THE APPLICATION OF DNA SEQUENCING TECHNOLOGIES AS AN AID IN THE METAANALYSIS OF THE RUMINAL MICROBIOME OF BEEF STEERS FROM A COMMERCIAL FEEDLOT

by

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(Under the Direction of Todd Callaway)

ABSTRACT

Next generation sequencing tools have revolutionized analysis of the rumen microbiome, but it is impossible to make equivalent predictions about the impact of microbial populations on host animal energy status solely based on their qualitative presence in the rumen, or their quantitative population numbers alone. To evaluate the rumen microbial population's effect on cattle production there must be a holistic understanding of the microbial ecosystem trophic niches, degradative activities, biosynthetic pathways, populations, and interactions between feedstuffs, ruminal microbes, and the ruminant animal. An evaluation of the association between the ruminal microbiota fermentation and cattle feed efficiency was conducted utilizing DNA sequencing. Although the sample size was small, findings suggested more efficient steers in the feedlot stage had ruminal microbial populations that were more involved in fiber degradation. A second study was conducted to assess the role of the rumen microbiome on the rumen metabolome utilizing DNA sequencing.

INDEX WORDS: Next generation sequencing, Rumen microbiome, Metanalysis, DNA sequencing, Cattle

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DEDICATION

I dedicate this thesis to James and Lorez, my amazing parents whose sacrifices made it possible for me to pursue my dreams and complete this body of work. They have instilled in me the virtues of perseverance and commitment and relentlessly encouraged me to strive for excellence. Thank you for being a source of motivation and strength during moments of despair and discouragement. I hope that this achievement will complete the dreams that you had for me all those many years ago.

I also dedicate this thesis to my loving fiancé and soon to be husband Trevor, for his loving support and many late-night coffee runs. Thank you for always pushing me in the direction of my dreams.

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CHAPTER 1

INTRODUCTION

Co-evolutionary pressures have caused the development of a symbiotic mutualistic relationship between the ruminant animal and the resident microbial population of the gut, especially in the rumen (Hungate, 1966). The relationship between the native microbial population of the rumen and ruminant animals has provided ruminants the ability to occupy an ecological niche by thriving on fiber rich diets that cannot be utilized by monogastric animals. While cattle themselves do not produce cellulose-degrading enzymes, they harbor bacteria, fungi, and protozoa that do degrade cellulosic forages (Russell and Rychlik, 2001). The ruminal microbiota catabolizes these low-quality feedstuffs to produce compounds used by the animal for energy through the fermentation process in the pregastric rumen. Although the presence of pregastric microbial fermentation makes ruminants unique and able to utilize different substrates than monogastrics, it comes at a cost of efficiency to the animal (Lobley, 1992). End products of fermentation that are important to the efficiency of the ruminant animal include volatile fatty acids (VFA), gases, microbial mass, heat of fermentation, lactate, ethanol, and ammonia (Russell and Hespell, 1980).

To analyze the composition of the ruminal microbial population, many studies have utilized a multiomics approach combining metagenomics, metatranscriptomics, metaproteomics and metabolomics to study the rumen microbial ecosystem and its impacts on the host animal (Shabat et al., 2016; Comtet-Marre et al., 2017; Shaani et al., 2018).

To holistically evaluate the rumen microbial population there must be a multifaceted understanding of the ecosystem niches, degradative activity, biosynthetic pathways, populations, and interactions amongst feeds, ruminal microbes, and the ruminant. While several strategies for optimizing the energy and nitrogen utilization by ruminants have been identified, a better understanding of the microbial composition and their underlying metabolic activities is essential before the rumen processes can be manipulated successfully. Next Generation Sequencing tools can serve as an aid in the analysis of the rumen microbiome, but it is impossible to draw equivalent predictions about the impacts of microbial populations on the host animal based solely upon their qualitative or quantitative presence in the rumen alone. Before predictions can be made about the rumen microbiome, many factors (the correlations between diet, the microbiome composition, the metabolome composition and animal production needs and demands) must be elucidated to further our understanding of the interactions between: feeds, the microbiome, fermentation end products (e.g., production and utilization of VFAs, ammonia, microbial crude protein, and other substrates), host animal physiology, production parameters, immune status, animal health, and food safety all of which must be considered individually and collectively (Carmichael and Callaway, 2019).

CHAPTER 2

LITERATURE REVIEW

The Ruminant and Microbial Fermentation

Mammals do not possess the carbohydrate degrading enzymes cellulase or hemicellulase that directly degrade the complex polymeric carbohydrates, cellulose and hemicellulose, that are the structural components of plant walls (Owens and Goetsch, 1988). The ruminant forestomach, the rumen, provides an anaerobic environment for the growth of bacteria, fungi, and protozoa which produce cellulase. This results in the production of monosaccharides and simple polysaccharides which are available for further microbial degradation (Hungate, 1966; Bryant, 1973; Russell and Cook, 1995). Ruminants have evolved a symbiotic relationship with their rumen microbiome, which allows them to occupy an ecological niche because the microbes can degrade these forages that are carbohydrate sources of low-quality for monogastrics (Hungate, 1966). Microbial degradation in the rumen, through the process of fermentation, produces end products such as, volatile fatty acids (VFA), methane, carbon dioxide, ATP, and heat (Hungate, 1966; Russell and Hespell, 1980). VFA are the primary energy sources for the ruminant animal, and the primary VFA of interest in the rumen are acetate, butyrate, and propionate (Hungate, 1966; Fulco, 1983). Ruminal carbohydrate fermentation can also affect dietary protein degradation. Dietary protein consumed by ruminants enters the rumen and is either degradable (ruminal degradable protein; RDP) or as undegradable (ruminal undegradable protein;

RUP). Microbes can degrade and ferment RDP to produce microbial crude protein (bacterial cells) or produce VFA or branched chain VFA (BCVFA) (van Houtert, 1993). Microbes ferment carbohydrates and proteins so that they can produce daughter cells which are composed of approximately 50% protein (MCP). MCP can be produced by direct assimilation of amino acids from dietary protein (or other microbes), or via de novo synthesis of amino acids from ammonia derived from either protein fermentation or non-protein nitrogen sources such as urea, which are combined with alpha-keto acids derived from carbohydrate fermentation (Johnson, 1976; Hoover and Stokes, 1991; Fessenden et al., 2019).

Evaluating the Rumen Microbiome Composition

Next Generation Sequencing (NGS) has provided a deeper and broader understanding of the microbial ecology of the rumen and intestinal tract of cattle (Stevens and Hume, 1998; Godoy-Vitorino et al., 2012; Petri et al., 2013). To date, the main NGS-based strategies that have been implemented for whole genome analysis include 16S ribosomal DNA (rDNA) sequencing and shotgun metagenomics (Dowd et al., 2008; Laudadio et al., 2019). 16s rRNA sequencing is a culture-independent molecular-based taxonomic assessment of the microbiota that utilizes polymerase chain reaction (PCR) to target and amplify portions of the hypervariable regions of the bacterial 16S rRNA gene through the use of degenerated primers and amplicon deep sequencing (Li et al., 2016; Laudadio et al., 2019). However, the exclusion of eukaryotic organisms and viruses from the analysis and the possible primer biases toward specific taxa are indicated limitations of 16S rRNA sequencing (Tremblay et al., 2015). Furthermore, while this powerful technology provides insight on phylogeny, it provides little information about their function

(Li et al., 2016; Wang et al., 2019b). Simply measuring microbial populations measures to describe the function of the rumen microbial ecosystem, ignores both the activities and ecological niches of the individual microbial populations (Carmichael and Callaway, 2019). In contrast, shotgun metagenomics relies on the sequencing of randomly sampled DNA fragments from a microbial community under a particular set of conditions that dictate function to provide insight on taxonomic composition and the metabolic activity (Gruninger et al., 2019). However, shotgun sequencing appears to be biased towards numerically abundant genes harbored by the most abundant microbial species, posing a limitation to this approach. Focusing on microbial population numbers does not tell the entire story of the impact of a microbial population in its environment. The rumen microbial community is very dense and diverse, and yet has largely remained a "black box" in terms of our understanding of composition and activity (Widder et al., 2016). Modern DNA sequencing technologies often use a hypervariable region of the highly conserved and universal 16S rRNA gene as a phylogenetic marker (Li et al., 2016; Laudadio et al., 2019) for microbiome analysis. The use of next generation sequencing (NGS) to accurately depict the ruminal microbial community composition requires a careful consideration of the bias introduced during sample processing (Knight et al., 2012). Bias introduced in the processing steps of DNA sequencing such as, PCR amplification (Pinto and Raskin, 2012), DNA extraction protocol (Feinstein et al., 2009) sequencing artifacts (Quince et al., 2009), DNA copy number (Kembel et al., 2012), and primer design (Andreson et al., 2008) can mask the true microbial community composition (Brooks et al., 2015). Therefore, it has been suggested that understanding systematic bias in analysis and processing will help give a more accurate depiction of microbial populations.

Based upon the lack of unified quality control methods in microbial analysis, a study was designed to quantify and characterize bias through the creation of small mock communities to understand how bias can affect NGS technology's depiction of microbial communities (Brooks et al., 2015); which demonstrated that models based on the analysis of small mock communities can improve our understanding of bias and enhance depiction of low diversity environments. However, in their utilization of a small population size, they concluded their methodology was impractical for larger, more diverse environments (i.e. rumen microbiome) due to the impractical number of mock communities necessary to represent the number of bacteria that need to be modeled.

A more recent study (Holman and Gzyl, 2019), suggested that by combining multiple 16S rRNA gene sequencing datasets and analyzing them together, large-scale patterns can be identified. In their meta-analysis they acquired 16S rRNA gene datasets from 53 different bovine gastrointestinal tract (GIT) microbiome studies to re-analyze archaeal and bacterial taxa in the GIT that may be shared among cattle regardless of experimental factors (Holman and Gzyl, 2019). This metanalysis included representation from both beef and dairy cattle from 29 different countries with different inclusions of positive and negative bias, and the use of more specific archaeal primer sets (V1 to V3, V3 to V4, V4 and V6 to V8) allowed them to identify an archaeal family that may be unique to those primers, and established bias toward rumen and fecal sampling. However, this also led them to hypothesize that other regions of the GIT may be underdefined and worth including in future microbial community studies.

Another limitation introduced by bias in DNA sequencing is that many rumen bacterial sequences cannot be reliably classified to the genus level, and sometimes not even at

the family level (Henderson et al., 2019). To improve the classification and resolution of rumen bacterial sequencing data, a recent study utilized the bacterial 16S rRNA gene sequence dataset created during the global rumen census (GRC) project (Henderson et al., 2015) to identify rumen bacterial groups in need of taxonomic refinement. PCR amplification bias has been demonstrated through the analysis of microbial composition amongst 16 ruminal samples via amplicon pyrosequencing of the V2 and V3 regions of the 16S rRNA gene (Jami and Mizrahi, 2012). Although bacterial communities (genus *Prevotella,* family *Lachnospiraceae,* and genus *Butyrivibrio*) were shared, their analyses comprised of a small sample of cattle with similar environmental exposures while utilizing only two primer sets.

Specific ruminal microbial taxa have been correlated with the production of specific ruminal metabolites (Taxis et al., 2015). Despite the establishment that rumen metabolites concentrations are based upon populations of ruminal bacteria, protozoa, fungi, archaea and bacteriophages populations, as well as composition of ingested feeds, and the host physiology (de Almeida et al., 2018), few studies have exclusively explored the rumen metabolome and microbiome in beef cattle. Metabolites produced by the microbiome affect host metabolic processes (Tang et al., 2019). While the utilization of metabolomics has been incorporated into the metanalysis of human microbiome studies (Wu et al., 2011; Koeth et al., 2013; Tang et al., 2019), only a few studies have incorporated modern metabolomics technologies to characterize the rumen microbiome. Furthermore, many of the studies focused on ruminal metabolites have focused on dietary effects on a single or few classes of molecules (e.g., amino acids, carbohydrates, or organic acids) rather than taking a broader, more holistic approach. A variety of methods have been utilized to explore the rumen metabolome and characterized ruminal metabolites such as

amino acids, carbohydrates, and organic acids (Saleem et al., 2012). Researchers comprehensively characterized the bovine ruminal fluid metabolome through various methods (NMR spectroscopy, inductively coupled plasma mass-spectroscopy, gas chromatography-mass spectrometry, direct flow injection, mass spectrometry and lipidomics with computer-aided literature mining to identify) to quantify and validate more than 200 different ruminal fluid metabolites (Saleem et al., 2013) which resulted in the creation of the Bovine Rumen Database (BRDB) (http://www.rumendb.ca) that contains 246 positively identified and quantified rumen metabolites. Metabolomics have also been used to assess ruminal (Wang et al., 2019a) metabolites' role in average daily gain (ADG) and indicated that 33 metabolites were associated with differences in ADG in beef cattle (Artegoitia et al., 2017). The higher levels of ruminal linoleic acid, alpha-linolenic acid and aromatic amino acids with lactic acids observed suggested the balance between microbial population and ruminal absorption of organic acids affects ADG of crossbreed beef steers. A more recent study (de Almeida et al., 2018), utilized untargeted metabolomics to provide a snapshot of the rumen fluid metabolome of 1,882 observed molecular features, including, amino acids, dicarboxylic acids, carboxylic acids, lactones, lignans, fatty acids derivatives and indole compounds, positively matching with the Global Natural Products Social Molecular Networking database. While the complexity of the rumen remains unknown, these findings suggest the potential of the rumen as a reservoir of novel compounds.

Metabolomic and metagenomic methodologies have the ability to combine evidence on the abundance of microorganisms and genes present in the rumen with information on the chemical composition of secondary microbial metabolites and expressed

proteins in the rumen (Henderson et al., 2015; de Almeida et al., 2018). While many studies have utilized a multi-omics approach combining metagenomics, metatranscriptomics, metaproteomics and metabolomics to study the rumen (Shabat et al., 2016; Comtet-Marre et al., 2017; Shaani et al., 2018), there must be a broad-based holistic understanding of the numerous factors that underlie the activities of specific population of microbiota in ruminants. Many approaches don't account for breed differences, environmental differences, dietary differences, and other outlying factors that decrease the repeatability and subsequent global application of studying the ruminal microbiome. Methodologies should be combined to further characterize interactions amongst feedstuffs, microbes of the rumen, and the ruminant animal to understand underlying activities of specific populations of microbiota and their metabolites in ruminants.

Degradation of Feed in the Rumen

Ruminants animals have the ability to take in large amounts of feed over short periods to thrive in competitive environments and avoid predation (Pérez-Barbería and Gordon, 1998). Cattle utilize their prehensile tongue to rip, pull, and tear forage for consumption (Baggett and Doran, 1971). Lower incisors, premolars, and molars are used to chew in a circular motion to grind and strip plant cell walls open. In ruminants chewing is critical to open plant cell walls via mechanical action to increase the microbial degradation of feedstuffs (Pond et al., 1984). It has been shown that steers fed various hay based diets were able to reduce ingested feed particle size up to 47% by chewing alone (Lee and Pearce, 1984). Saliva aids in chewing and swallowing, by lubrication and contains enzymes for the breakdown of lipids, recycling nitrogen to the rumen, and most importantly acts as a ruminal pH buffer (McDougall, 1948). Ingested feed and saliva form a bolus

which rapidly enters the rumen to be further degraded in the process of rumination. The process of rumination entails regurgitation of the feed from the ruminoreticulum, remastication, reinsalivation, and finally reswallowing (Fails et al., 2018). Indicated in Figure 1,

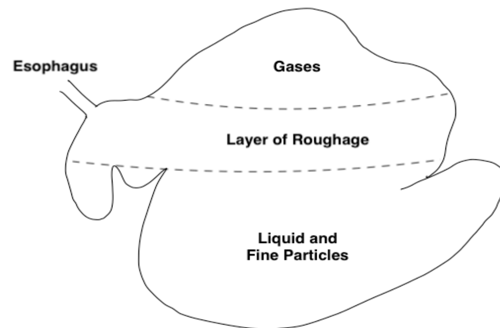

Figure 1. Stratification of rumen contents

as ingested feedstuff enters the rumen, heavier, finer, and more digested particles sink to the bottom or are solubilized in the ventral sac, while less dense (e.g., higher specific gravity) feeds sit on a top layer called the hay mat (layer of roughage). The dorsal sac is the upper compartment of the rumen that contains gases (e.g., CH_4, CO_2) produced by ruminal fermentation. Factors including intake, particle size, diet composition, and concentration of solids can influence the liquid dilution rate and particulate passage rate through the ruminoreticulum (Hungate, 1966; Church, 1969; Bruining and Bosch, 1992). “Scratch factors”, such as contact of roughage with the wall of the rumen account for the major stimulus for rumination, so that cattle average about 8 hours a day ruminating, with each rumination cycle requiring 1 minute (Fails et al., 2018).

