which uses a combination of mechanical and enzymatic methods to optimize DNA extraction. Briefly, this procedure uses 0.33 g of sample placed in 2-mL Lysing Matrix E tubes (MP Biomedicals LLC, Irvine, CA, USA) which are homogenized in a FastPrep 24 Instrument (MP Biomedicals LLC, Irvine, CA, USA) to disrupt the cells. Enzymatic inhibition was achieved by using InhibitEX Tablets (QIAGEN, Venlo, Netherlands), and DNA elution and purification were carried out using an automated robotic workstation (QIAcube; QIAGEN, Venlo, Netherlands). Determination of DNA concentration and purity in the resulting eluate was performed spectrophotometrically using the Synergy H4 Hybrid Multi-Mode Microplate Reader in conjunction with the Take3 Micro-Volume Plate (BioTek Instruments Inc; Winooski, VT, USA). Samples with a minimum volume of 20 microliter (μL) and 10 nanogram (ng)/μL of DNA were stored at 4°C until the following day. Samples that failed to meet these requirements were rejected and subjected to a new DNA extraction cycle.

Following DNA extraction, samples were taken to the Georgia Genomics and Bioinformatics Core (https://dna.uga.edu) for library preparation and 16S rRNA gene sequencing. The library preparation step included PCR replications using the forward: S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′); and reverse: S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) primer pair (Klindworth et al., 2013). Sequencing was performed using an Illumina MiSeq v3 2 x 300 base pairs kit (Illumina Inc., San Diego, CA, USA).

Sequencing data and diversity analyses

Sequencing data were demultiplexed and converted into FASTQ files. Pair-end reads were set and then merged using BBMerge Paired Read Merger v37.64, and files were

further analyzed using the QIIME pipeline v1.9.1 (Caporaso et al., 2010). Files were quality-filtered, merged into one single file, and converted into the FASTA format. The sequences were then clustered into operational taxonomic units (OTU) at 97% similarity using the Greengenes database (gg_13_8_otus), and singleton OTU were excluded from the analysis. The sequencing depth for further analysis was set at 17,542 sequences per sample.

Several alpha and beta diversity indices were computed; however, only two alpha diversity indexes are shown here: Chao1 richness estimator and Shannon diversity index. For beta diversity, we present results only from the weighted UniFrac distance matrix. The interested reader can refer to the supplemental material to visualize the other calculated indexes. Chao1 was selected as our microbial richness estimator (estimator of total number of species) because it compensates for potentially low sampling depths (Magurran, 2013). Shannon index was the metric chosen to represent microbial diversity because it is widely used in describing microbial communities (Kim et al., 2017). For beta diversity, the weighted UniFrac distance matrix was chosen because it accounts for phylogenetic relationships when calculating diversity (Lozupone and Knight, 2005; Lourenco et al., 2019).

Analysis of volatile fatty acids

Three grams of each sample was placed into 15-mL conical tubes and diluted with 9 mL of distilled water. Tubes were vortexed for 30 seconds and 2.0 mL of the mixture was transferred to centrifuge tubes, which were then centrifuged at 10,000 x gravitational force (*g)* for 10 minutes. One mL of the resulting supernatant was aliquoted into new centrifuge tubes, along with 0.2 mL of a metaphosphoric acid solution (25%

weight/volume), and samples were frozen overnight. Samples were thawed and centrifuged at 10,000 x *g* for 10 minutes. The supernatant was transferred into polypropylene tubes and mixed with ethyl acetate in a 2:1 ratio of ethyl acetate to supernatant. Tubes were vortexed for 15 seconds and allowed to settle for 5 minutes before 0.5 mL of the supernatant was transferred to screw-thread vials for VFA analysis using a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionization detector and a capillary column (Zebron ZB-FFAP; 30 m x 0.32 millimeter (mm) x 0.25 micrometer (μm); Phenomenex Inc., Torrance, CA, USA). Sample injection volume was set as 1.0 μL, and helium was used as the carrier gas. Column temperature was initially set at 110°C and gradually increased to 200°C. Injector and detector temperatures were set at 250 and 350°C, respectively.

Statistical Analysis

Statistical analyses were performed using the software Minitab (v18.1) and R (v3.3.3). Animal performance data, the alpha diversity indexes, and bacterial relative abundances at the phylum, family, and genus levels were analyzed using one-way ANOVA with feedlot RFI category as a main effect (i.e. high- or low-RFI). Differences in beta diversity were accessed by two-sample t-tests in which the p-values were corrected by Bonferroni's method for multiple comparisons. Additionally, multiple correlations were calculated between alpha diversity indices, bacterial groups at different phylogenetic levels, RFI performance, and VFA concentrations. For all statistical tests, results were considered significant at $P \leq 0.05$, and trends were recognized at $0.05 < P \leq 0.10$.

Results

After the quality filtering steps, the microbiome sequencing data resulted in 2,184,938 sequences, with an average count of 36,415.6 sequences per sample. Results from the 10 most efficient and 10 least efficient steers in terms of RFI value were used; however, only 9 ruminal samples were used for each group due to technical problems with sample processing.

Animal Performance

Table 2 demonstrates the growth performance of high and low RFI steers during the feedlot-finishing period. At the beginning of the feedlot period, the high and low efficiency steers were not different in body weight ($P = 0.53$). Although numerical differences in body weight gain were observed between the two groups of steers, these differences were not significant ($P = 0.22$). However, the most efficient steers (low-RFI) had a lower daily dry matter intake (DMI), higher gain:feed ratio, and lower RFI value compared to the less efficient (high-RFI) steers ($P < 0.001$).

Alpha and Beta Diversities

Beta diversity in the samples collected from each GIT location (rumen, cecum, and feces) for both groups of steers combined is shown in Fig. 1 and was different ($P < 0.001$) between the ruminal samples, and the lower GIT samples. Beta diversity differences for high- and low-RFI steers across all three GIT locations are shown in Fig. 2. Gastrointestinal location (rumen, cecum, or feces) had a strong impact ($P < 0.001$) on the composition of microbial communities, regardless of RFI status.

