

**Microbial and Metabolomic Biomarkers of Feed Efficiency in Angus
Cattle**

**A Dissertation Presented for the
Doctor of Philosophy
Degree
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DEDICATION

This dissertation is dedicated to my amazing family, including my husband, Josh, and beautiful baby boy, Asher. None of this would have been possible without you.

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ABSTRACT

In Tennessee and the United States, cattle account for a significant proportion of the livestock agricultural sector. In the beef cattle industry, 40-70% of the total cost of production comes from feed costs, resulting in billions of dollars going towards feeding cattle. Thus, identifying methods for improving feed efficiency is imperative for reduced feed input costs and more effective use of environmental and economic resources. The rumen microbiome contributes a vast amount of nutrients for the host ruminant, including approximately 70% of energy precursors as well as vitamins, proteins, and other nutrients. These microbes produce metabolites that are then available to the host and may influence host feed efficiency phenotypes. The microbiota and metabolites may be used as biomarkers of feed efficiency in beef cattle. In this dissertation, growing steers and heifers were used to identify potential biomarkers of feed efficiency using a residual feed intake (RFI) model of feed efficiency. In the steers, eight metabolites were greater in low-RFI steers compared to high-RFI steers ($P \leq 0.05$) and unassigned protozoal taxa were present in greater abundance in high-RFI compared to low-RFI steers ($P = 0.03$). In the heifers, no metabolites differed by RFI ($P > 0.05$) after false discovery rate correction in the serum or rumen fluid. Furthermore, no microbial populations differed by RFI in the heifers, though several numerical differences did occur and were examined. In both the steers and heifers, rumen metabolites associated with nucleic and amino acid turnover appeared to be indicators of feed efficiency, though additional studies confirming this should be conducted. Additionally, the heifer study did not reach adequate statistical power to delineate differences in microbial populations after false discovery rate correction, but still may serve as biomarkers of feed efficiency because numerical differences were biologically relevant. Future studies should examine other sexes and production stages (primiparous and multiparous cows) to determine other microbial and metabolomic biomarkers of feed efficiency in beef cattle.

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INTRODUCTION

In the next 30 years, the agricultural sector is tasked with increasing food production by 50% to feed an expected global population of over 10 billion people (Alexandratos and Bruinsma 2012). Beef cattle can provide a great source of animal protein. However, with feed costs accounting for 40-60% of the total cost of production in the beef cattle industry in the United States, novel methods to improve feed efficiency and nutrient utilization in cattle are becoming increasingly critical (McBride 2011). Given the importance of the beef industry in the United States and around the world, as well as the increasing cost of product from feed alone, researchers are charged with finding novel methods of improving production without increasing production costs and in an environmentally-ethical manner.

The rumen microbiome is responsible for much of the feed breakdown for the host animal as well as most of the energy precursors, B vitamins, and other nutrients (Hungate 1975; Young 1977). There are four different kingdoms of microbes that reside in the rumen, including fungi, protozoa, bacteria, and archaea (Hungate 1966), each possessing different functions. For instance, rumen bacteria are the most genetically and functionally diverse kingdom of microbiota that live in the rumen, and are thus the most widely studied rumen microbes with regards to feed efficiency (Creevey et al. 2014; Henderson et al. 2015). Archaea account for approximately 2% of the genetic material of the rumen microbiota and are much less functionally diverse than bacteria (Janssen and Kirs 2008). Additionally, the archaea in the rumen are methanogenic, and are thought to contribute to decreased feed efficiency by shunting carbon from use in nutrients to methane, a waste product that is eructed by the host animal (Janssen and Kirs 2008). Ciliate protozoa are found in the rumen as well and account for approximately 50% of the microbial biomass in the rumen of cattle (Newbold et al. 2015). The rumen protozoa have been less studied than archaea or bacteria, but have been implicated as consumers of bacteria and reduction of available microbial crude protein for the host animal (Newbold et al. 2015). Finally, the rumen microbiome includes fungi, which are able to break through the tough cell exterior and aid in fiber digestion (Akin and Borneman 1990). Elucidating the complex dynamics among the various kingdoms of microbes with the host ruminant is key to understanding how manipulation of the rumen microbiome could lead to improvements in feed efficiency in cattle.

The rumen microbiota break down the feedstuffs that are consumed by the host ruminant and release products available to host and other microbes. It was previously thought that the microbial metabolites released predominantly served solely as energy precursors, but recent work has demonstrated the wide-reaching impacts of these metabolites. For instance, in monogastric species, conjugated secondary bile acids are able to directly alter host transcription, and the type and effect of the secondary bile acids on the host are dependent upon the gut microbiota (Just 2017). Additionally, glutamate can be metabolized by the rumen microbiota, and glutamate and similar molecules can serve as potent neuromodulators (Newbold et al. 2005; Luine, Grattan, and Selmanson 1997; Wu 2014). Therefore, there is a need to not only understand the complexities of the relationship among the microbes, but also the multidirectional crosstalk between the ruminant host and its rumen microbiome.