Further enzymatic breakdown of ingested feedstuff is carried out by symbiotic microorganisms. The rumen provides a favorable environment for the growth of the ruminal consortium that is comprised of bacteria, fungi, protozoa, and archaea (Wang et al., 2017). Several physiological processes help maintain the constancy of this environment including the continual input of fermentable substrates, carbonate buffers in saliva, absorption of end products such as VFA, osmotic pressure, anerobic environment, stable temperature, eructation, and the outflow of end products (Callaway et al., 2010; Janssen, 2010). The continual input of fermentable material stimulates rumen motility and a complex series of ruminal contractions and processes resulting in rumination, eructation, and the passage of digesta to the omasum (Pearce and Moir, 1964). Saliva contains a carbonate buffer that helps to maintain a proper pH in the rumen (Allen, 1997), which normally ranges from 5.5 to 6.8 (Van Soest, 1994; Janssen, 2010) and varies with diet. The absorption of end products such as VFA and ammonia help maintain the rumen pH, as well as the frequency of feeding. The rumen is anaerobic and highly reduced and maintains a very low redox potential (Eh) (Falkowski et al., 2008). The consumption of oxygen by facultative anaerobic bacteria and other aerobic microorganisms helps maintain an anaerobic environment and low redox potential reducing conditions which are essential for anaerobic bacteria (Hungate et al., 1964).

Composition of the Rumen Microbiome

While calves are born essentially as monogastric animals, maternal contact quickly colonizes the calf with maternal microbes. Milk spilled from the esophageal groove (as well as any hay or grain consumed) can also provide a substrate in the rumen for bacterial fermentation (Anderson et al., 1987). As the ruminal microbial population

receives more nutrients, the increase in microbial fermentation produces VFAs, resulting in an increase in the growth and allometric development of the rumen (Harrison et al., 1960). However, recent research has demonstrated that the calf may not be born as "sterile" as was previously thought (Edrington et al., 2016; Hanson et al., 2016), and is seeded before birth with a diverse microbiota, but the microbiota changes rapidly postnatally (Conroy et al., 2009; Malmuthuge et al., 2015; Alipour et al., 2018), and continues to change as cattle grow and develop and are fed different diets at different stages of life.

Bacteria

Bacteria are thought to be the most abundant and metabolically active microbes in the rumen with over $10^{(9-10)}$ CFU/ml (Krause and Russell, 1996). Although ruminal bacteria are the most well characterized, only 15% of the total species have been cultured, and only 70 species are available in pure culture from public repositories (Morgavi et al., 2013; Creevey et al., 2014). It is known that the rumen microbial ecosystem is dominted by a core community composed mainly of the genera *Prevotella, Butyrivibrio,* and *Ruminococcus* as found by the Global Rumen Census (Henderson et al., 2015). Bacteria can be assigned to different ecologically functional groups, such as cellulolytic, amylolytic, and proteolytic species (Deusch et al., 2017). The rumen bacterial composition changes throughout the animal's life based on factors including diet, substrate balance, ruminal pH, physiological status, and metabolic end product availability (Anderson et al., 1987). Amylolytic bacteria are thought to be the most abundant bacterial population and play a major role in the degradation of readily fermentable starch and sugars that are the main component in high grain diets (Deusch et al., 2017). High forage diets are more favorable for gram negative bacterial populations, and high grain diets are more favorable for gram

positive bacteria (Hungate, 1966). As amylolytic rapidly ferment available starch, the ruminal pH can decrease enough to inhibit acid-tolerant cellulolytic and favor the growth of lactate-producing bacteria, such as *Streptococcus bovis* and *Lactobacillus sp*., (Khafipour et al., 2009). This can also lead to an increase in feed intake, the hosts' energy retention, and the risk of acidosis increases. Acidosis is associated with an increased accumulation of organic acids in the rumen due to the microbial fermentation of starch. Ruminal acidosis continues to be a common ruminal disorder in beef cattle that can result in marked reductions in cattle performance (Nagaraja and Titgemeyer, 2007).

Cellulolytic bacteria degrade the primary structural carbohydrates that comprise plant cell walls, cellulose and hemicellulose (Bryant, 1973; Leschine, 1995). *Fibrobacter succinogenes, Ruminococcus albus, Ruminococcus flavefaciens and Butyrivibrio fibrisolvens* are the key cellulolytic bacteria that are associated with the degradation of cellulose and hemicellulose in the rumen (Morgavi et al., 2013); they are also sensitive to low levels of ruminal pH (e.g., below 6.0) (Nagaraja and Titgemeyer, 2007). Fermentation end products from the activity of cellulolytic bacteria include acetate, butyrate, propionate, and CO_2, H_2, ethanol, succinate, lactate, and formate (Bauchop and Mountfort, 1981) some of which are utilized by other bacteria, protozoa, and archaea. *Methanobrevibacter ruminantium* is an anaerobic archaea that can utilize CO_2, H_2, and formate as substrates for methane production (Balch et al., 1979). Feeding high forage diets shifts the ruminal VFA profile in favor of cellulolytic bacterial acetate production at the expense of propionate (Beauchemin et al., 2009). This increase in cellulolytic bacterial populations, coin-

cides with an increase in the availability of H_2, ruminal acetate, ruminal pH, and thus ruminal methane concentrations all of which have previously been observed on forage-based diets (Van Kessel and Russell, 1996).

While it was long understood many proteolytic bacteria degrade peptides and amino acids, prior to further studies an individual bacterium could not be identified to explain the rate of ammonia production in mixed ruminal culture without the utilization of carbohydrates (Bladen et al., 1961). Eventually a specialized group of hyper amino acid fermenting (e.g., obligate amino acid fermenters) bacterial species were isolated and identified (Russell et al., 1988). These proteolytic organisms carry out primary hydrolysis of protein, peptides, and amino acids for their fermentation processes without carbohydrate fermentation allow many of them to occupy an ecological niche of obligate peptide and amino acid fermentation (Attwood et al., 1998).

Fungi

Fungi are thought to compose approximately 6% of the ruminal biomass (Lourenco et al., 2010), but they are poorly understood. Prior to the 1970s, rumen fungi were classified as zooflagellates (Akin and Borneman, 1990). Further investigations (Orpin, 1975, 1976) indicated they were actually fungal zoospores due to their obligately anaerobic nature, the presence of chitin in their cell walls, and their colonization of fibrous material. In vivo studies have observed that ruminal fungi possess a life-cycle alternating between a motile flagellated form (zoospore) and a non-motile, reproductive form (sporangium or zoosporangium) (Orpin, 1975, 1976). Fungi are thought to have a vital role in fiber degradation as their initial colonization and degradation of fibrous plant material allows feedstuff to be more accessible for bacteria (Akin and Borneman, 1990).

Rumen fungi produce high levels of cellulases, hemicellulases, and estrases that are responsible for degradation of cellulose and hemicellulose (Qi et al., 2011). Fungal fermentation of cellulose results in CO_2, H_2, lactate, and acetate (Akin and Borneman, 1990). Due to H_2 production, fungal abundance in the rumen has been found to increase in the presence of hydrogen-utilizing organisms, such as methanogenic archaea (Marvin-Sikkema et al., 1990).

Protozoa

Rumen protozoa were first described in 1843 (Gruby, 1843), but a true understanding of their role in the rumen still remains unclear (Williams et al., 2008). Protozoa are classified as fauna and are much larger than bacteria and account for 50% of the ruminal biomass (Williams and Coleman, 1997). The majority of ruminal protozoa are ciliates, although flagellates can be found more in the cecum and rumen of animals that lack ciliates (Williams, 1986). Ciliated protozoa are defined by the presence of cilia, hair-like organelles used for locomotion and feeding (Parry, 2004). Common protozoan genera found in the rumen include, *Entodinium, Epidinium, Polyplastron, Eudiplodinium, Isotrich, and Dasytricha* (Dehority, 1986; Shah et al., 2016). Due to the lack of culture collections and in vitro culture techniques for rumen ciliated protozoa, microscopic identification and counting have been the main means of protozoal community identification in the rumen (Williams, 1992; Dehority, 1993). Previous studies have utilized alternative techniques to identify and diversify protozoa including, 18S rRNA sequencing (Embley et al., 1995; Wright et al., 1997), denaturing gradient gel electrophoresis (DGGE) (Kittelmann and Janssen, 2011), and real-time PCR (Belanche et al., 2010; Kittelmann and Janssen, 2011). However, these alternative techniques for examining rumen ciliated

protozoa have limitations. Furthermore, rumen ciliated protozoa have two kinds of nuclei, micronucleus and macronucleus (Newbold et al., 2015), and the extremely high copy number of rDNA found in the macronucleus across different protozoal genera may skew the observed proportions of genera in a sample during molecular analysis (Medinger et al., 2010).

Protozoa prey upon bacteria in the rumen, and have been shown to selectively ingest or reject certain bacterial species (Williams and Coleman, 1997). Engulfed bacteria comprise the main source of nitrogenous compounds for protozoal growth (Williams, 1992). Protozoa degrade the engulfed microbial protein and either incorporate the amino acids directly into protozoal crude protein or ferment the amino acids and release amino acids and keto acids. However, this decreases the amount of microbial crude protein available to the animal. Defaunation leads to a decrease in ruminal ammonia production, but creates an increase in the efficiency of proteosynthesis resulting in an increase in the duodenal flow of microbial crude protein available to the animal (Williams, 1992; Gebeyehu and Mekasha, 2013). Previous studies support this as it has been shown that decreased ruminal ammonia concentration can partially be attributed to the decrease in ruminal recycling of bacterial nitrogen resulting from the lower protozoal populations on high concentrate diets (Hristov et al., 2001). Further experiments with defaunated animals indicate the significant role that ruminal protozoa play in nitrogen recycling and ammonia concentrations in the rumen (Bird and Leng, 1978; Jouany et al., 1988; Hristov et al., 2001).

Protozoa are frequently found in close association with methanogens (as endosymbionts and exosymbionts) and increasing methane emissions in the rumen (Vogels et al.,

1980). Methanogens utilize H_2 and CO_2 produced by protozoa during fermentation to produce methane and it has been estimated that between 9 and 37% of ruminal methane production can be attributed to methanogens associated with protozoa in the rumen (Finlay et al., 1994). Although the effect of defaunation on methane production in the rumen remains mixed, it has been suggested that the utilization of saponins, tannins, essential oils, or vaccines can decrease rumen methane emissions (Wright et al., 2004; Tavendale et al., 2005; Hook et al., 2010). While ruminal methanogens and fungi appear to have a symbiotic relationship, there is an antagonism between ruminal fungi and protozoa. Previous studies have indicated this is a result of competition for fiber substrates, protozoal enzymatic degradation of fungal cell walls (Gruninger et al., 2014), or the protozoal consumption of fungal spores (Hsu et al., 1991).

Protozoa exhibit diurnal population cycles which benefits the animal by preventing acidosis due to their ability to engulf and sequester starch from the activity of ruminal bacteria. Protozoa engulf starch granules and store them internally in the form of glycogen (Williams and Coleman, 1997), preventing the rapid bacterial fermentation of highly fermentable carbohydrates that would otherwise be used to produce lactate which leads to pH reduction and potential acidosis (Mackie et al., 1978). Furthermore, it has been suggested that the protozoal sequestration of starch (Karnati et al., 2009) may divert more carbon toward the slower VFA production in protozoa instead of using energy for fatty acid synthesis and ultimately increase VFA production (Newbold et al., 2015).

Archaeal Methanogens

Archaea are single cell obligate anaerobic organisms that compose up to 13.3 % of 16S and 18S rRNA in the rumen (Janssen and Kirs, 2008), and in the rumen the archaea are primarily methanogens. Most ruminal methanogens are hydrogenotrophic rather than acetoclastic (Janssen and Kirs, 2008), which means most ruminal methanogens metabolize hydrogen as an energy source of energy (Enzmann et al., 2018). Methanogens in the rumen can either live freely in the liquid portion, be part of the biofilms on feedstuffs or tissues, or act as endo/exo-symbionts with protozoa or fungi (Tokura et al., 1997). Only eight species of ruminal methanogens have been grown in pure culture: *Methanobacterium formicicum*, *Methanobacterium bryantii*, *Methanobrevibacter ruminantium*, *Methanobrevibacter millerae*, *Methanobrevibacter olleyae*, *Methanomicrobium mobile*, *Methanoculleus olentangyi*, and *Methanosarcina* barkeri (Janssen and Kirs, 2008). Most isolates have been from the family Methanobacteriacae, and this low methanogen diversity reflects how the rumen environment has selected for specific methanogens.

Interaction of methanogens with the ruminant and other rumen microbes

In the rumen methanogens frequently interact with other ruminal microbes, including protozoa, bacteria, and fungi through interspecies H_2 transfer (Wolfe, 1971; Balch et al., 1979). Interspecies H_2 transfer is a critical process during the catabolic process of fermentation because H_2 is produced by some microbes and utilized by others in a syntrophic process that maintains a low NADH/NAD ratio that makes anaerobic catabolism energetically favorable (Wolfe, 1971; Krause et al., 2013). Interspecies H_2 transfer has been demonstrated in co-cultures of methanogens with *Ruminococcus albus* (Wolin

et al., 1997), *Ruminococcus flavefaciens* (Latham and Wolin, 1977), and *Selenomonas ruminantium* (Scheifinger et al., 1975). The interaction between rumen bacteria and methanogens affects energy conservation, VFA profiles, and methane production by the rumen microbiome. Demonstrated by Bryant et al. (1967), in a pure culture of *Ruminococcus albus* there is a yield of 3 ATP, while when *Ruminococus albus* is grown in a mixed culture with a methanogen, such as *Methanobacterium ruminantium,* there is a yield of 4 ATP. *Methanobacterium ruminantium* is able to utilize the H_2 produced by *Ruminococus albus* via catabolism, along with carbon dioxide to produce methane. In return NADH and $FADH_2$ are regenerated at a faster rate allowing *Ruminococus albus* to produce more VFAs and ultimately ATP.

A small portion of ruminal methanogens are symbionts, either exosymbionts or endosymbionts with protozoa or fungi. Many methanogens that have endosymbiotic relationships with protozoa are attracted to them due to the presence of the hydrogenosome, which is a membrane bound organelle that produces H_2 via malate oxidization (Muller, 1993). Hydrogen generated by rumen protozoa could be utilized by these protozoa-associated methanogens, which benefits both parties (Wrede et al., 2012). Methanogens have also been observed as ectosymbionts (Valle et al., 2015), whose populations can be affected by the relative contribution of H_2 production by rumen ciliate protozoa and H_2-producing bacteria (Sharp et al., 1998). It is important to note however, it is difficult to distinguish engulfed methanogens from true endosymbiotic methanogens. As an example, methanogens were previously detected along with some rumen fungal isolates, but it was not reported if the methanogens and fungi had any physical association as either ectosymbionts or endosymbionts (Patra et al., 2017).