Microbial richness and diversity for the two RFI groups of steers are shown in Fig. 3. Ruminal Chao1 richness was lowest in the most efficient cattle ($P = 0.01$), but greatest

in the cecum ($P = 0.01$), and numerically greater in the feces ($P = 0.13$) of the efficient (low-RFI) steers compared to the less efficient (high-RFI) steers. Shannon diversity index values were similar ($P = 0.34$) across efficiency groups in the rumen, but the more efficient steers had greater diversity in the cecum ($P = 0.01$) and feces ($P < 0.001$) than the less efficient steers.

Bacterial Abundance

The overall taxonomic profiles of the samples at both the phyla- and family-levels are shown in Fig. 4. Both the phyla- and family- level analyses show that the rumen environment was different from the cecum and feces, with the two latter ones being very similar. Actinobacteria was higher ($P = 0.03$) in the high-RFI steers compared to the low-RFI steers at the phyla-level when comparing samples in the feces. At the family-level, *Ruminococcaceae, Rikenellaceae*, as well as unidentified bacterial families in the order *Clostridiales* and *Bacteriodiales* were higher ($P < 0.05$) in the low-RFI steers versus the high-RFI steers when comparing samples in the feces and cecum. RF16 and *Mogibacteriaceae* were higher ($P \leq 0.033$) in the low-RFI steers versus the high-RFI steers in the cecum. On the other hand, *Bifidobacteriaceae* was higher ($P = 0.03$) in the high-RFI steers than the low-RFI steers in the feces. *Lactobacillaceae* populations were higher ($P = 0.044$) in the high-RFI steers than in the low-RFI steers when comparing cecal samples.

The 10 most abundant bacterial families for the high and low efficiency steers at the three different GIT locations are shown in Table 3 and comprised 70%, 80%, and 78% of the total bacterial populations in the rumen, cecum, and feces, respectively. Five of the most abundant families found at high proportions across all three GIT locations were *Ruminococccaceae, Lachnospiraceae,* S24-7, *Paraprevotellaceae,* and *Clostridiaceae*;

however, their relative abundances differed between GIT locations. *Prevotellaceae* populations varied from the most abundant in the rumen to not being one of the 10 most abundant cecal and fecal families. Cecal and fecal microbial populations were most similar and were distinct from the ruminal populations with five of the most abundant bacterial families not being found in the rumen (*Peptostreptococcaceae, Bacteroidaceae, Turcibacteraceae, Bifidobacteriaceae*, and *Lactobacillaceae)*.

The composition of the top 10 ruminal bacterial families did not differ ($P \geq 0.24$) based on RFI status of steers. In both cecal and fecal samples, *Ruminococcaceae* was more abundant ($P = 0.01$) in the most efficient steers; however, *Bifidobacteriaceae* was more abundant ($P = 0.03$) in the feces of the less efficient steers (Table 3). As can be visualized in Fig. 5, the abundance of *Ruminococcaceae* remained relatively constant across the rumen, cecum, and feces in highly efficient animals, yet *Ruminococcaceae* populations was lower ($P = 0.01$) in the cecum and feces of less efficient cattle. Whereas populations of *Bifidobacteriaceae* increased distally in both group of steers, the increase was greater in low efficiency steers than in the high efficiency steers, and *Bifidobacteriaceae* populations were higher ($P = 0.03$) in the feces of less efficient steers compared to more efficient steers (Fig. 5).

Volatile Fatty Acid Concentrations

Concentrations of acetate, propionate, butyrate, and total VFA in the rumen, cecum, and feces are shown in Table 4. In the rumen, the molar proportion of butyrate was greater ($P = 0.04$) in the more efficient steers than in the high RFI steers. Cecal acetate molar proportions were higher ($P = 0.05$) and butyrate molar proportions were lower ($P = 0.05$) in the low-RFI (more efficient) steers than their high-RFI counterparts. However, no

differences in total VFA production or their molar proportions were observed in the fecal samples of steers from both groups.

Correlations with Feed Efficiency

No ruminal bacterial family abundances were correlated with RFI in the rumen ($P \geq 0.07$; Table 5); however, six bacterial families were significantly correlated ($P \leq 0.05$) with RFI in the cecum and feces. Four bacterial families (*Ruminococccaceae, Mogibacteriaceae, Christensenellaceae,* and BS11) were negatively correlated with RFI ($P \leq 0.05$) in both the cecum and feces. *Succinibrionaceae* in the cecum ($r = 0.445$, $P = 0.05$) and *Bifidobacteriaceae* in the feces ($r = 0.478$, $P = 0.03$) were the only bacterial families positively correlated with RFI.

Discussion

Animal Performance

As expected, the more efficient (low RFI) animals consumed less feed during the feedlot period, which translated into a higher gain:feed ratio (G:F). In fact, in order for the less efficient steers to gain 1 kilogram (kg) of body weight they required an extra 5.3 kg of feed per day, on average, compared to the more efficient steers (15.6 vs. 10.3 kg feed intake/day; data not shown). This is consistent with reports on steer performance of the first three selected generations for this cattle population (Pringle et al., 2019). Improving feed efficiency is critical to improving food production sustainability because feed costs are the greatest driver of profitability of beef production (Lancaster et al., 2009). Therefore, it is imperative that we select for cattle that have adequate body weight and carcass size and quality but consume less feed.

Bacterial Diversity

Biodiversity cannot currently be reduced to a single index (Magurran, 2013); therefore, we present two alpha diversity and one beta diversity indices which revealed that the ruminal bacterial population was distinct from the cecum and fecal populations. Moreover, in the rumen, more efficient steers (low-RFI) had lower species richness and numerically lower microbial diversity compared to less efficient cattle. Shabat et al. (2016) reported that the ruminal microbiomes of more efficient dairy cows had lower alpha diversity but produced more relevant metabolites (e.g., VFA) which are usable by the host animal. Conversely, the microbiome from less efficient cattle had greater diversity, resulting in the production of more end products that were not well utilized by the host animal for energy (Shabat et al., 2016). Although not significant, the present study detected a numerically greater concentration of total VFA in the rumen of the most efficient steers compared to the less efficient steers (107.38 versus 92.90), which corroborate the findings of Shabat et al. (2016).

Unlike our ruminal results, greater phylogenetic diversity and richness were found in the cecum of the most efficient steers. Because both microbial richness and diversity were different in the ceca of efficient and inefficient steers, we hypothesized that a greater array of carbohydrate-active enzymes (CAZyme) were present in the ceca of the more efficient steers. The hypothesized greater cecal CAZyme diversity likely resulted in more cecal carbohydrate fermentation and consequently greater nutrient utilization in the lower GIT of more efficient steers, which would have not otherwise been available, as it was likely the case in the less efficient steers. Alpha and beta diversity demonstrated that fecal and cecal populations were similar, as has been previously shown (de Oliveira et al., 2013;

Myer et al., 2017). In the present study, microbial richness and diversity were similar in the fecal and cecal compartments, with higher diversity in the feces of more efficient steers.

Bacterial Abundances

Unlike other studies that found differences in ruminal bacterial population composition of animals with different feed efficiency (Hernandez-Sanabria et al., 2012; Shabat et al., 2016), the present study did not observe any correlations between RFI and ruminal bacterial families. This discrepancy may be partially explained because the steers were fasted for approximately 20 hours before sample collection. This fasting may also explain why ruminal VFA concentrations in our steers were lower than anticipated. Previous studies have shown that ruminal VFA concentrations have a rapid decrease 4-6 hours post-feeding (Stewart et al., 1958; Bryant, 1964) and a continued decrease with sustained fasting.

Ruminococcaceae was found in greater abundance in the cecum and feces of the most efficient steers. The family *Ruminococcaceae* contains many species which are cellulolytic and/or hemicellulolytic and which produce acetate, formate, and hydrogen (Biddle et al., 2013); therefore, the higher abundance of *Ruminococcaceae* likely resulted in increased degradation of fibrous carbohydrates in the lower gut of the most efficient steers, which in turn allowed them to harvest more energy from their diets, likely in the form of acetate which is readily absorbable by the host (Myer et al., 2015). Furthermore, the greater molar proportion of acetate found in the cecum of the most-efficient (low-RFI) steers supports this hypothesis. One unique aspect of the fecal bacterial populations compared to the ceca was the higher abundance of *Bifidobacteriaceae* in the inefficient steers.

Bacterial Population Correlations with Feed Efficiency

As previously mentioned, there were no significant correlations between the abundance of any ruminal bacterial family and RFI in this study, which highlighted the differences in bacterial populations between the rumen and lower gastrointestinal tract. More efficient steers had higher populations of *Ruminococcaceae* in their ceca and feces, indicating a negative correlation between RFI and *Ruminococcaceae*. Several other bacterial families in both the ceca and feces were also negatively correlated to RFI including the following: *Mogibacteriaceae, Christensenellaceae,* and BS11. *Christensenellaceae* has been found in the feces of adult humans as an indication of a "healthy digestive system" and has been tied to reduced adipose tissue accumulation (Goodrich et al., 2014). BS11 is a family of hemicellulose degraders found in the GIT of mammals that produce acetate and butyrate and is thought to contribute to gut health (Solden et al., 2017). In the cecum, the family RF16 was also negatively correlated to RFI, and this family produces acetate, succinate, and propionate and has been isolated from the large intestines and feces of cattle (Kong et al., 2010; Jeong et al., 2011; Abe et al., 2012; de Oliveira et al., 2013; Myer et al., 2015). *Rikenellaceae* populations of the feces were also found in this study to be negatively correlated to RFI. Collectively this supports our hypothesis that the more efficient steers contained microbial populations that produced higher proportions of end products (e.g., succinate and acetate) that could be absorbed by the host.

In the cecum, only *Succinivibrionaceae* abundance was positively correlated with RFI, meaning that the abundance of this family was higher in the less efficient steers. Interestingly, fecal *Bifidobacteriaceae* abundance was also positively correlated to RFI, so

the steers with more fecal *Bifidobacteriaceae* consumed more feed and were less efficient. This is contrary to the findings of Mitsuoka (1990) and Perea et al. (2017) who reported this acetate-producing bacterial group to be higher in more efficient cattle.

Overall, our findings indicated that the rumen environment was distinct in terms of both diversity indices and bacterial abundances, compared to the cecum and feces. In addition, we found no differences in rumen bacterial family abundances regarding feed efficiency status, and there was no correlation between ruminal bacterial abundances and feed efficiency. Cecal and fecal populations were quite similar in terms of diversity and bacterial family abundances. Several bacteria were correlated with RFI in both the cecal and fecal samples, particularly for the family *Ruminococcaceae*, which was consistently found in greater abundance in the hindgut samples of the most efficient (low-RFI) steers. Collectively, our present findings suggest that additional nutrient fermentation and energy absorption occurring in the hindgut can play an important and underappreciated role in feed efficiency of feedlot cattle.

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Table 5.1. Composition of the diets used during the transition and finishing periods of the feedlot steers.