End Products of Microbial Fermentation

Carbohydrate Fermentation

Carbohydrates in feedstuff comprise the major proportion of the feed of ruminants, with forages usually being a large portion of the feed in dairy diets, but grains comprising a larger portion of beef diets (Dynes et al., 2003; Nafikov and Beitz, 2007). Forage cell walls contain highly digestible contents (e.g., soluble sugars) encased by a less digestible cell wall (Jung and Allen, 1995), composed of polymeric carbohydrates including cellulose, hemicellulose, and pectin. Cellulose is an insoluble polysaccharide comprised of chains of glucose monomers with β 1-4 linkages (Choct, 1997),whereas hemicellulose is a more heterogenous polysaccharides that is characterized by β 1-4 linked backbones (Wong et al., 1988).

Starch is a reserve energy store commonly found in grains and can be almost completely and uniformly digested in the gastrointestinal tract when adequately processed (Nocek and Tamminga, 1991). Starch is a readily mobilized polysaccharide reserve for the plants, that can be found in leaves, tubers, legume seeds, and cereal grain endospores (Ashford and Gubler, 1984). Starch is composed of amylose and amylopectin. Amylose, making up a smaller portion of starch, is a linear molecule with many α 1-4 linkages and very few branched α 1-6 linkages. Amylopectin is a larger and less dense molecule with more branched α 1-6 linkages and fewer α 1-4 linkages (Imberty and Pérez, 1989). The amylose: amylopectin ratio in a feedstuff impacts its starch digestibility. Feedstuff with a greater percentage of amylopectin have greater digestibility due to the increased surface area from branched α 1-6 linkages. Environmental factors such as

temperature can influence the amylose: amylopectin ratio, with higher temperatures increasing the amount of long amylopectin chains (Bertuzzi et al., 2012).

Soluble carbohydrate fermentation in the rumen relies on microbial enzymes for degradation (Huntington, 1997)(Figure 2). Following the catabolism of complex carbohydrates into oligosaccharides and ultimately to simple sugars, metabolism of these disaccharide or monosaccharides sugars by the microorganisms produces ATP that is utilized by the microbe (Walker, 1968). The end products of fermentation are primarily VFA, methane, CO_2, H_2, and heat; but other end products include formate, ethanol, lactate and succinate that are produced in smaller fractions (Bauchop and Mountfort, 1981). Microbial fermentation allows ruminants to utilize polysaccharides that monogastrics cannot, however this advantage comes at a cost of efficiency for ruminants because some of these end products are not utilizable by the host animal, and the process of fermentation is inherently inefficient. Cattle are deemed inefficient as they can lose 2 to 12% of their gross energy intake as CH_4 emissions (Johnson and Johnson, 1995).

Volatile Fatty Acid production and absorption

VFA are the primary energy source for ruminants and are produced in the rumen via the microbial fermentation of carbohydrates (Mallèvre, 1891; Baker, 1942). While acetate, propionate, and butyrate contribute about 97% of the total VFA production, branched chain VFA such as isobutyrate, valerate and caproate are also produced (Allison, 1978). The ratios of acetate, propionate, and butyrate (Cummings et al., 1987) generated by the ruminal fermentation varies widely and is affected by the type of feedstuff fed, and the species and quantity of rumen bacteria that can utilize the diet as well as other environmental conditions (Alstrup et al., 2016; Wang et al., 2020). The rate

of VFA absorption across the ruminal epithelium is influenced by factors such as pH, osmolality of fluid, effective surface area, and type and concentration of VFA (Bergman, 1990).

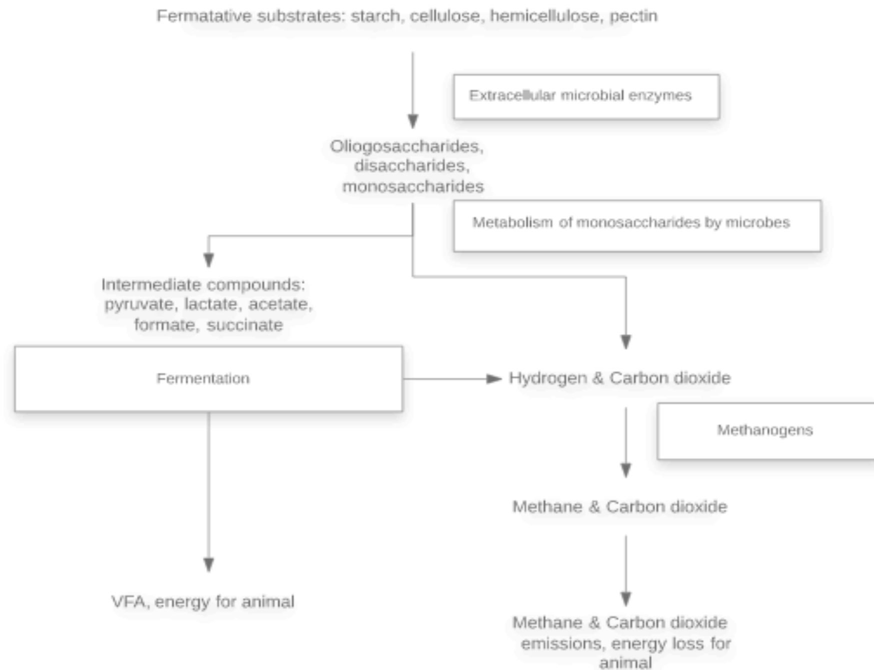

Figure 2: Carbohydrate Fermentation

Passive diffusion of nonionized VFA across ruminal epithelium is the most common ruminal absorption theory followed by the mechanism of bicarbonate exchange (Dijkstra et al., 1993; Gäbel et al., 2002). VFA are weak acids (with a pK_a of < 4.8) therefore, when the ruminal pH drops below 5.8, VFA are rapidly absorbed from the rumen in the nonionized form (Bergman, 1990). When the ruminal pH is above 5.8, most of the VFA occur in the ionized state, while the absorption of nonionized (protonated) VFA by the rumen epithelium occurs passively (Bugaut, 1987).The shift in favor of the absorption

of ionized VFA can occur with the accumulation of bicarbonate within the rumen contents, as well as the absorption of ionized VFA with no associated appearance of bicarbonate in the rumen (Masson and Phillipson, 1951; Stevens et al., 1980; Gäbel et al., 2002). Absorption of ionized VFA by the rumen epithelium occurs in exchange for bicarbonate secreted from the epithelial cells (Gäbel et al., 2002), acting as a major factor moderating ruminal pH. It has been estimated that salivary buffers neutralize from 15% (Gäbel et al., 2002) to 40% (Allen, 1997) of the VFA produced in the rumen. An increase in saliva secretion can help buffer the rumen and reduce the time in which pH drops below 5.8 following meals (Gäbel et al., 1991). In comparison to more fibrous diets, high concentrate diets cause a decrease in saliva production (Dijkstra et al., 1992), resulting in decreased rumination and ruminal pH. Bouts of low pH can occur when VFA production is rapid and exceeds the capacity of the rumen to maintain equilibrium (McAllister et al., 2011). The drop in ruminal pH enhances the absorption of VFA from the rumen, and earlier studies have shown hydration of ruminal CO_2 provides a source of hydrogen ions for the production of nonionized VFA (Dobson, 1984).

Highly fibrous, low energy feed consumption by ruminants result in microbial populations that produce higher ratios of acetate to propionate and higher levels of methane and CO_2 (Beauchemin et al., 2009). In the rumen, acetate (two carbons) is the major VFA produced (Clark and Cronan, 2005). Absorbed acetate is primarily oxidized by the host animal through the citric acid cycle and provides energy to the animal that allows the conservation of glucose for other functions (Wolfe, 2005). Acetate is also a source of acetyl CoA for lipogenesis.

Feeds high in rapidly fermentable carbohydrates (e.g., starch) result in microbial populations that produce relatively more propionate and butyrate than acetate. Propionate production subsequently results in less energy lost to methane and CO_2 production (Lana et al., 1998; Russell, 1998). Absorbed propionate (three carbons) is metabolized to glucose in the liver, via gluconeogenesis (Young, 1977). The fairly constant supply of propionate allows for a continual production of glucose in the blood. In contrast, when there are insufficient propionate levels available to cattle, body fat can mobilize to meet the energetic needs, decreasing animal body condition.

Butyrate is primarily produced from fermentation of forage and is a 4-carbon acid. When butyrate is absorbed it is primarily converted in the epithelial tissue to the ketone beta-hydroxybutyric acid during absorption (Masson and Phillipson, 1951). Beta-hydroxybutyric acid is a significant source of energy for metabolic functions in epithelial tissues as well as to the mammary gland. Butyrate levels in the hindgut are linked with increased tight junction integrity, and low levels of butyrate are correlated with "leaky gut syndrome" in cattle (Kvidera et al., 2017).

Methane

CO_2 and methane produced during fermentation are either removed through the rumen wall or by eructation (Johnson and Johnson, 1995). The highly reduced, anaerobic rumen is a nearly ideal environment for methanogens. Methane production is a concern for its contribution to the accumulation of greenhouse gases in the atmosphere (McAllister and Newbold, 2008). Greenhouse gases such as carbon dioxide, methane, ni-

trous oxide, and ozone contribute to climate change and global warming through their absorption of infrared radiation in the atmosphere (Solomon et al., 2007; McAllister and Newbold, 2008; Smith et al., 2008).

Diet impacts the ruminal microbial population as well as the fermentation end products which in turn affects the ruminant's relationship with its native population of methanogens (Johnson and Johnson, 1995). The greatest methanogen inhibition or reduction in methane production usually occurs when cattle are fed a high concentrate diet (Sauvant and Giger-Reverdin, 2007). The acetate to propionate ratio is generally lower when cattle are fed cereal grains than forages (Russell, 1998). Starch-degrading bacteria produce more propionate, while fiber degraders tend to produce more acetate. As propionate levels in the rumen increase, acetate levels decrease, which is typically is accompanied by a decrease in ruminal pH due to the higher levels of lactic acid production from starch fermentation, typically from *Lactobacillus* activity. The methanogen population and methanogenesis also decrease, but so does the host feed intake and energy retention (Hegarty et al., 2007). While there have been attempts to mitigate methane production by methanogens (Smith et al., 2008; Beauchemin et al., 2009), the ecology of the rumen reverts back to initial levels of methane production through adaptive mechanisms and redundancy within the ecological niches (McAllister and Newbold, 2008).

Microbial crude protein

Bacterial cells are composed of 50% protein, and cell production is the primary goal of microbial fermentation. Microbial crude protein has a similar amino acid profile to meat, thus reducing the need for supplemental limiting amino acids (Agricultural Research Counsil, 1980; Storm and Orskov, 1983). Intake protein enters the rumen as

undegradable protein (RUP) or degradable protein (RDP) (Sloan et al., 1988). RUP is unavailable to rumen microbial fermentation and passes through to the abomasum where it is denatured and hydrolyzed to be made available to the animal (Chalupa, 1975). In addition to true protein, nitrogen in the feed can occur in the form of non-protein nitrogen (NPN) (Kertz, 2010). As NPN (e.g., urea) enters the rumen, microbial urease hydrolyzes it into ammonia and carbon dioxide and the resultant ammonia can be combined with an alpha-keto acid carbon skeleton from carbohydrate fermentation to produce microbial protein. Once synthesized, MCP leaves the rumen and travels through the omasum, and reaches the abomasum where it is degraded and denatured by the hosts' enzymes, pepsin and HCl (Stern et al., 1985). Amino acids released from this hydrolysis are absorbed in the small intestine (Crawford et al., 1978; van Dijk et al., 1983; Stern et al., 1985). Utilization of NPN results in an upgrade of nitrogen sources provided to the ruminant animal which is mediated by the microbial population and which decreases the exogeneous amino acid requirement for ruminants (Kung and Rode, 1996).

Carbohydrate availability is the greatest driver of ruminal NPN utilization (Hristov and Ropp, 2003). Microbial growth in the rumen is proportional to the intake and extent of carbohydrate fermentation coupled with an adequate nitrogen source available to the microbes (Russell et al., 1992). The most common source of NPN in cattle rations is urea, which is 292% CP (Plumlee, 2004). While urea is not required in the diet of ruminant animals, the primary reason for the utilization of urea as a substitute for other common high-protein ingredients is the lower cost for protein supplementation. As of April 2020, soybean meal was priced at $363.83 USD per metric ton, while urea was priced at $235 USD per metric ton (© 2020 CME Group Inc).

Utilizing urea as a cheaper alternative to increase nitrogen supplementation has been widely utilized in feedlots (Burkhardt, 1971), which demonstrated a benefit in early weight gains and feed efficiency. Although urea is commonly used in ruminant diets, it is recommended that urea concentrations be limited to 40 g/100kg of animal body weight to avoid urea toxicity (Santos, 2011). As urea is degraded, excess ammonia can be absorbed through the rumen wall and eventually drained via the portal vein to the liver (Pisulewski et al., 1981). In the liver ammonia is detoxified by being incorporated in urea, which can be excreted by the kidneys as waste, or recycled back into the rumen via transfer through saliva. Salivary N recycling ensures there is a constant source of nitrogen in the rumen in cases of nitrogen limiting diets. Protein supplementation to cattle consuming low-quality forage increases forage utilization and improves animal performance, by providing N sources to capture carbon in the form of MCP. Wickersham et al. (2008), reported that supplementation of urea increased forage utilization and nitrogen recycling in steers that were fed low quality forage.

Feedlot Cattle Production

Cattle production is the most important agricultural industry in the United States, accounting for $67.1 billion in cash receipts in 2018 (USDA, 2019). Within the U.S beef industry, feedlots are the most common finishing stage for beef cattle and focus on feeding a predominantly grain diet that favors improved feed efficiency, and efficient bone, muscle, and fat tissue growth (USDA, 2019). Cereal grains (e.g., corn) are a major dietary source for cattle due to their high concentrations of starch, a component that is almost completely and uniformly digested in the gastrointestinal tract when adequately processed (Nocek and Tamminga, 1991). Ultimately on a high grain diet, energy density is

increased and therefore production can be optimized in a well-managed intensive feedlot system (Huntington, 1997). Feed costs are the largest variable expense associated with producing beef cattle (Lancaster et al., 2009), accounting for approximately 75% of the cost of producing finished cattle (Hill, 2012). Therefore, improving efficiency will decrease feeding costs, reduce days spent in the feedlot and all associated expenses, and decrease environmental impacts of beef and dairy production.

Impact of Diet on the Ruminant and Rumen Microbiome

Most calves are born in the spring and are weaned at about six to ten months of age (450 to 700 pounds). They are either retained to replace older animals or to expand the herd, or steers and excess heifers are sold (USDA, 2019). To further growth and development, steers and heifers may enter a backgrounding program where they continue to graze on grass for 3-4 months along with some grain supplementation (Pelletier et al., 2010). After backgrounding, cattle enter the feedlot where they are fed a high concentrate diet to gain on average 2.5 to 4 kilograms per day while using approximately 6 kilograms of dry-matter feed per kilogram of gain (USDA, 2019)

Outside of the feedlot, forages are the main feed source in ruminant diets, and can represent up to 83% of beef cattle diets (Barnes, 2003). Higher fiber content diets lead to an increase in ruminal microbial diversity, however a more diverse microbial population produces a more diverse array of metabolites that results in a decrease in energetic efficiency (Shabat et al., 2016). Saliva production is increased when cattle are fed forage diets which increases rumination and the liquid dilution rate (Allen, 1997). Increasing rumination and salivation ultimately aids in the maintenance of the normal range of pH in the rumen (5.7-6.7) and results in an increase in the acetate to propionate ratio

High concentrate diets are often fed to beef cattle to increase yield and efficiency (Yang et al., 2012). The addition of grain to forage diets decreases ruminal pH and passage rate due to an increase in starch and soluble sugars resulting in shifts in fermentation end products and microbial composition (Russell, 1998). Although grain feeding causes a decrease in ruminal pH, the increase in propionate concentrations increase decrease methane production and animal efficiency.