	Transition Diet	**Finishing Diet**
Ingredient, % DM		
Corn	41.12	56.20
Dried distillers grains	22.18	19.54
Corn gluten feed	-	7.08
Soybean Hulls	15.80	-
Barley Straw	6.15	4.36
Vitamin/Mineral Premix	4.47	4.76
Corn Silage	10.27	8.05
Total	**100.00**	**100.00**
Nutrient, % DM		
Dry Matter, %	62.00	62.00
NEm, Mcal/kg	2.02	2.10
NEg, Mcal/kg	1.37	1.43
Crude Protein, %	14.63	14.51
Roughage, %	16.43	12.40
Rough NDF, %	9.28	6.92
Fat, %	5.11	5.28
Calcium, %	0.75	0.70
Phosphorus, %	0.39	0.45
Potassium, %	0.90	0.71
Magnesium, %	0.22	0.21
Sulfur, %	0.25	0.26
Added Salt, %	0.21	0.22

Table 5.2. Performance of high- and low-RFI steers (n=10/ group) during the feedlot-finishing period.

Item	High-RFI	Low-RFI	SEM	*P*-value
Initial weight, kg	491.80	480.20	18.4	0.53
Average daily gain, kg/day	0.87	0.97	0.07	0.22
Daily dry matter intake, kg	13.40	9.76	0.53	**< 0.001**
Feed:Gain ratio	15.6	10.3	0.97	**< 0.001**
Residual feed intake, kg/day	1.97	-1.93	0.33	**< 0.001**

Table 5.3. Most abundant bacterial families detected by Illumina MiSeq Sequencing in the rumen, cecum, and feces of high- and low-RFI steers (n=10 steers/treatment*).

	Abundance, %			
Bacterial Families	**High-RFI**	**Low-RFI**	**SEM**	***P*-value[1]**
Rumen				
Prevotellaceae	20.9	20.7	0.42	0.94
Ruminococcaceae	16.7	18.0	1.64	0.56
Lachnospiraceae	15.3	13.8	1.78	0.51
S24-7	4.9	5.1	0.57	0.81
Veillonellaceae	4.2	3.0	0.14	0.24
Paraprevotellaceae	2.1	2.1	0.71	0.93
Coriobacteriaceae	2.2	1.9	0.07	0.32
Spirochaetaceae	1.7	2.3	0.33	0.31
Mogibacteriaceae	1.7	1.6	0.10	0.83
Clostridiaceae	1.2	1.4	1.59	0.52
Cecum				
Ruminococcaceae	14.0	22.1	1.64	**0.01**
Lachnospiraceae	17.4	15.4	1.78	0.59
Peptostreptococcaceae	15.5	15.4	1.52	0.96
Clostridiaceae	13.7	9.5	1.59	0.20
Bacteroidaceae	4.8	4.1	1.80	0.84
Turicibacteraceae	3.8	4.1	0.56	0.80
Paraprevotellaceae	3.2	3.4	0.71	0.90
S24-7	3.1	2.7	0.57	0.75
Bifidobacteriaceae	3.8	1.1	0.79	0.09
Lactobacillaceae	2.1	0.8	0.33	**0.04**
Feces				
Ruminococcaceae	15.0	22.6	1.45	**0.01**
Lachnospiraceae	18.3	13.8	1.92	0.25
Peptostreptococcaceae	12.7	11.7	1.39	0.73
Clostridiaceae	11.8	7.9	1.11	0.08
Paraprevotellaceae	4.1	5.1	1.00	0.65
Bacteroidaceae	3.1	5.2	1.05	0.34
Turicibacteraceae	3.9	3.4	0.70	0.72
Bifidobacteriaceae	5.7	1.2	1.06	**0.03**
S24-7	3.2	3.3	0.43	0.90
Lactobacillaceae	3.5	0.6	0.77	0.07

*Rumen values reflect n=9 steers/treatment.

[1] *P*-value for the contrast between inefficient and efficient steers.

Table 5.4. Total VFA production (millimolar [m*M*]), Acetate: Propionate ratio, and VFA proportions (mol/100 mol) in the rumen, cecum, and feces of high- and low-RFI feedlot steers (n=10 steers/treatment*).

Item	High-RFI	Low-RFI	SEM	*P*-value[1]
Rumen				
Acetate	62.11	61.70	5.87	0.87
Propionate	20.70	19.85	3.88	0.75
Butyrate	9.03	11.01	1.38	**0.04**
Total VFA	92.90	107.38	11.00	0.37
Acetate:Propionate	3.37	3.27	0.37	0.85
Cecum				
Acetate	60.19	63.75	5.24	**0.05**
Propionate	22.88	20.70	1.61	0.15
Butyrate	12.18	9.34	1.69	**0.05**
Total VFA	108.92	97.52	8.06	0.33
Acetate:Propionate	2.75	3.09	0.17	0.15
Feces				
Acetate	66.97	66.20	4.45	0.78
Propionate	19.85	19.35	1.40	0.73
Butyrate	8.95	8.78	1.03	0.90
Total VFA	84.68	72.51	6.17	0.18
Acetate:Propionate	3.47	3.55	0.25	0.82

* Rumen values reflect n=9 steers/treatment.

[1] *P*-value for the contrast between inefficient and efficient steers.

Table 5.5. Correlation between RFI of feedlot steers (n=10 steers/treatment*) and bacterial families** at different GIT locations.

Bacterial Family	Pearson Correlation	*P*-value
Rumen		
None[1]		
Cecum		
Ruminococcaceae	-0.674	0.001
Mogibacteriaceae	-0.647	0.002
Succinivibrionaceae	0.445	0.05
Christensenellaceae	-0.455	0.04
BS11	-0.528	0.02
RF16	-0.492	0.03
Feces		
Ruminococcaceae	-0.725	<0.001
Bifidobacteriaceae	0.478	0.03
Mogibacteriaceae	-0.494	0.03
Rikenellaceae	-0.538	0.01
Christensenellaceae	-0.493	0.03
BS11	-0.497	0.03

* Rumen values reflect n=9 steers/treatment

**Only bacterial families that had correlation ($P \leq 0.05$) with cattle RFI are shown.

[1]There were no ruminal bacterial families correlated with RFI ($P \geq 0.07$).

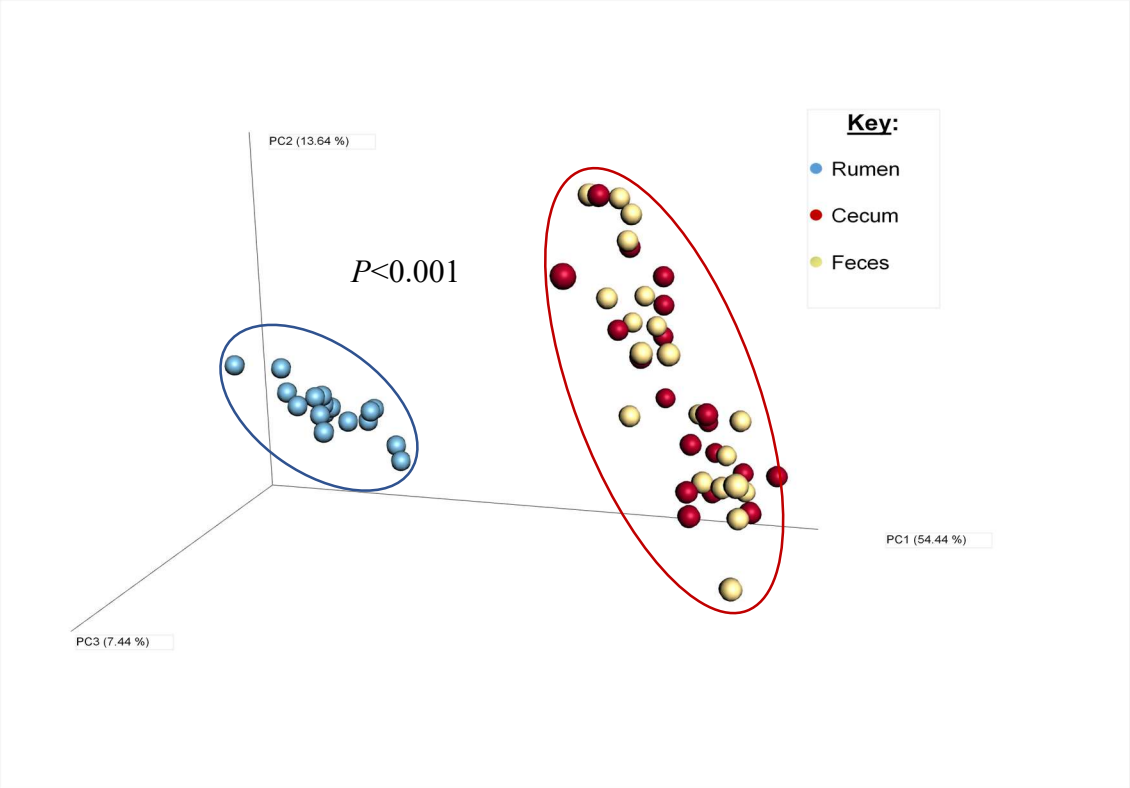

Fig. 5.1. Principal Coordinate Analysis plot of Beta diversity of bacterial populations from rumen, cecum, and feces from feedlot steers (n=20 steers *) P-value indicates a difference in beta-diversity between samples at each GIT location. *Ruminal samples reflect n= 18 steers.