Implications

As the global human and livestock productions continue to increase (USDA, 2020), the development of advanced methods to manipulate the rumen microbiome will be necessary for the improvement of ruminant nutrition, productivity, and efficiency whilst reducing ruminant environmental impacts (Yáñez-Ruiz et al., 2015). The previous utilization of "omics" approaches, traditional quantitative genetics approaches, dietary interventions, or analysis of relationships with feed efficiency, identified that the influence of the gut microbiome on food safety, animal health, production parameters, metabolomics, and host immunity are more complex and vastly more interconnected that we previously appreciated (Carmichael and Callaway, 2019). There is still a need to deeply understand the rumen microbiome holistically, in addition to identifying factors that affect the efficiency of beef cattle (Clemmons et al., 2019). Diet is considered the single largest expense in most commercial beef production operations, suggesting that any effort at improving the efficiency of feed use will help reduce input costs (Arthur et al., 2001). The use of Next Generation Sequencing has allowed for deeper and broader characterizations of the microbial ecology of the rumen. While the potential to manipulate the rumen mi-

crobiome and predict global livestock challenges has been investigated using NGS technologies, further understanding of the complex interrelationships that underlie the activities of specific populations of microbiota and their metabolites in ruminants is necessary. Utilization of multiple methodologies in combination can further characterize interactions amongst feedstuffs, microbes of the rumen, and the ruminant animal based upon the specific dietary requirements that provide substrates for microbial metabolism and host animal needs. While this combination cannot provide all ruminant and rumen microbiome causal mechanisms, it can serve as an aid in understanding the interconnectivity of host genetics, microbial functional genomics, microbial ecology and fermentation, and host performance assessment when developing microbial manipulation methods for improving cattle feed efficiency.

CHAPTER 3

THE APPLICATION OF DNA SEQUENCING TECHNOLOGIES AS AN AID IN THE METAANALYSIS OF THE RUMINAL MICROBIOME OF BEEF STEERS FROM A COMMERCIAL FEEDLOT[1]

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Abstract

The ruminal microbiota of cattle allows them to convert low quality feedstuffs such as forages into higher quality human foods via the process of fermentation. The activity and end products of the ruminal fermentation can affect cattle feed efficiency. The present study assessed the relationship between cattle feed efficiency and their ruminal microbial populations across their feedlot period. Angus steers (n=65) steers were fed a feedlot-finishing diet for 82 days and their growth performance was evaluated, including: dry matter intake (DMI), average daily gain (ADG), and residual feed intake (RFI). Steers were rank ordered based upon their RFI, and the 5 lowest RFI (most efficient) and 5 highest RFI (least efficient) were selected. Ruminal samples were collected at the beginning (d 0) and end (d 82) of the feedlot-finishing period and microbial DNA was extracted, and the 16S rRNA gene was sequenced. There were no significant differences ($P = 0.82$) in ADG between the two groups of steers during the 82-d feedlot period; however, the most efficient animals did have had a lower ($P = 0.03$) DMI and lower ($P = 0.003$) RFI. The less-efficient (High RFI) steers had higher ($P = 0.01$) ruminal *Methanobrevibacter* populations ($P = 0.01$) of in their rumens during the feedlot period and tended ($P = 0.09$) to have a greater in the abundance of *Methanosphaera* during the feedlot period. In the rumen of the high-efficiency steers, abundance of *Ruminococcaceae* was increased ($P = 0.04$) over the course of the study. Findings suggest that the most efficient steers ruminal microbial populations favored structural carbohydrate degradation, resulting in more ruminal catabolism during the feedlot phase, which resulted in more energy harvested from their diets and improved feed efficiency.

Introduction

The symbiotic relationship between ruminant animals and their resident ruminal microbial consortium allows ruminants, such as cattle, to occupy a specific ecological niche as consumers of cellulosic fiber (Hungate, 1966). The presence of a native ruminal consortium allows ruminant animals to convert fibrous feedstuffs into high quality food products via microbial fermentation (Owens and Goetsch, 1988). Because mammals do not produce fiber degrading enzymes, ruminants are entirely dependent on the degradative capacity of the ruminal bacteria, fungi, and protozoa to degrade cellulose and hemicellulose (Russell and Rychlik, 2001).

Feed costs are the largest variable expense associated with producing beef cattle (Lancaster et al., 2009), accounting for approximately 75% of the cost of producing finished cattle (Hill, 2012). Within the U.S beef cattle production industry, feedlots utilize a predominantly grain diet to ensure improved feed efficiency, and efficient bone and muscle growth (USDA, 2019). Cereal grains are a major energy source for cattle due to their high concentrations of starch, a component that is almost completely and uniformly digested in the gastrointestinal tract when adequately processed (Nocek and Tamminga, 1991). When cattle are fed a grain-based diet, energy density is increased and therefore production can be optimized in a well-managed intensive feedlot system (Huntington, 1997). Improving food efficiency will both decrease both feeding costs and decrease environmental impacts of beef and dairy production (Nkrumah et al., 2006).

Residual Average Daily Gain (RADG) is a feed efficiency selection tool adopted by the American Angus Association in their breeding selection programs because of its high degree of heritability (North, 2010; Association, 2015)(~0.3 (Association, 2019), RADG

can be a predictor of a sire's genetic ability for postweaning gain in future progeny compared to that of other sires, given a constant amount of feed consumed, allowing producers to select for genetics that increase the efficiency of finishing cattle in a feedlot environment. Previous studies have shown a close association between the ruminal microbiota composition and fermentation end products and cattle feed efficiency (Nkrumah et al., 2006; Hernandez-Sanabria et al., 2012; Shabat et al., 2016). Therefore, the present study was designed to compare the ruminal microbiome of beef cattle with different feed efficiencies at the beginning and end of the feedlot phase. We hypothesized that animals with different feed efficiencies would experience different changes in their microbial compositions over the course of their finishing period.

Materials and Methods

Animals, experimental design, and management

All procedures involving animals were verified and approved by the University of Georgia's Office of Animal Care and Use (AUP #A2012 11-006-R1). This study was conducted as a smaller portion of a more expansive study (Detweiler et al., 2019). Briefly, for five generations, commercial Angus cows were bred to high and low efficiency bulls to determine the effect of selection using RADG and marbling expected progeny differences (EPDs) on productivity, performance, and carcass quality of cattle grown from this selected herd. The current study utilized the fifth generation of steers born to that selection program and analyzed their performance in a grain fed feedlot-finishing system. The commercial feedlot (Ridgefield Farm L.L.C.; Brasstown, North Carolina; 35.0391° N, 83.9576°W) was used from March 8, 2018 to May 29, 2018. A total of 65 steers were included in the feedlot study, and steers were rank ordered based upon

their residual feed intake (RFI) data collected in the feedlot period, and the 5 most efficient and 5 least efficient steers were selected for the present analysis of their ruminal microbiomes.

Steers were adapted to the high grain feedlot finishing ration for 21 d, and all rations were formulated to meet the nutrient requirements for finishing cattle are shown in Table 3.1 NRC (2000). Steers were fed the transition diet from d 0-21, and the finishing diet from d 22-82. Over the 82-d feeding trial daily feed intake was recorded using a GrowSafe feed intake monitoring system (GrowSafe Systems® Ltd., Calgary, Canada), that monitors individual feed intake. Steers were weighed at the beginning, mid-point, and end of the feedlot experimental period (d 0, 41, and 82, respectively). Using the individual dry matter intakes (DMI) and body weight gains for each steer, RFI was calculated as the difference between actual and expected DMI. The expected DMI was the residual estimate for each steer, following linear regression of midpoint (41-d) metabolic body weight and average daily gain (ADG) on actual DMI.

Sample collection, DNA extraction and sequencing

Ruminal samples were collected– upon entering (d0) and exiting (d82) the feedlot using methodology described previously by Lourenco et al. (2019). All samples were immediately placed on wet ice and transported to the laboratory, where they were frozen and stored at -20°C until further processing. DNA was extracted from the rumen fluid samples according to the semi-automated extraction protocol (Rothrock et al., 2014). Briefly, the procedure started with 0.5 mL of homogenized rumen fluid and achieved extraction of DNA through a combination of mechanic and enzymatic methods. The mechanic portion of the protocol used a FastPrep 24 Instrument (MP Biomedicals LLC, Ir

Table 3.1: Composition of the diets used during the transition and finishing periods of the feedlot trial. Transition diet was fed for 21 d prior to the Finishing diet which was fed for d 22-82

	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
Feedlot NEm, Mcal/cwt	91.84	95.17
Feedlot NEg, Mcal/cwt	62.10	65.00
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
Trace Mineral Salt, %	0.21	0.22

vine, CA, USA) to disrupt the cells. The enzymatic portion used InhibitEX® Tablets (QIAGEN, Venlo, Netherlands). Elution and purification of DNA were performed using an automated robotic workstation (QIAcube; QIAGEN, Venlo, Netherlands). Determination of DNA concentration and purity were performed spectrophotometrically using the Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments Inc; Winooski, VT, USA).

After DNA extraction, samples were taken to the Georgia Genomics and Bioinformatics Core facility (https://dna.uga.edu) for library preparation and 16S rRNA gene sequencing. Library preparation 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). Samples were sequenced using the Illumina MiSeq system (Illumina Inc., San Diego, CA, USA).

Sequencing data and statistical analyses

Sequencing data were demultiplexed and downloaded as FASTQ files. Pair-end reads were 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 converted into FASTA files and quality filtered. The sequences were clustered into operational taxonomic units (OTUs) at 97% similarity according to the Greengenes database. Singleton OTUs were excluded from the analysis. A sampling depth of 28,662 sequences per sample was used for the diversity analyses. The computed alpha-diversity indexes were: number of observed OTUs, Chao1, species evenness, Shannon index, and Faith's phylogenetic diversity.

Statistical analyses were performed using the software Minitab® v18.1. The alpha-diversity indexes and bacterial relative abundances were analyzed by ANOVA within each individual group of steers (i.e. low- vs. high-RFI), and feedlot period (beginning or end) was used as factor. Animal performance data was analyzed across the RFI groups (high or low). Results were considered significant when $P \leq 0.05$ and were categorized as trends when $0.05 < P \leq 0.10$.

Results and Discussion

Steer growth and efficiency performance

Feedlot cattle are typically fed high grain rations to maximize their rate of gain, as well as to increase intramuscular marbling and carcass quality (Nocek and Tamminga, 1991; Huntington, 1997; Benchaar et al., 2001). Grain diets typically are high in starch, a readily fermentable substrate which can alter the microbial population composition of the rumen (Nocek and Tamminga, 1991; Ferraretto et al., 2013). Performance and efficiency of each steer were individually assessed throughout the 82-d feedlot period by measuring DMI, ADG, feed:gain ratio, RFI, and hot carcass weights (HCW; Table 3.2). The most efficient steers (low-RFI) had a lower average DMI ($P = 0.03$) than did the least efficient (high-RFI). In addition, the average difference in RFI between the least and the most efficient groups was 1.85 kg DMI/day ($P = 0.003$). It was expected that more efficient animals would have a lower DMI and lower RFI, which has been previously demonstrated in both dairy and beef cattle (Elolimy et al., 2018).

Table 3.2. Animal performance data observed during the finishing period according to Residual Feed Intake (RFI) classification in feedlot steers (n=5/group).

	RFI Classification		
Item	**High**	**Low**	***P*-value[1]**
Dry matter intake (DMI), kg/day	13.02	10.89	**0.03**
Residual feed Intake (RFI), kg	0.76	-1.09	**0.003**
Feed:gain ratio, kg	12.43	11.27	0.37
Average daily gain (ADG), kg/day	1.05	1.02	0.82
Hot carcass weight (HCW), kg	367.9	378.6	0.62

[1] P-value for the contrast between high and low-RFI steers.

Bacterial populations

Analysis of rumen fluid samples identified the 10 phyla with relative abundance equal or greater than 0.2% in both high-RFI (low efficiency) and low-RFI (high efficiency) steers (Tables 3.3 and 3.4). The relative abundance of the predominant phyla varied both over the course of the 82-d feedlot period, as well as between the high- and low-RFI steers. At the beginning of the feedlot period, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* were the most predominant phyla for both high- (Table 3.3) and low-RFI (Table 3.4) steers, accounting for more than 90% of the total OTU in these steers. However, at the end of the feedlot period (d 82), the top 3 phyla (Table 3.3 and 3.4) were *Firmicutes*, followed by *Bacteroidetes* and *Proteobacteria*, which collectively composed more than 88% of the OTU.

Table 3.3 Bacterial abundance* (phyla level) observed at the beginning (d0) and end (d82) of the finishing period for high-residual feed intake (RFI) Angus steers (n = 5 steers).

Bacterial Phyla	Feedlot-Finishing Period		Average Abundance	*P*-value[1]
	Beginning	End		
Firmicutes	41.34	60.37	50.86	0.08
Bacteroidetes	47.34	25.98	36.66	0.09
Actinobacteria	3.01	1.56	2.28	0.19
Proteobacteria	0.97	2.27	1.62	0.12
Euryarchaeota	1.24	2.01	1.62	0.01
TM7	1.05	2.12	1.59	0.09
Spirochaetes	1.40	0.53	0.97	0.06
Tenericutes	0.32	0.89	0.61	0.14
Cyanobacteria	0.49	0.44	0.46	0.81
Planctomycetes	0.35	0.39	0.37	0.82
Other Phyla	2.49	3.43	2.96	0.07
Firmicutes:Bacteroidetes ratio	1.14	2.80	1.97	0.05

* Only phyla with average abundance ≥ 0.2% are shown.

[1] P-value for the contrast between beginning and end of the feedlot-finishing period.

The populations of *Firmicutes* were higher at the end of the finishing period compared to the beginning in the most efficient animals (i.e. low-RFI; $P = 0.02$; Table 3.4); as well as in the least efficient animals (high-RFI; Table 3.3), although the increase was not as large ($P = 0.08$). Additionally, there was a trend ($P = 0.09$) for *Bacteroidetes* to be lower in abundance in the rumen of the least efficient animals during the feedlot period. Interestingly, an archaeal phylum with low abundance in the rumen, *Euryarchaeota*, was significantly higher at the end of the finishing period than at the beginning in the least efficient steers. The phylum *Euryarchaeota* contains a diverse group of obligate anaerobic methanogens (Holmes and Smith, 2016). The increase in the abundance of *Euryarchaeota* subsequently resulted in an increase in families *Methanobrevibacter* and *Methanosphaera* within this phylum (Figure 3.2).

Table 3.4. Bacterial abundance* (phyla level) observed at the beginning (d0) and end (d82) of the finishing period for low-residual feed intake (RFI) Angus steers (n= 5 steers).

	Feedlot-Finishing Period			
Bacterial Phylum	Beginning	End	Average Abundance	*P*-value[1]
Firmicutes	34.95	55.14	45.04	0.02
Bacteroidetes	48.73	33.04	40.88	0.12
Actinobacteria	7.21	1.29	4.25	0.34
TM7	1.81	2.06	1.94	0.85
Proteobacteria	1.48	2.31	1.90	0.43
Euryarchaeota	1.28	1.39	1.34	0.83
Spirochaetes	1.31	0.81	1.06	0.42
Cyanobacteria	0.39	0.33	0.36	0.74
Tenericutes	0.16	0.46	0.31	0.20
Planctomycetes	0.21	0.27	0.24	0.55
Other Phyla	2.46	2.91	2.69	0.55
Firmicutes:Bacteroidetes ratio	0.78	1.96	1.37	0.10

* Only phyla with average abundance ≥ 0.2% are shown.