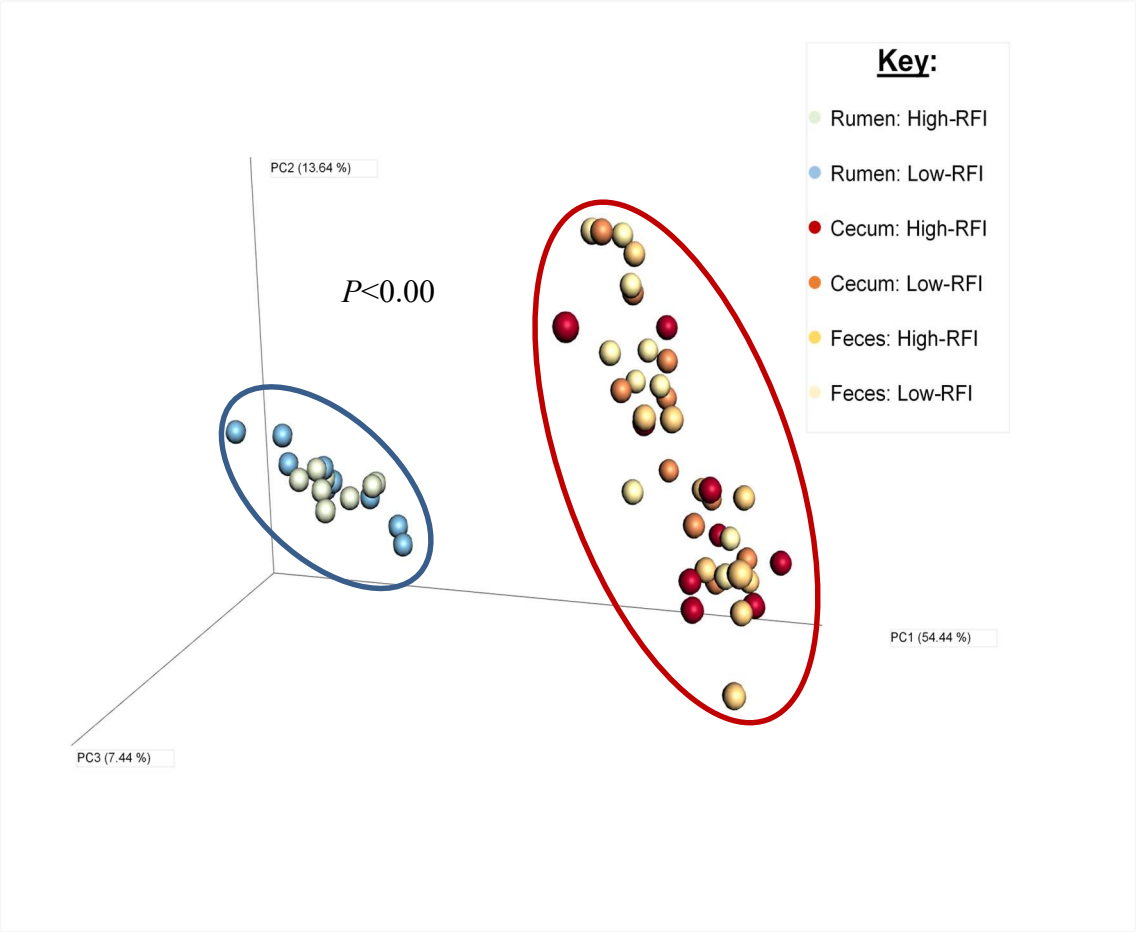

Fig. 5.2. Principal Coordinate Analysis plot of Beta diversity of bacterial populations from rumen, cecum, and feces of high- and low-RFI feedlot steers (n=10 steers/treatment*) P-value indicates a difference in beta-diversity between samples at each GIT location.

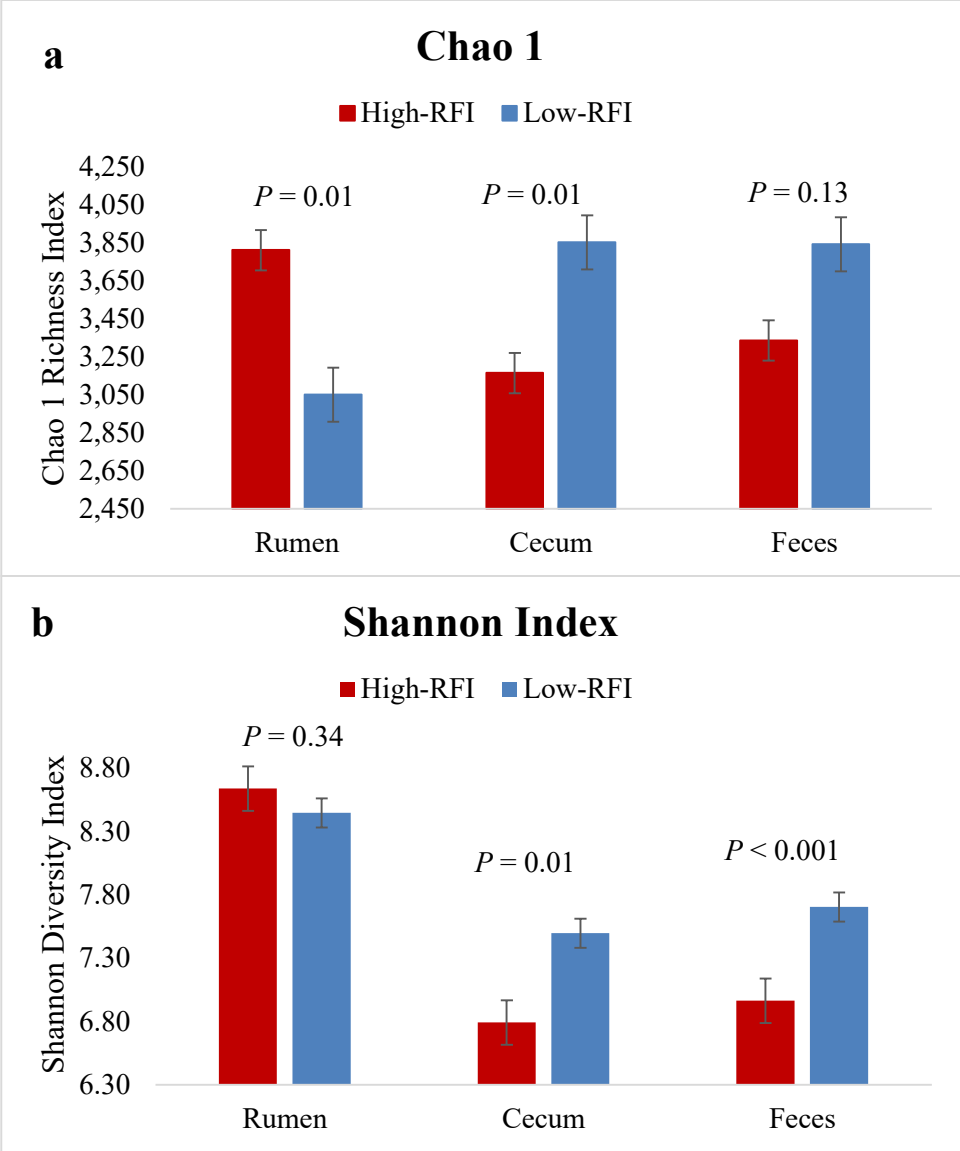

Fig. 5.3. Indices of bacterial richness (a; Chao 1) and diversity (b; Shannon Index) calculated for the ruminal, cecal, and fecal environments of high- and low-RFI feedlot steers (n=10 steers/treatment*). *P*-values indicate the significance of the difference between the efficiency of groups. Error bars indicate the standard error. *Ruminal samples reflect n=9 steers/treatment.

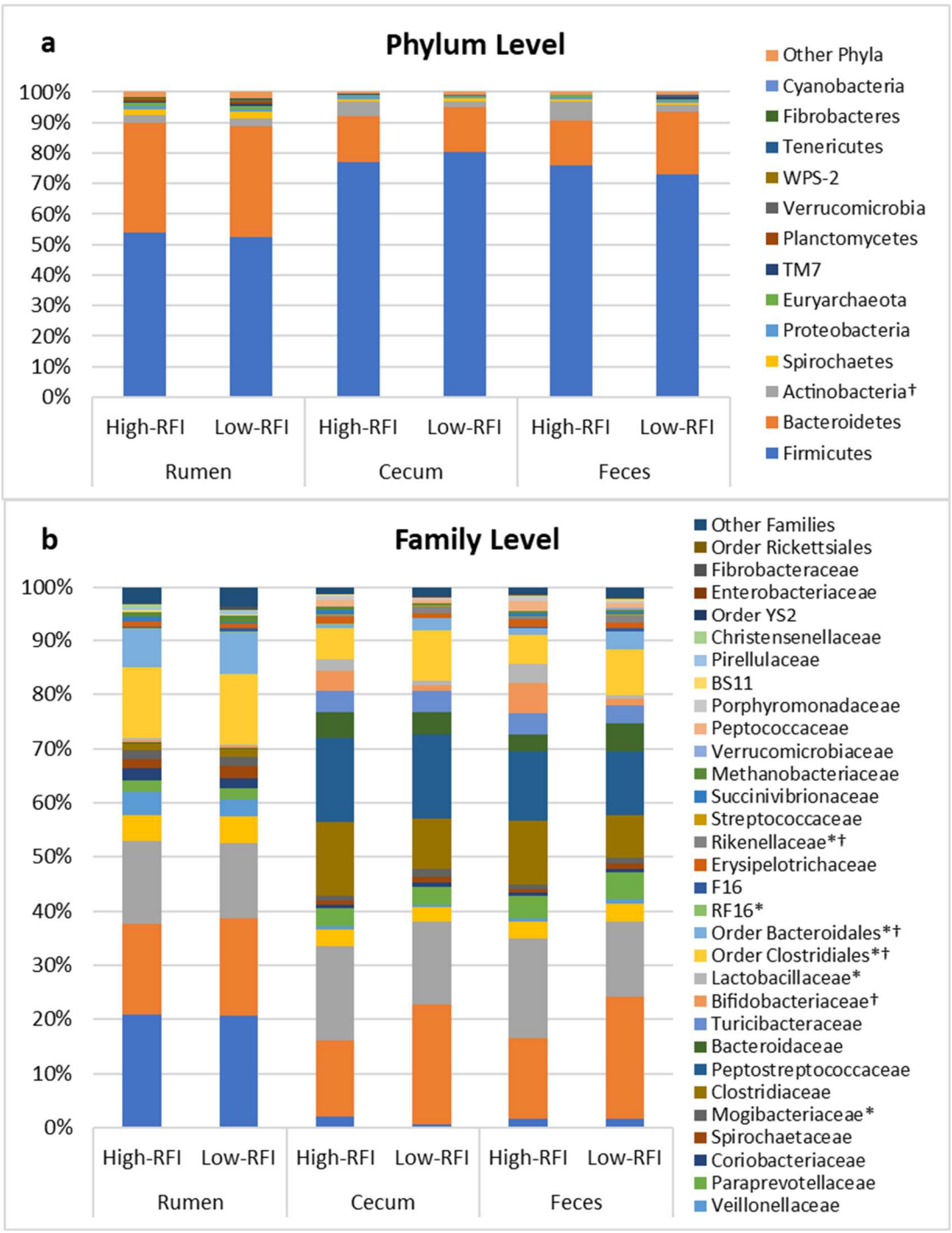

Fig. 5.4. Taxonomic profiles of the abundance at the phylum-level (**a**) and family-level (**b**) of high- and low- RFI steers from the cecum and feces of feedlot steers (n=10 steers/treatment[#]). Significant differences between efficiency groups are indicated by * in the cecum and † in the feces. ($P \leq 0.05$); [#] Ruminal samples reflect n= 9 steers/treatment.