[1] P-value for the contrast between beginning and end of the feedlot-finishing period.

The *Firmicutes:Bacteroidetes* increased (*P*= 0.05) in the least efficient steers (Table 3.3) and tended (*P*= 0.10) to increase in the more efficient steers (Table 3.4) from the beginning to the end of the feeding trial. A classical study identified a link between the *Firmicutes:Bacteroidetes* ratio and obesity in mice (Turnbaugh et al., 2006), indicating that this ratio affected the efficiency by which those animals harvested energy from their diets. However, the importance of this ratio in the microbiome action and host physiology is still controversial, poorly understood, and should not be overemphasized (McCann). The increase in *Firmicutes:Bacteroidetes* during the 82-d feedlot period, suggests that in both efficiency groups the ruminal microbial populations changed to harvest more energy from the diet. The limited numbers of steers used to maximize the differences between the two groups poses a clear limitation to the power of the present study.

Prevotellaceae was the most prevalent family identified in both groups of steers at the beginning of the feedlot period, with relative abundances averaging approximately 38% of the total population for both RFI groups (Figure 3.1). However, at the end of the feedlot period, *Prevotellaceae* were significantly decreased (*P* = 0.05) in the rumen of both high- and low-RFI steers (to 14 and 20%, respectively; Figure 3.1). The family *Prevotellaceae* consists of amylolytic gram negative bacteria that proliferate rapidly in the presence of high-starch diets such as feedlot diets (Mackie and Gilchrist, 1979; Slyter, 1986; Goad et al., 1998) Thus, the decrease of *Prevotellaceae* observed in both groups of steers during the feedlot phase when cattle were fed a high starch containing diet was unexpected. However, the family *Ruminococcaceae* contains ruminal fiber degrading bacteria

(Leschine, 1995; Rainey, 2009), and the abundance of *Ruminococcaceae* numerically increased in both groups of steers, which could be explained by environmental niche-filling from the decrease in Prevotellaceae, and could also have some potential mitigation of the effects of low ruminal pH associated with the starch containing feedlot diet (Cotta, 1992; Matsui et al., 2000). *Ruminococcaceae* were increased in the most efficient animals (Figure 3.1; $P = 0.04$), whereas, in the least efficient animals, this increase was less extensive ($P = 0.17$). We hypothesized that pH stabilization would allow more extensive fiber (NDF) degradation to occur in the low RFI group, which could be one of the mechanisms leading to the difference in efficiency between groups.

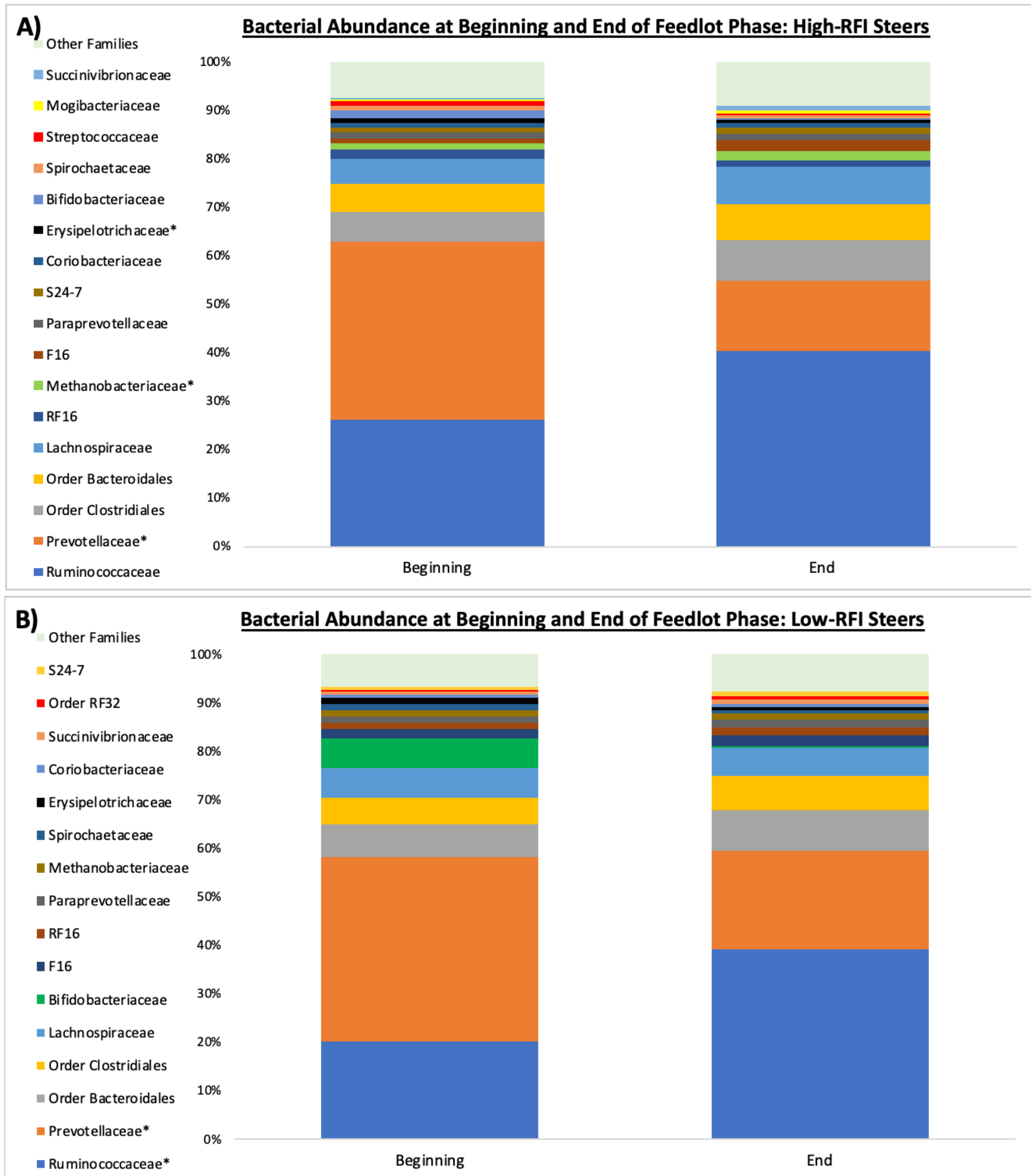
More changes occurred at the family level throughout the feedlot period, but these changes occurred in bacterial families with smaller relative populations and were limited to occurring in the least efficient (high-RFI) steers. For example, the abundance of *Erysipelotrichaceae* decreased (beginning: 1.2%, end: 0.7%; ($P = 0.01$), whereas the family *Methanobacteriaceae* increased ($P = 0.01$) in abundance (beginning: 1.2%, end: 2.0%). Previous studies have shown a greater abundance of the Erysipelotrichaceae family in low-methane emitting animals in which less hydrogen and thus less methane is formed (Kittelmann et al., 2014), and therefore it was hypothesized that the enrichment of the Erysipelotrichaceae family is associated with higher ruminal turnover rates of low-methane emitting ruminants that favors rapid heterofermentative starch degrading microorganisms such as *S. azabuensis* (Morita et al., 2008; Kamke et al., 2016).

Two archaeal genera were identified in the rumen of the present steers in the feedlot: *Methanobrevibacter* and *Methanosphaera* (Figure 3.2). In the least efficient steers (high-RFI), the *Methanobrevibacter* population increased ($P = 0.01$; Figure 3.2A) and the *Methanosphaera* population tended to increase ($P = 0.09$; Figure 3.2C) during the 82-d feedlot period. In the most efficient (low- RFI) steers however, *Methanobrevibacter* and *Methanosphaera* populations were not different ($P \geq 0.83$) over the 82-d feedlot period. A previous study has indicated a shift in *Methanobrevibacter* and *Methanosphaera* populations can be a result of a decrease in the relative abundance of Thermoplasmata levels (Poulsen et al., 2013), a group of Archaea classified as methanol and methylamine users (methylotrophic) for the purpose of methanogenesis (Seedorf et al., 2014). Although this smaller archaeal population might not be the only factor attributable to shifts in cattle efficiency and methane production, further examination should be conducted to assess their impact on rumen fermentation.

In addition to volatile fatty acids (VFA), ruminal microbial fermentation also produces H_2, CO_2, and CH_4, which are not utilized by the host (Hungate, 1966; van Houtert, 1993). Methane is released by the animal primarily through eructation and can represent a loss of up to 12% of the dietary feed energy to cattle (Johnson and Johnson, 1995; Arndt et al., 2015). Thus, this potent greenhouse gas poses both an environmental threat and negatively impacts host productivity since it is a waste of energy. Archaea are metabolically diverse organisms that in the rumen are often associated with bacteria, fungi, protozoa (Williams and Coleman, 1997) by providing a reducing equivalent sink that allows NADH to be disposed of within the bacteria or protozoa in the highly reduced ruminal environment (Rothschild and Mancinelli, 2001). Interspecies hydrogen transfer was

demonstrated using pure cultures of *Ruminococcus albus* compared with a co-culture was demonstrated using pure cultures of *Ruminococcus albus* compared with a co-culture of *Ruminococcus albus* and *Methanobacterium ruminantium*, the fermentation endproducts shifted from ethanol formation to an additional acetate, and increased ATP yield from 3

Figure 3.1: Bacterial abundance at the family level in ruminal samples collected at the beginning and end of the feedlot period in high-RFI Angus steers (n = 5) (A); and low-RFI Angus steers (n = 5) (B). * Indicates significant differences ($P \leq 0.05$).



to 4 ATP (Bryant et al., 1967). *Methanobacterium ruminantium* is able to utilize the $NADH_2$ produced by *Ruminococus albus* along with CO_2 to produce methane, and it allows *Ruminococcus albus* to regenerate reducing equivalents (Bryant et al., 1967). Therefore, ruminal *Methanobrevibacter* and *Methanosphaera* populations increasing in the least efficient steers (high-RFI) suggests that there was potentially a greater waste of dietary energy intake in the form of methane in these steers, contributing to their reduced feed efficiency.

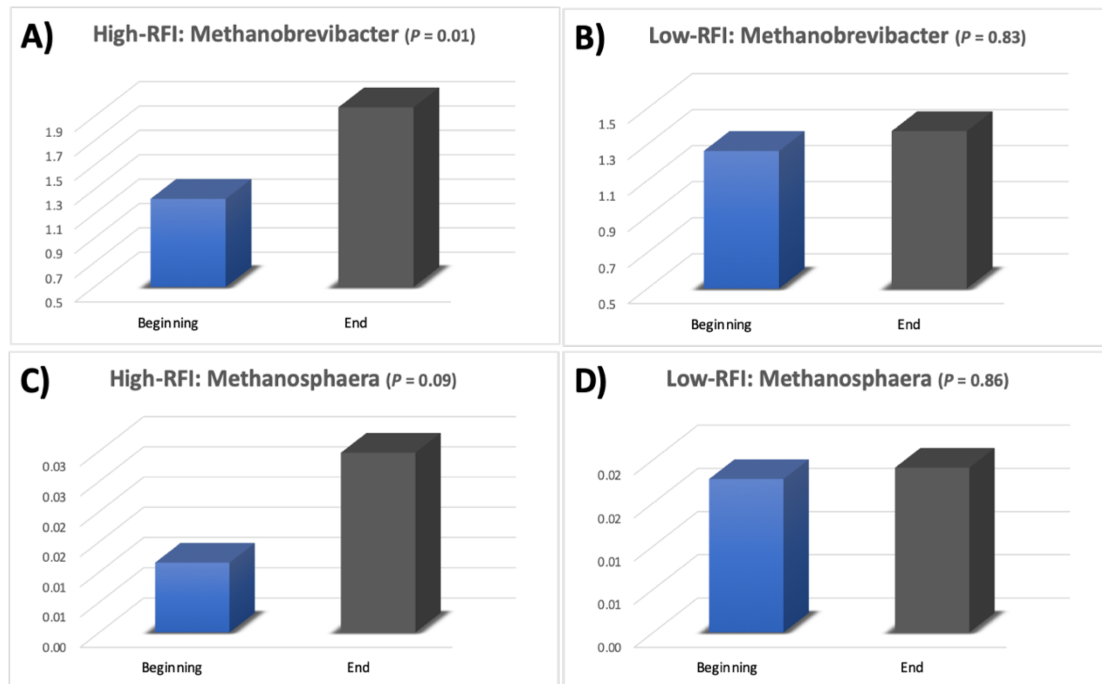


Figure 3.2. Abundance of *Methanobrevibacter* and *Methanosphaera* in ruminal samples (n=5 for high-RFI and 5 for low-RFI) collected at the beginning (d0) and end (d82) of the feedlot period in high-RFI steers (A, C); and low-RFI steers (B, D).

Differences in feed efficiency occur due to many factors, including ruminal and gastrointestinal production of methane, and feed intake levels affect methane production (Johnson and Johnson, 1995). Ruminal methane production can represent a loss of carbon and energy up to 12% of the dietary energy intake of the animal (Johnson and Johnson,

1995). Therefore, in the current study, the higher proportion of methane-producing microorganisms detected in the rumen of high-RFI (lower efficiency) steers at the end of the feedlot period (Figure 2) was potentially related to their lower feed efficiency. Moreover, more efficient steers (low-RFI) had a lower feed:gain ratio than did the lower efficiency steers (high-RFI), although this was not statistically significant ($P = 0.37$). HCW, and ADG were very similar ($P \geq 0.49$) between the two efficiency groups of steers.

Metabolomics have been utilized to assess the metabolism of rumen fluid in dairy cows (Ametaj et al., 2010; Saleem et al., 2012), as well as to assess phenotypic differences on residual feed intake in association with ruminal microbes and specific metabolic pathways in dairy cattle (Shabat et al., 2016). Furthermore, changes in ruminal microbial communities have been associated with differences in feed efficiency of beef cattle (Hernandez-Sanabria et al., 2012; Jami et al., 2014; Myer et al., 2015) and more recently linkages between active rumen microbiomes and feed efficiencies in beef cattle have been studied utilizing metagenomics and metabolomics approaches (Li and Guan, 2017; Li et al., 2019). While these studies utilized RFI to define feed efficiency, it has also been suggested that when residual feed intake is utilized to define feed efficiency there is a difference in feed intake between the groups that can greatly influence the microbiota (Artegoitia et al., 2017). To avoid changes associated with feed intake, metabolomics have also been used to assess ruminal metabolites' role in average daily gain (ADG). Artegoitia et al. (2017) indicated that 33 individual metabolites were associated with differences in ADG in beef cattle. The higher levels of ruminal linoleic acid, alpha-linolenic acid and aromatic amino acids with lactic acids observed suggested the balance between microbial population and ruminal absorption of organic acids affects ADG of crossbreed

beef steers. More recently, de Almeida et al. (2018), utilized untargeted metabolomics to provide a snapshot of the rumen fluid metabolome. They observed 1,882 molecular features, with 67 molecular features including, amino acids, dicarboxylic acids, carboxylic acids, lactones, lignans, fatty acids derivatives and indole compounds, positively matching with the Global Natural Products Social Molecular Networking database (GNPS). While the complexity of the rumen remains unknown, these findings suggest the potential of the rumen as a reservoir of novel compounds. The production traits of beef cattle have been improved through animal selection using traditional quantitative genetics approaches (Berry et al., 2011), however, there is still a need to understand the physiological mechanisms that contribute to variation in feed efficiency, including impacts of changes in the ruminal microbial population.

Alpha diversity

The number of observed OTUs increased numerically in both low and high RFI groups during the 82-d feedlot period, however, those differences were not statistically significant ($P \geq 0.14$; Table 3.5). The Chao1 index estimates microbial richness while accounting for rare species present (Chao, 1984) increased in both efficiency groups of steers during the feedlot phase, especially in high-RFI steers, where a tendency for greater microbial richness was observed ($P = 0.07$). Microbial diversity asses through the Shannon index revealed a trend for increased diversity in the rumen of high-RFI steers tended to increase ($P = 0.10$) over the course of the feedlot phase; however, when microbial diversity was expressed as Faith's Phylogenetic Diversity index, no differences were observed ($P = 0.21$) in this group of animals. In the most efficient steers (low-RFI), no

differences ($P \geq 0.20$) in microbial diversity were observed during the feedlot-finishing phase, regardless of the measure evaluated.

Table 3.5. Alpha diversity metrics observed during the finishing period according to Residual Feed Intake (RFI) classification.

	Feedlot Finishing Period		
Item	**Beginning (d0)**	**End (d82)**	***P*-value[1]**
High-RFI Steers (n= 5)			
Number of OTUs	1,587	1,732	0.14
Chao1	2,366	2,575	0.07
Faith's Phylogenetic Diversity	92.1	97.6	0.21
Shannon Index	7.19	7.89	0.10
Low-RFI Steers (n = 5)			
Number of OTUs	1,498	1,787	0.21
Chao1	2,284	2,727	0.22
Faith's Phylogenetic Diversity	89.0	100.8	0.20
Shannon Index	7.14	7.74	0.41

[1]P-value for the contrast between beginning (d0) and end of the feedlot-finishing period (d82).

Conclusions

Steer growth performance was impacted by shifts in the ruminal microbial composition and resulting activity. Despite the limitations of sample size in the present study, we observed an increase in abundance of the family *Ruminococcaceae* in the rumen of the most efficient steers over the course of the finishing phase, suggesting that more slow carbohydrate degradation occurred. This could result in a more stable pH over the course of the day, providing a healthier ruminal environment resulting in potentially more energy harvested from the diet by the microbial population, and increasing steer efficiency. Conversely, the less efficient steers contained a greater population of the methanogenic archaea *Methanobrevibacter* and *Methanosphaera* over the course of the feedlot period,

suggesting a greater loss of energy as ruminal methane in the low efficiency steers. Further studies are required to investigate the smaller microbial populations and to more deeply understand the rumen microbiome holistically. Additionally, further integration of metabolomics approaches can be useful strategies to understand how shifts in the ruminal microbial populations affect feed efficiency in beef cattle. As we develop a more comprehensive understanding of the rumen microbiome and metabolome interactions, further advancements can be made towards decreasing production costs, improving management strategies, and increasing energetic efficiency.

CHAPTER 4

THE EFFECT OF UREA SUPPLEMENTATION ON THE IN VITRO FERMENTATION OF CATTLE DIETS BY MIXED RUMINAL MICROORGANISMS[2]

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Abstract

The ruminal microbial population requires nitrogen for protein synthesis just as the host animal does. The activity of the ruminal microbiota of cattle allows them to utilize non-protein nitrogen (NPN) sources to synthesize microbial crude protein. Microbes degrade NPN to ammonia and transaminate this onto keto acids to produce amino acids for the inclusion in microbial protein. The amino acid composition of MCP more closely mimics the amino acid profile of muscle tissue than do plant-based feedstuffs. Microbial de novo synthesis of amino acids decreases the amino acid requirement of ruminants. The objective of this study was to assess the effect of urea addition to in-vitro mixed-ruminal-microorganism fermentation of typical beef cattle feedstuffs on dry matter digestibility (DMD), microbial population composition, ruminal pH, ruminal ammonia concentrations, volatile fatty acid production (VFA), and microbial crude protein (MCP). Urea was added to the in vitro fermentations to reach final concentrations of 0, 1, or 2% (w/w) followed by the addition of dietary substrates prior to the addition of the mixed ruminal microorganisms. While there were significant differences between dietary %IVDMD (0% P = 0.001; 1% P= 0.001; 2% P= 0.001), ammonia concentrations (0 % P = 0.005; 1% P = 0.180; 2% P = 0.052), MCP concentrations (0% P = 0.31; 1% P = 0.018; 2% P = 0.526), and total VFA concentration (0 % P = 0.013; 1% P = 0.003; 2% P = 0.072), these differences can mostly be attributed to dietary differences as urea had variable impacts on fermentation characteristics. However, in the case of the ground cracked corn fermentations (CC), %IVDMD increased linearly (P= 0.004) with increasing urea concentrations and had the highest %IVDMD in the presence of all three levels of urea. Results suggest that

when fed in the presence of available carbohydrate sources, urea supplementation can improve low quality forage utilization as Bermuda grass hay (BGH) fermentations experienced a decrease in ammonia concentrations and an increase in MCP concentrations (P = 0.06) as urea was added to the diet. Further data from microbial genome sequencing will be utilized to analyze microbial composition shifts as diet and urea supplementation changes.

Introduction

Ruminants have evolved a symbiotic relationship with their resident ruminal microbiome that allows them to occupy an ecological niche due to the microbial fermentation of low-quality feedstuff composed of cellulose and hemicellulose (Hungate, 1966). Ruminal microorganisms can combine ammonia with alpha-keto carbon skeletons derived from carbohydrate metabolism to synthesize microbial crude protein (MCP) (Chalupa, 1975). Microbial crude protein is the source of the majority of amino acids that are presented to the ruminant animal, and MCP has a similar amino acid profile to muscle tissue (Owens and Bergen, 1983). Microbial amino acid synthesis results in an apparent upgrade of dietary protein from plant based feedstuffs, and reduces the dietary amino acid requirement for ruminants (Hoover and Stokes, 1991).

Dietary nitrogen in ruminant rations typically falls in two categories: true protein, or part of a broad category termed non-protein-nitrogen (NPN). NPN is broadly defined as nitrogen from a source other than protein that can be used by microorganisms in the rumen of cattle and includes nucleic acids, and urea (Loosli and McDonald, 1968). One of the most common NPN sources fed to ruminants is urea, which is hydrolyzed by microbial urease into ammonia and carbon dioxide (Pearson and Smith, 1943). Microbes

can utilize this ammonia along with alpha-keto acid carbon skeletons derived from dietary carbohydrate fermentation to produce amino acids for incorporation into microbial crude protein (MCP) (Huber and Kung, 1981). After MCP leaves the rumen and reaches the abomasum it is denatured and degraded by gastric acid and enzymes prior to small intestinal absorption of the constituent amino acids (Stern et al., 1985).

Urea is included in rations as a substitute for other more expensive high-protein ingredients for protein supplementation (Pearson and Smith, 1943). Urea is a cheaper protein alternative that is often used in a feedlot setting (Burkhardt, 1971), when its benefits include improvements in weight gains and feed efficiency. The level of dietary urea inclusion affects microbial utilization of NPN which must be matched with available fermentable carbohydrate to capture the N as MCP (Johnson, 1976). When urea is fed continuously at low levels it is incorporated more efficiently into amino acids (Huber and Kung, 1981). Urea concentrations are recommended to be limited to 40 g/100kg of animal body weight to avoid urea toxicity (Santos, 2011), and if provided with proper levels of starch availability (e.g., temporal alignment), urea can be added to beef cattle rations at levels up to 80 g/100 kg body weight without toxicity.

Feeding RDP in excess of cattle requirements could require the use of additional energy to metabolize excess protein, and to synthesize and excrete urea (Tyrrell, 1970; Poos et al., 1979), while diets deficient in RDP have been shown to adversely affect MCP synthesis and rumen fermentation (Satter and Roffler, 1975; Broderick, 1987). Excess ruminal ammonia is absorbed through the rumen wall and drained via the portal vein to the liver where it is converted to urea. A portion of the urea produced by the liver is excreted

in the urine, while a portion is recycled back to the GIT through blood across the epithelial tissue or via saliva (Reynolds and Kristensen, 2008). The ratio of rumen-degradable organic matter **(**RDOM**)** to rumen degradable protein (RDP) plays an important role in determining MCP synthesis efficiency (Oldham, 1984). The excretion of N from excess rumen degradable protein (RDP) as urinary urea reduces the efficiency of N utilization as the synthesis of urea is an energetic cost to the animal (Oldham, 1984; Sinclair et al., 2014).

In addition to being an environmental pollutant, ammonia volatilization has been linked to human illnesses as well as being a source of social problems in areas of agricultural/residential interaction (McCubbin et al., 2002; Fowler et al., 2013). On farm ammonia mitigation strategies includes implementing feeding strategies and manure management strategies that reduce the amount of nitrogen lost (Bussink and Oenema, 1998; Johnson et al., 2016). Specifically in feedlot cattle operations dietary crude protein is often underfed early and overfed late in the feedlot period (Vasconcelos, 2007), therefore it is critical to reduce ammonia loss to the environment and improve animal production efficiency and profitability.

Therefore, the objective of this in vitro study was to assess the effect of urea as a source of NPN on dry matter digestibility (DMD), microbial composition, ruminal pH, ruminal ammonia concentrations, volatile fatty acid production (VFA), and microbial crude protein (MCP) synthesis in mixed ruminal microorganism fermentations of different substrates.

Materials and Methods

Animals, experimental design, and management

All procedures involving animals were verified and approved by the University of Georgia's Office of Animal Care and Use (AUP #A2012 11-006-R1*).* Experiments were conducted at The University of Georgia (Edgar L. Rhodes Center for Animal and Dairy Science located in Athens, Georgia; 33.5610°N 83.2206°W). The experiment was performed to assess the impact on the fermentation parameters of diets by mixed ruminal microorganism fermentations supplemented with different concentrations of urea.

Sample collection, DNA extraction and sequencing

In the present study, three urea concentrations were utilized along with three feedstuff substrates, in a 3 x 3 factorial design. In vitro mixed ruminal microorganism fermentations were performed in 160 mL serum bottles in triplicate along with controls.

Dietary components were obtained from Double Bridges Dairy (Winterville, Georgia; 34.1250°N 82.5114°W), and transported back to the laboratory. Samples were ground in a Wiley Mill (Thomas Scientific, Swedesboro, NJ) to pass through a 1 mm screen. Ground diets were separated, weighed, and recorded before and after drying in an oven (48 hours at 60°C) to determine dry matter composition (DMW). The three diets that were utilized included: ground dry-rolled corn (CC), Bermudagrass hay (BGH), and mixed corn and Bermudagrass Hay (50:50 w/w; CBG). Crystalline powder urea (Urea, ≥99.5%, Molecular Biology Grade, Thermo Scientific™) was added to the in vitro fermentations to reach final concentrations of 0, 1, or 2% (w/w) immediately before mixed

ruminal fluid addition to each fermentation bottle. Dietary substrates with final concentrations of 100, 99, or 98% (w/w) were added (totaling 0.5 g) to the fermentation bottles prior to the addition of the mixed ruminal microorganisms.

Ruminal fluid inoculum donors were housed at the J. Phil Campbell Sr. Research and Education Center (33.5220°N 83.2531°W). Rumen fluid was collected from a ruminally cannulated steer consuming a forage-based diet that contained no supplementary urea or other form of added NPN. Rumen fluid was collected prior to the morning feeding from five different locations within the rumen and squeezed through a fine nylon mesh strainer (Reaves and Co., Durham, NC), and transferred to a preheated 1.0-L thermos; pH was immediately measured and found to be 6.7 The rumen fluid inoculum was placed on wet ice and immediately transported to the laboratory approximately 20 min away. Mixed ruminal bacteria were separated from the large feed particles via gas production by the ruminal contents which were incubated at 39°C for 30 min in an incubator so that gas production buoyed feed particles to the top of the flask and protozoa sedimented to the bottom. The middle layer of ruminal fluid was utilized as the inoculum for the present study.

Ruminal fluid was added (33% v/v) to anoxic media (Cotta and Russell, 1983; Callaway and Martin, 1996). Media composition was: 292 mg of K_2HPO_2, 240 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4000 mg of Na_2CO_3, and 600 mg/L of cysteine. The mixed ruminal microorganism media was continuously sparged with O_2-free CO_2 for 10 minutes prior to anaerobic transfer (100 mL) to O_2-free CO_2 flushed fermentation bottles (160 mL volume) which were subsequently sealed with butyl rubber stoppers and aluminum crimps. Bottles

were placed in an incubator (39°C) for 24 hours and periodically mixed. After 24 h of fermentation the bottles were unsealed and the pH was measured immediately after uncapping using a portable pH meter (Starter 300; Ohaus Instruments Co. Ltd, Shanghai,China). Following pH measurement samples were collected from each fermentation bottle and aliquoted into 2 ml vials, and centrifuged at 10,000×*g* for 10 min. The supernatant was removed and stored at -20°C prior to the determination of VFA and ammonia concentrations in the sample. Particulate samples were collected after intense shaking of the serum bottles to ensure that representative portions of liquid and solid fractions could be obtained and were immediately stored at -80°C for subsequent DNA extraction.

DNA was extracted from the rumen fluid samples according to the semi-automated extraction protocol (Rothrock et al., 2014). Briefly, the procedure started with 0.5 mL of homogenized rumen fluid and achieved extraction of DNA through a combination of mechanic and enzymatic methods. The mechanic portion of the protocol used a FastPrep 24 Instrument (MP Biomedicals LLC, Irvine, CA, USA) to disrupt the cells. The enzymatic portion used InhibitEX® Tablets (QIAGEN, Venlo, Netherlands). Elution and purification of DNA were performed using an automated robotic workstation (QIAcube; QIAGEN, Venlo, Netherlands). Determination of DNA concentration and purity were performed spectrophotometrically using the Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments Inc; Winooski, VT, USA). After DNA extraction, samples were taken to the Georgia Genomics and Bioinformatics Core facility (https://dna.uga.edu) for library preparation and 16S rRNA gene sequencing. Library preparation 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′-GAC-TACHVGGGTATCTAATCC-3′) primer pair (Klindworth et al., 2013). Samples were sequenced using the Illumina MiSeq system (Illumina Inc., San Diego, CA, USA).

In vitro dry matter disappearance

The substrate DM content was determined by drying at 60°C in a forced-air oven for 48 h followed by hot weighing, and the fraction of %IVDMD for each bottle was calculated by subtracting the dry residue weight (corrected for the blank) from the dry substrate weight and dividing by the dry weight of substrate:

$$\%\ \text{IVDMD} = 1 - \frac{[(\text{Dry residue weight} + \text{filter paper}) - \text{filter paper}] - \text{blank}}{(\text{Substrate weight})\text{x}(\text{DM})}$$

$$\text{Blank} = (\text{Blank residue weight} + \text{filter paper}) - \text{filter paper}$$

In vitro ammonia determination

Upon removal from the freezer, samples were thawed and centrifuged at 10,000 x *g* for 10 min and the supernatant was analyzed for ammonia concentrations (Chaney and Marbach, 1962). Concentrations were analyzed from absorbance by spectrophotometry at 630 nm (Thermo Scientific™ GENESYS™ 30 Visible Spectrophotometer).

In vitro volatile fatty acid production determination

Samples were thawed at 20 °C prior to VFA analysis following the procedure described by (Cottyn and Boucque, 1968) with some modifications. Samples were centrifuged at 10,000 x *g* for 10 min and 1.0 ml of the resulting supernatant was added to 0.200 ml of 25% (w/v) metaphosphoric acid and pipetted into vials. Vials were stored at -18°C for 12 h. The vials were thawed for 2 h then centrifuged at 10,000 x *g* for 10 min. The resulting supernatant fluid was mixed with ethyl acetate in a ratio of 1:2 and vortexed. Then

0.5 ml of the supernatant was transferred to glass vials to be analyzed using gas chromatography, equipped with Flame Ionization Detector (FID) and capillary column (DB-FFAP, 122–3232). The standard solutions were prepared with distilled, deionized water and acids purchased from commercial sources and contained 0.2% acetic acid, 0.2% propionic acid, 0.1% butyric acid, 0.02% isovaleric acid, 0.02% valeric acid, and 0.02% caproic acid.