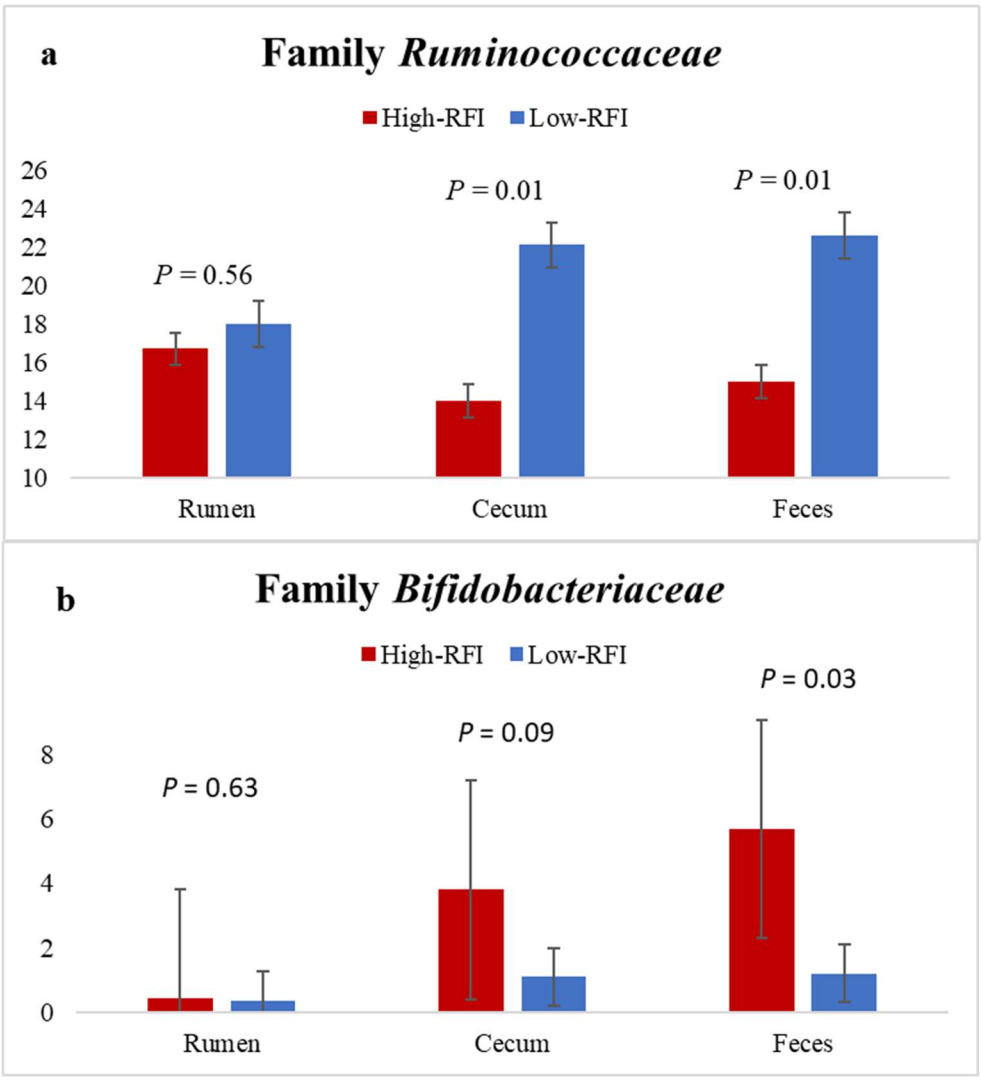

Fig. 5.5. Abundance of the bacterial families *Ruminococcaceae* (a) and *Bifidobacteriaceae* (b) in the rumen, cecal, and feces of low and high-RFI steers (n=10 steers/treatment*). Differences between high and low-RFI steers are represented by the *P*-values indicate the significance of the differences between high- and low-RFI steers. Error bars indicate the standard error. * Ruminal samples reflect n= 9 steers/treatment.

CHAPTER 6

CONCLUSION

After evaluating groups of Angus steers from the fifth generation of genetic selection for feed efficiency and marbling from weaning to slaughter, results indicate that the gastrointestinal microbiome experiences fluctuations based on changes in diet, fasting, and gastrointestinal location. However, despite the effects of environmental factors, specific bacterial families were consistently correlated with a divergence in feed efficiency. Additionally, the hindgut microbiota was found to be important in the divergence of host feed efficiency. Not only was an increase in bacterial diversity in the hindgut correlated with feed efficiency, but also, there were many bacterial families related to the host's feedlot feed efficiency which were identifiable from weaning to slaughter.

In the first study, our results indicated that even with fluctuations in the fecal microbial population due to dietary changes at weaning, yearling, and slaughter, there were still specific bacterial families found to be consistently different in steers divergent in feedlot residual feed intakes. *Rikenellaceae* was increased in more efficient steers at all three sampling timepoints. *Ruminococcaceae* and *Christensenellaceae* were also increased in efficient steers at yearling and slaughter. Conversely, *Bifidobacteriaceae* and *Lactobacillaceae* were numerically increased in inefficient steers throughout their lives. These results highlight the strong correlation between feedlot feed efficiency and specific bacteria within the hindgut. Therefore, our results indicate that producers may be able to

predict the feedlot feed efficiency of their steers as early as weaning with a sample that can be completed via an easy-to-collect fecal grab.

The results from the second study demonstrated that pre-slaughter fasting impacts the ruminal microbial consortium. After steers were fasted prior to slaughter, there were more bacterial species present, and they were more equally represented, resulting in an increase in bacterial diversity. These changes happened mainly as a result of *Prevotellaceae* and *Ruminococcaceae* (the two most abundant bacterial families in their rumens) whose populations decreased after fasting. This decrease left a void in the ruminal niche that was filled with more than 30 other families which were increased after fasting. Some of the bacterial genera that increased were *Blautia*, *Methanosphaera*, *Campylobatcer*, and *Treponema* which all contain species known to be deleterious to the host animal. Results suggest that when the selection pressure applied to the ruminal microbiota by the diet is replaced with a low nutrient environment, the most dominant bacterial species no longer have their competitive advantage, resulting in an open niche in the ruminal ecosystem which can potentially be filled with opportunistic pathogens.

The third study evaluated the ruminal, cecal, and fecal microbial populations at slaughter, and the results indicated that many bacterial species were either positively or negatively correlated with residual feed intake (RFI) found in the hindgut. The number of bacterial species present in the cecum and the overall bacterial diversity in both the cecum and the feces increased in the efficient steers compared to inefficient steers. *Ruminococcaceae* was found to be more abundant in both the cecum and the feces of low-RFI (efficient) steers compared to high-RFI (inefficient) steers and was correlated with feed efficiency. Conversely, *Bifidobacteriaceae* was increased in the feces of high-RFI

steers compared to low-RFI and, was inversely correlated with feed efficiency. Furthermore, correlations between RFI and *Mogibacteriaceae*, *Christensenellaceae*, and BS11 were found in the cecum and feces along with RF16 in the cecum and *Rikenellaceae* in the feces. Therefore, these results highlight the importance of the hindgut microbial consortium to the overall feed efficiency of the host. The additional microbial fermentation and energy absorption occurring in the hindgut can play an important role in the overall efficiency of the host animal.

Collectively, our results indicate that regardless of environmental factors (e.g., diet, fasting, gastrointestinal location) there are many bacterial families important in terms of the host animal's feedlot feed efficiency, specifically in the hindgut. Moreover, these bacterial families' abundances can begin to diverge as early as weaning. However, this study was limited to the bacterial family-level, so future research should be conducted to identify which species within these families are contributing to the correlation with RFI. Additionally, further research is needed to determine if the bacteria are contributing to the overall feed efficiency of the host or vice versa. If the specific bacteria are contributing to the host's feed efficiency, then they could be manipulated in order to improve the feed efficiency of beef cattle.