In vitro microbial crude protein determination

Protein concentrations from each sample were determined (Lowry et al., 1951). Absorbance was measured at 660 nm and compared with a standard curve to determine protein concentrations produced compared to controls for each feedstuff.

Sequencing data and statistical analyses

Sequencing data were demultiplexed and downloaded as FASTQ files. Pair-end reads were 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 converted into FASTA files and quality filtered. The sequences were clustered into operational taxonomic units (OTUs) at 97% similarity according to the Greengenes database. Singleton OTUs were excluded from the analysis. The computed alpha-diversity indexes were number of observed OTUs, Chao1, species evenness, Shannon index, and Faith's phylogenetic diversity.

Statistical analyses were performed using the software Minitab® v19. Results were analyzed by ANOVA within each treatment of urea (i.e. 1%- vs. 2% urea) and fermentation parameters (VFA production, pH, ammonia concentration, MCP production, dry

matter digestibility (DMD), and rumen microbial composition) were used as factors. Individual dietary substrate data was analyzed using each fermentation parameter (VFA production, pH, ammonia concentration, MCP production, dry matter digestibility (DMD), and rumen microbial composition) as a factor. Results were considered significant when $P \leq 0.05$ and were categorized as trends when $0.05 < P \leq 0.10$.

Results

In vitro dry matter disappearance and rumen pH

Table 4.1: Final pH of in vitro mixed ruminal microorganism fermentation of cracked corn (CC), Bermudagrass Hay (BGH), or a 50/50 mix of corn and hay (CBG)

Diet	**Urea DM%**		
	0	1	2
CC	6.35	*6.03	*6.14
BGH	6.75	*6.32	*6.61
CBG	6.23	*6.4	*6.32

* Indicates significant dietary differences within diet fermentations based on urea supplementation ($P \leq 0.05$).

Urea supplementation resulted in lower pH at both 1% ($P = 0.020$) and 2% ($P = 0.036$). Although substrate addition had no significant impact on final pH, the BGH diet tended to be higher compared to other diets. %IVDMD by diet and by urea supplementation is shown in Figure 3.

Corn fermentations had the highest in vitro dry matter disappearance, and BGH had the lowest (Figure 3). Urea supplementation increased %IVDMD (0% $P = 0.001$; 1% $P= 0.001$; 2% $P= 0.001$) in all diets. For CC diets %IVDMD was linearly increased ($P= 0.004$) as levels of urea increased (Figure 3). BGH fermentations were less impacted by urea addition (P= 0.271) but tended to be highest in the 2% urea treatments.

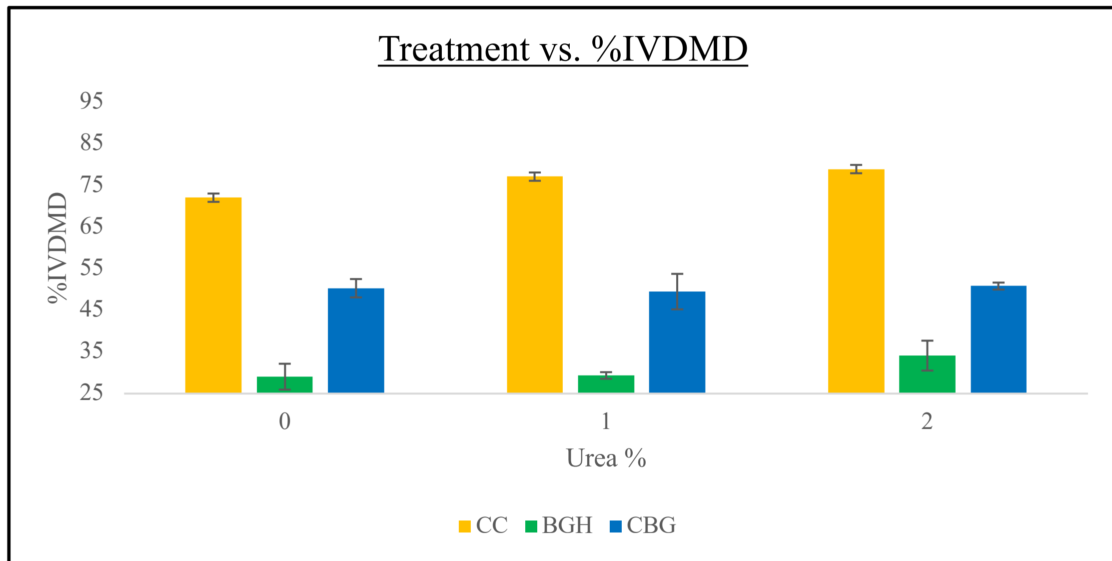

Figure 4.1: In vitro dry matter disappearance percentage in samples based on dietary treatment and urea supplementation

In vitro ammonia concentrations

Ammonia concentrations tended to be different (0% $P = 0.005$; 1% $P = 0.180$; 2% $P = 0.052$; Figure 4) across dietary fermentations on all levels of urea respectfully. CC fermentations tended to have the lowest ammonia concentration at all levels of urea addition. The addition of urea to the BGH fermentation caused an increase in ammonia concentrations ($P \leq 0.05$) at 1% and 2% urea supplementation. Ammonia concentrations were not increased ($P = 0.143$) in CBG diet fermentation except when 2% urea was added.

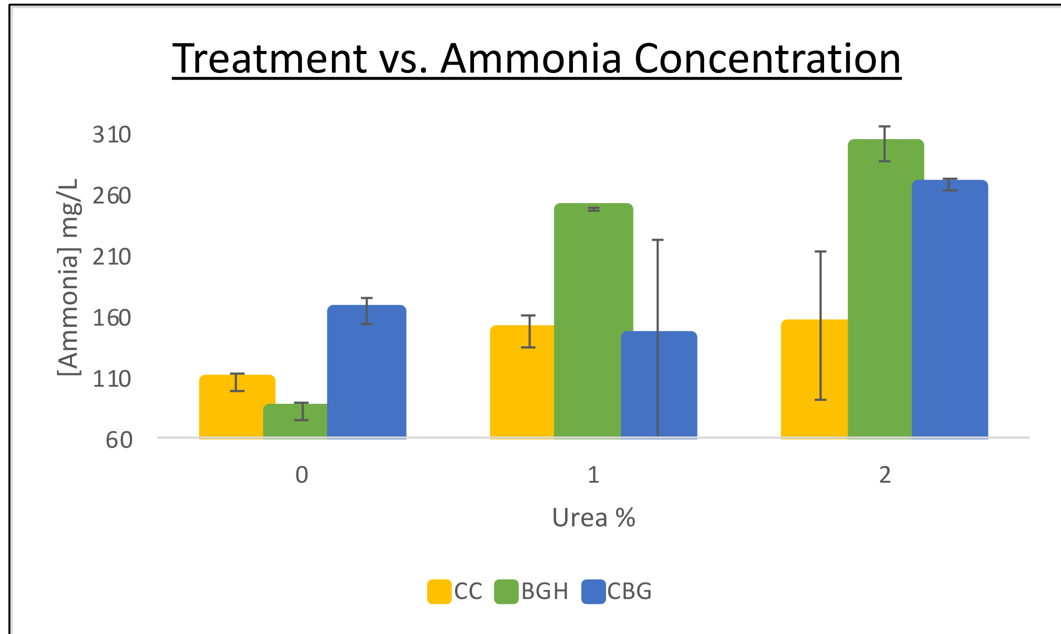

Figure 4.2: Ammonia concentration from in vitro mixed microorganism fermentation with three diets and three levels of urea addition. *P*-value for urea supplementation across diets; 0 % urea *P* = 0.005; 1% urea *P* = 0.180; 2% urea *P* = 0.052.

Microbial crude protein

Microbial crude protein concentrations were similar in fermentations of all three diets in the absence of urea (Figure 5). The addition of 1% urea caused the diets to become distinct (*P* = 0.018) from each other in terms of MCP. The addition of 2% urea resulted in no dietary differences in MCP. Generally, as urea supplementation increased, MCP concentrations tended (*P* = 0.066) to increase the most in CBG diets, followed by CC diets (*P* = 0.660). The BGH diets had the lowest MCP concentration in the sample as urea supplementation increased (*P* = 0.066).

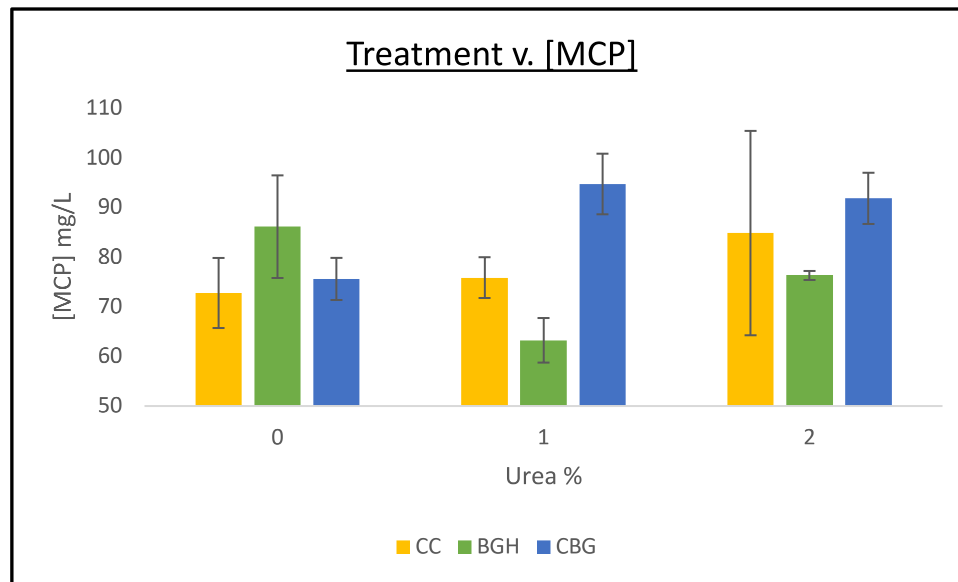

Figure 4.3: Microbial crude protein concentration from in vitro mixed microorganism fermentation with three diets and three levels of urea addition. P-value for the contrast between dietary MCP concentration and urea supplementation; 0 % urea P = 0.31; 1% urea P = 0.018; 2% urea P = 0.526. P-value for the contrast between dietary microbial crude protein concentration and urea supplementation by diet; CC P = 0.660; BGH P = 0.086; CBG P = 0.06.

Volatile fatty acids

Corn fermentations yielded the highest total VFA concentrations with all levels of added urea (Figure 6), likely reflective of the high level of IVDMD%. CC fermentations had the lowest acetate to propionate ratio at all levels of added urea, whereas BGH had the highest acetate to propionate ratio and the lowest total VFA concentrations on all levels of added urea (0% $P < 0.005$; 1% $P = 0.002$; 2% $P = 0.011$; Figure 7). As urea supplementation increased, acetate concentrations became less significantly different between diets (0% $P = 0.031$; 1% $P = 0.009$; 2% $P= 0.165$). Butyrate concentrations were significantly different between all three diets on all three levels of urea (0% $P = 0.002$; 1% $P <$ 0.005; 2% $P = 0.004$). Acetate to propionate ratios were significantly different between

diets before ($P < 0.005$) and after (1% $P = 0.002$; 2% $P = 0.011$) supplemental urea addition. Dietary isobutyrate and isovalerate concentrations differed the most after supplementation with 1% urea ($P = 0.009$; $P = 0.006$; Figure 8), but there was no urea effect on BCVFA concentrations within the diets (CC $P = 0.62$; BGH $P = 0.214$; CBG $P = 0.593$). CBG diets had the highest BCVFA concentration at 1% urea supplementation (isobutyrate: $P = 0.009$; isovalerate: 1% $P = 0.006$), while BGH diets tended to have the lowest BCVFA concentrations amongst the diets.

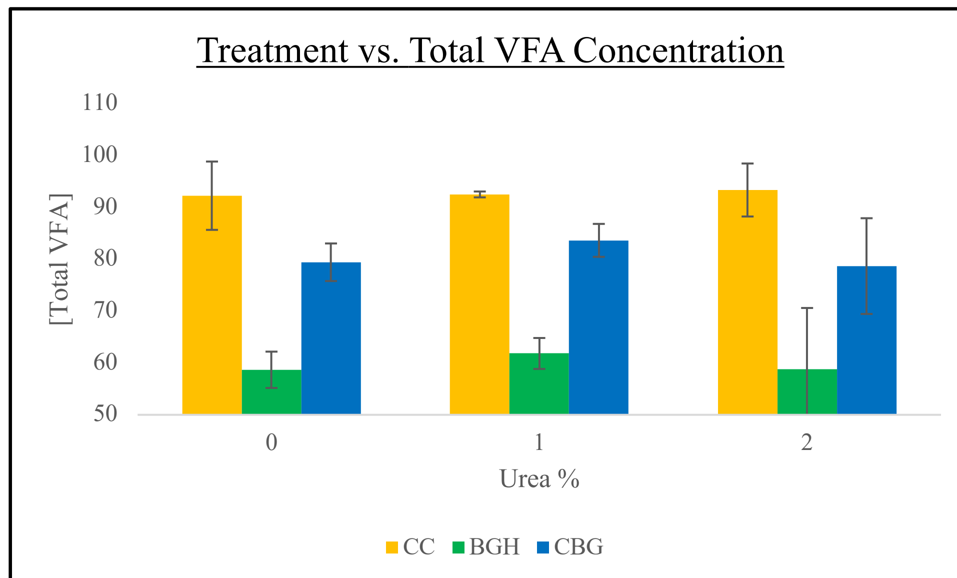

Figure 4.4: Total VFA concentrations from in vitro mixed microorganism fermentation with three diets and three levels of urea addition. *P*-value for the contrast between dietary VFA concentration and urea supplementation; 0 % urea $P = 0.013$; 1% urea $P = 0.003$; 2% urea $P = 0.072$.

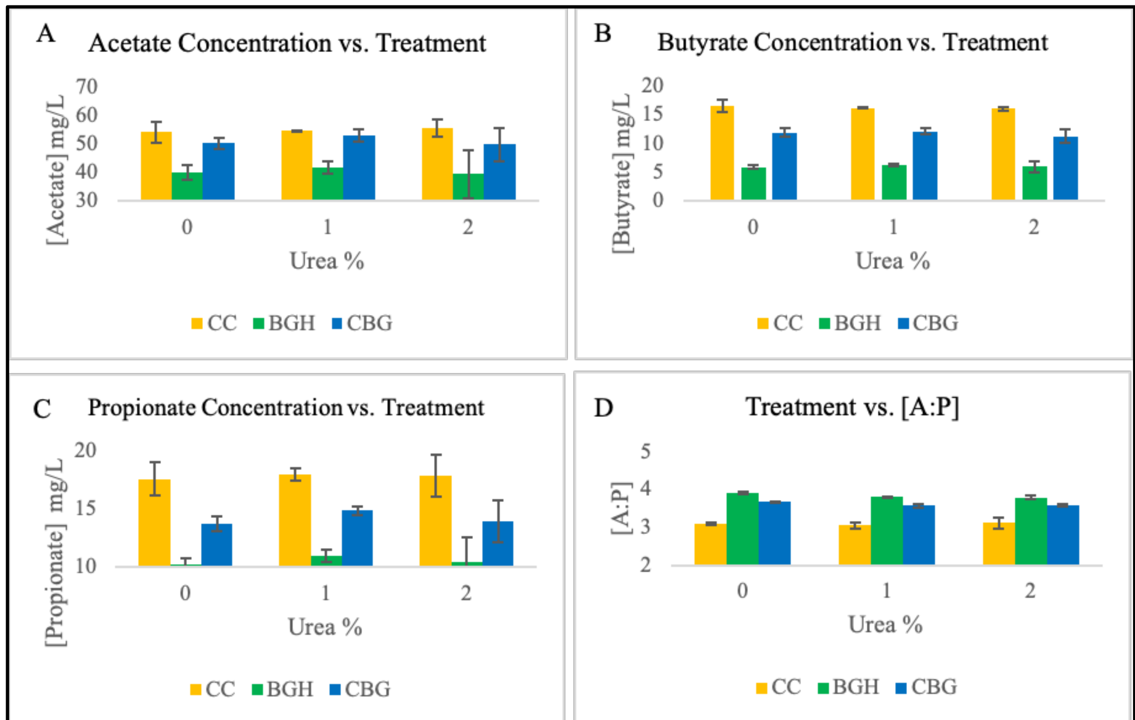

Figure 4.5: Acetate concentrations from in vitro mixed microorganism fermentation with three diets and three levels of urea addition. (A); propionate concentrations in samples based on dietary treatment and urea supplementation (B); butyrate concentrations in samples based on dietary treatment and urea supplementation (C); acetate to propionate ratio concentrations in samples based on dietary treatment and urea supplementation (D).

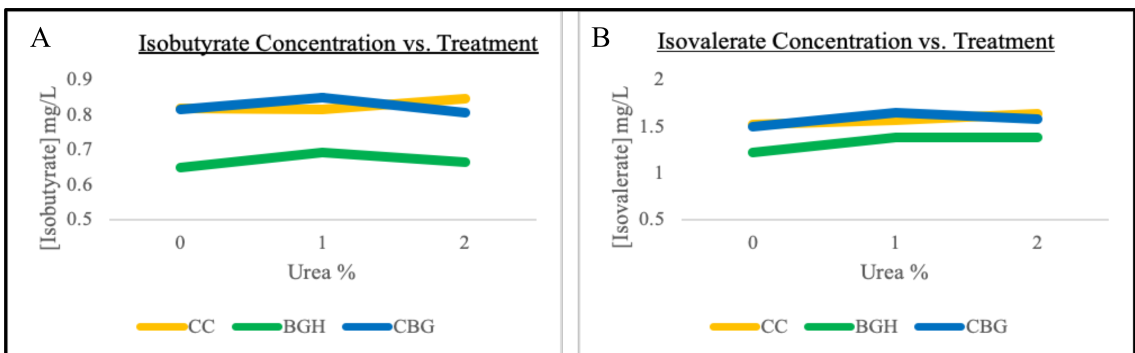

Figure 4.6: Isobutyrate (A) and isovalerate (B) concentrations from in vitro mixed microorganism fermentation with three diets and three levels of urea addition. *P*-value for the contrast between dietary isobutyrate concentration and urea supplementation; 0 % urea *P* = 0.066; 1% urea P = 0.009; 2% urea *P* = 0.212. *P*-value for the contrast between dietary isovalerate concentration and urea supplementation; 0 % urea *P* = 0.300; 1% urea *P* = 0.006; 2% urea *P* = 0.115.

Discussion

Animals depend on dietary protein for tissue growth and maintenance, and have developed numerous evolutionary mechanisms to maximize the utilization and conservation of nitrogen for survival (Stewart and Smith, 2005). Dietary nitrogen comes primarily in the form of protein-derived dietary amino acids and peptides, however non-protein nitrogen (NPN) such as urea are a source of nitrogen that can be utilized by ruminant animals (Pearson and Smith, 1943). Mammals do not produce the ureolytic enzyme urease, but bacteria do; thus the ruminal microbial population can hydrolyze urea allowing ruminants to utilize urea as a nitrogen source (Mobley et al., 1995). Ruminant animals secrete urea in their saliva, which ensures a constant low level of N that can be used by the microbial population of the gut, and this has been described as 'urea nitrogen salvaging'(Fuller and Reeds, 1998), or nitrogen recycling. Ruminal ammonia concentrations are determined largely by the availability of nitrogen sources and fermentable carbohydrates which impact MCP formation, ruminal ammonia uptake, and liquid dilution rate (Xu et al., 2019).

If the rate of ammonia production exceeds the microbial ability to utilize it to form amino acids, there is an accumulation of ruminal ammonia. Ammonia absorbed through the rumen wall eventually drains out through the portal vein where it is detoxified by the liver via the urea cycle. Once in the form of urea, it can be excreted by the kidneys as waste, or recycled back into the rumen by passive absorption or transfer through salivary secretions. Ureagenesis in the liver depends on the supply of N to the or-

nithine cycle from either mitochondrial ammonia or cytosolic aspartate for the assimilation of two N atoms (Parker et al., 1995; Milano et al., 2000). Previous in vivo studies (Cooper et al., 1987; Meijer et al., 1990) have suggested that in the case of excess urea, the mitochondrial supply of ammonia may not be enough to supply both N atoms for ureagenesis and amino acid N is sequestered instead. This ultimately suggests a metabolic cost of amino acid deamination in favor of ammonia detoxification. Excess ammonia and related amines from NPN sources can be a further burden on the metabolism of cattle causing a decrease in feed intake and animal performance (Van Soest, 1994) and simply costing the animal more energy to dispose of excess N. The ability of the ruminant animal to utilize nitrogen recycling with the help of the rumen microbiome provides a mechanism to impact animal efficiency through the increase of nitrogen retention and decrease of nitrogen excretion. Feeding N at concentrations that meets animal requirements for production efficiency is one of the most practical methods to increase nitrogen retention and decrease nitrogen excretion (Rotz, 2004). However, despite previous literature's findings pertaining to energy and protein requirements in beef cattle, there is still a lack of information about the effect of excess or required dietary N on energy use efficiency. Furthermore, several studies have investigated the impact of NPN inclusion in ruminant diets on important metabolic parameters (Capelari and Powers, 2017; Hall, 2017; Nur Atikah et al., 2018; Moraes et al., 2019), but few have assessed the role of NPN on the microbial population and on feedstuff degradation by ruminal microbes that contributes to the efficiency of the animal. In the present study, it was hypothesized that ammonia concentra-

tions would be affected by the rate of feedstuff degradation which would ultimately determine microbial crude protein synthesis, pH, and VFA concentration's contributions to animal efficiency.

In vitro dry matter disappearance and pH

Urea had little impact on fermentation end products in the present study. Although there were differences between Dry Matter Disappearance for each level of urea, the primary differences were due to feedstuff. Dry Matter Disappearance percentage increased with increasing urea in only corn fermentations (Table 1; Figure 3; $P = 0.004$), and has been reportedly higher (72.7%) compared to cottonseed meal fermentations (Ezequiel et al., 2001). The source of rapidly fermented nonstructural carbohydrates is a profound influencer of nitrogen utilization in the rumen (Rotger et al., 2006). Structural and non-structural carbohydrates comprise the major portion of the feed of ruminants, whether they are fed forage or grain diets Forage cell walls are composed of cellulose, hemicellulose, and pectin (Owens and Goetsch, 1988). Starch is the predominant form of non-structural carbohydrates, and corn grain is the most common energy source fed to ruminants in the U.S due to its high starch content (Huntington, 1997). High starch diets are rapidly fermented and produce strong acids such as lactic acid which result in a decreased ruminal pH (McAllan et al., 1994), decreased ruminal ammonia concentrations (Hristov et al., 2005), and decreased fiber digestibility (Archimède et al., 1996; Serment et al., 2011), while increasing dry matter between 6.1 to 6.8 (Van Soest, 1994), and the lower starch content of the BGH and CBG diets resulted in a higher pH (Table 1; Figure 3), but came with a lower total digestibility. The previously noted increase in rumen pH

associated with increasing urea supplementation in the diet (Polan et al., 1976; Bernard et al., 2001; Aschenbach et al., 2011; Spanghero et al., 2017; Xu et al., 2019), was not evident in the current study. Supplemental NPN can improve forage digestion due to ammonia availability to cellulolytic bacteria (Titgemeyer and Löest, 2001). Our present results however, agreed with previous results that linked urea supplementation with decreased fiber digestibility, potentially as a result of reduced availability of free amino acids or peptides for cellulolytic bacteria rather than requiring de novo synthesis from ammonia (Burroughs et al., 1951; Russell et al., 1992). The synchronized rate of degradation of dietary carbohydrate and N release improved in vitro fermentation in corn fermentations.

In vitro ammonia concentrations and microbial crude protein synthesis

Rumen bacteria rapidly hydrolyze urea to ammonia and CO_2 at a higher rate than they can utilize ammonia (Patra, 2015), and protein fermentation has the potential to produce more ammonia than microbes can utilize (Russell et al., 1992). In the present study, the diet with the highest ruminal ammonia concentration (BGH) had the lowest in vitro dry matter disappearance which agreed with previous research that suggested that a factor other than ammonia availability had a greater impact on ruminal digestion at high levels of urea supplementation (Köster et al., 1997). In the present study the greater level of carbohydrate fermentation and ammonia release is evident by the improved MCP yield when urea increased from 0% to 2% in CC and CBG diets. Although the CC fermentations had the lowest ammonia concentration, they had an intermediate level of MCP yield (Figure 4; Figure 5). The lower pH observed with the CC fermentations for each urea supplementation level may have decreased the efficiency of microbial protein synthesis (Hoover, 1986; Strobel and Russell, 1986) as microbial protein synthesis is reported to be lower in

animals fed high levels of concentrate (NRC, 1996). The ATP generated from carbohydrate fermentation can be utilized for microbial growth, but ATP is also directed towards maintenance, synthesis of reserve carbohydrates, or wasted by energy spilling (Hackmann and Firkins, 2015). Energy spilling occurs in rumen cultures administered excess glucose (Russell, 1986), which resulted in energy dissipation as heat when ATP was in excess and N was limited following bacterial charging of metabolic energy pools in preparation for growth when the N limitation was alleviated (Russell and Cook, 1995; Portais and Delort, 2002).

Increasing starch availability with low quality forage based diets can improve supplemental nitrogen utilization and ultimately improve MCP yield (Huber and Kung, 1981). This is a potential explanation for the increased MCP concentrations observed in CBG diets as supplemental urea was added in the present study, similarly demonstrated by Czerkawski (1976) where sheep fed a mixed hay and concentrate diet had greater microbial growth in the rumen compared to those fed concentrate and hay separately. The concentrate to forage ratio in the CBG diet potentially slowed the rate of carbohydrate fermentation, increased the pH, and created a more favorable ruminal environment. Urea appeared to have the greatest effect on MCP yield on all diets at the 1% level. This may reflect the 1% urea supplementation as the upper limit for microbial fermentative activity and subsequent change in ammonia accumulation in samples. In contrast, decreased MCP concentrations are indicative of a depression in microbial protein synthesis consistently observed when the starch availability in forage-based diets decreases (Broderick and Reynal, 2009). The present sharp decline in MCP concentrations with supplemental urea

in BGH fermentations were similar to those of Broderick and Reynal (2009). These results demonstrate that low microbial crude protein yields from low quality forage based diets cannot be resolved solely by supplementing with concentrates (Agricultural Research Counsil, 1980).

All three diets produced different ammonia concentrations in the absence of urea supplementation with the lowest concentrations occurring in corn fermentations, but these fermentations produced an intermediate level of MCP. The BGH fermentations had the highest concentrations of ammonia treated with both 1% and 2% supplementary urea, which could be a result of a decrease in the availability of readily fermented starch found in forage-based diets. In the absence of available carbon skeletons, ammonia accumulates (Russell et al., 1992). Corn fermentations tended to have the lowest ammonia concentration, consistent with previous reports that ammonia concentrations decrease with the addition of a rapidly fermentable carbohydrate to diets (Stern et al., 1985). Cellulolytic microorganisms utilize ammonia as their primary source of N (Hoover and Stokes, 1991). The excess ammonia concentrations produced from urea supplementation in BGH diets suggests a limitation of microbial growth and ammonia capture (Nolan and Dobos, 2005) for cellulolytic microorganisms and limiting urea supplementation on forage-based diets can decrease ammonia accumulation and wastage.

In vitro volatile fatty acid concentrations

In the context of urea supplementation, total VFA concentrations and increased ammonia concentrations have been correlated with the increased rumen pH along with increasing urea supplementation in the diet (Lu et al., 2019; Xu et al., 2019). In the current study, acetate, propionate, and total VFA concentrations were unaffected by urea

(Currier et al., 2004; Xu et al., 2019). Corn fermentations had the highest total VFA concentrations on all three urea levels, while the CBG diet had the highest branched-chain volatile fatty acid (BCVFA) concentrations on all three urea levels. Branched-chain volatile fatty acids (BCVFA) such as isobutyrate, isovalerate, are naturally produced in the GIT of ruminants from the degradation products of amino acids (Liu et al., 2008), and can be utilized for the synthesis of amino acids (Andries et al., 1987). Optimal levels of BCVFA in the rumen are required for efficient carbohydrate fermentation and subsequent synthesis of branched chain amino acids for microbial crude protein as part of the nutrient crossfeeding common in the rumen (Iannotti et al., 1973). While not statistically significant, the decrease in BCVFA from BGH fermentations in the present study can be potentially attributed to a decrease in protein catabolism by microbes in the de novo synthesis of amino acids from NPN (Allison and Bryant, 1963). Cline et al. (1958) and Windschitl and Stern (1988) have previously found a decrease in isobutyrate and valerate concentrations from valine (Blackbrun, 1965), lysine, and arginine catabolism in the rumen (Blackbrun, 1965) in diets with lower RDP, potentially indicating certain amino acids may be preferentially deaminated due to their higher potential to generate ammonia N (Griswold et al., 2003). These results suggest levels of certain VFA and BCVFA may serve as a way to monitor N utilization and protein deamination for N requirements.

Conclusion

Understanding the effects of NPN on the ruminal microbial degradation of feedstuffs and its contribution host efficiency can help refine the nutrient requirements of beef cattle. Fermentation by mixed ruminal microorganisms is affected by the type of dietary substrate and urea supplementation. The addition of NPN on low quality forage diets can

limit microbial growth and ammonia capture which can result in excess ammonia accumulation and excretion. The extent of the accumulation of ammonia's energy cost to the animal remains unclear, and according to the results of our present study, monitoring BCVFA levels to measure amino acid deamination in response to ammonia detoxification requirements in vivo can further understanding of N utilization. Present results also suggest that dietary NPN fed at or above beef cattle requirements can have positive impacts on the fermentation efficiency and MCP synthesis from low-quality forages when supplemented with readily fermentable carbohydrate. While higher levels of urea supplementation at 2% on mixed corn and bermudagrass hay diets can improve MCP production and decrease ammonia accumulation, 1% urea supplementation gave optimal MCP yields for all three diets. It is known that factors such as dietary starch content and ruminal ammonia concentrations influence MCP synthesis and blood urea nitrogen, but the total amount of urea utilized is likely determined by microbial requirements. Further analysis of the ruminal microbial composition may be critical to further determine the effect of supplemental urea on N utilization and fermentation parameters in beef cattle.

CHAPTER 5

CONCLUSIONS

To analyze the composition of the ruminal microbial population, many studies have utilized a multi-omics approach combining metagenomics, metatranscriptomics, metaproteomics and metabolomics to study the rumen microbial ecosystem and its impacts on the host animal (Shabat et al., 2016; Comtet-Marre et al., 2017; Shaani et al., 2018). Individual "omics" approaches only give snapshots of the microbiome. More conclusions can be drawn from multi-omics approaches to maximize our understanding of the interactions between diet, the microbiota, the metabolome to understand underlying activities of specific populations of microbiota and their metabolites in ruminants. In doing this, a more holistic evaluation of the rumen microbial population can provide a multifaceted understanding of the ecosystem niches, degradative activity, biosynthetic pathways, populations. This can lead to the development of several strategies for optimizing the energy and nitrogen utilization by beef steers from commercial feedlots.

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