Recent advances in technology may allow researchers to bridge the gap between correlation and causation. Untargeted metabolomic techniques provide global analysis of metabolites without the

previously cumbersome method of measuring individual metabolites using assays (Scalbert et al 2009). Hundreds of known metabolites can be identified in a single sample on one run of liquid chromatography-mass spectrometry (LC-MS) (Scalbert et al 2009). Given the ability to interrogate biological samples deeply, untargeted metabolomics techniques are becoming a useful tool to examine physiological differences that occur among various phenotypes, including those in livestock.

To date in beef cattle, most studies regarding feed efficiency have been focused on either physiology aspects or the rumen microbiome, without integration of these different factors. The central objective of this dissertation was to identify potential microbial and biochemical biomarkers of feed efficiency in Angus steers and heifers by incorporating novel -omics technologies such as metagenomics and metabolomics, and traditional livestock nutritional measurements, such as body weight and feed intake. Furthermore, few studies have examined the relationship of the rumen microbiome and feed efficiency in female beef cattle, although they have greater capacity to impact phenotypes of subsequent generations of cattle in a herd than male cattle typically. Thus, this dissertation focuses on both steers and heifers, and the relationship among host physiology, the rumen microbiome, and feed efficiency.

CHAPTER I

LITERATURE REVIEW

A version of this chapter has been published by Brooke A. Clemmons, Brynn H. Voy, and Phillip R. Myer:

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Abstract

The beef cattle industry represents a significant portion of the USA’s agricultural sector, with beef cattle accounting for the most red meat consumed in the USA. Feed represents the largest input cost in the beef industry, accounting for approximately 70% of total input cost. Given that, novel methods need to be employed to optimize feed efficiency in cattle to reduce monetary cost as well as environmental cost associated with livestock industries, such as methane production and nitrogen release into the environment. The rumen microbiome contributes to feed efficiency by breaking down low-quality feedstuffs into energy substrates that can subsequently be utilized by the host animal. Attempts to manipulate the rumen microbiome have been met with mixed success, though persistent changes have not yet been achieved beyond changing diet. Recent technological advances have made analyzing host-wide effects of the rumen microbiome possible, as well as provided finer resolution of those effects. This manuscript reviews contributing factors to the rumen microbiome establishment or re-establishment following rumen microbiome perturbation, as well as host-microbiome interactions that may be responsible for possible host specificity of the rumen microbiome. Understanding and accounting for the variety of factors contributing to rumen microbiome establishment or re-establishment in cattle will ultimately lead to identification of biomarkers of feed efficiency that will result in improved selection criteria, as well as aid to determine methods for persistent microbiome manipulation to optimize production phenotypes.

Introduction

With feed costs representing greater than half of the total cost of production in the beef cattle industry in the USA, novel methods to improve feed efficiency and nutrient utilization in cattle are becoming increasingly critical (McBride 2011). Additionally, agriculture represents one of the largest anthropomorphic producers of greenhouse gas emissions globally, with cattle representing the largest portion of livestock methane emissions (Alexandratos and Bruinsma 2012). Methane production alone is estimated to reduce feed efficiency by 2 to 12%, shunting potential carbon sources for the host to methane emissions (Johnson and Johnson 1995; Crutzen, Aselmann, and Seiler 1986; Alemu, Ominski, and Kebreab 2011). Increasing efficiency in the beef cattle industry will not only reduce greenhouse gas emissions and reduce natural resources required to meet expected animal protein needs but will also decrease input costs.

The rumen microbiome is responsible for the successful breakdown of low-quality feedstuffs into usable energy for ruminants, providing approximately 70% of energy to the host animal (Seymour,

Campbell, and Johnson 2005). Spanning several kingdoms, including Bacteria, Archaea, Protozoa, and Fungi, the rumen microbial ecosystem fulfills several functional niches, including proteolytic, fibrolytic, and lipolytic functions. Following the degradation of forages or concentrates, a number of metabolites are produced and released, including volatile fatty acids (VFA), biohydrogenated lipids, and other metabolites. These microbes also provide microbial crude protein (MCP), an important source of protein in the ruminant. The metabolites produced are either absorbed across the rumen epithelium or in the lower gastrointestinal tract and can then enter the bloodstream to be available to the host (Weston and Hogan 1968; Hout 1970; Kempton, Nolan, and Leng 1977).

Divergences in rumen microbial communities are associated with a number of host phenotypes, including feed efficiency (Myer et al. 2017), diseased states (Khafipour et al. 2009), and methane emissions (Shi et al. 2014). Rumen microbes produce glucogenic, lipogenic, and aminogenic precursors that partake in or regulate energy metabolism in cattle (Hungate 1975). Differences in relative abundances of different microbial populations have been associated with varying levels of methane emissions in the rumen of goats and sheep (Shi et al. 2014), whereas other phenotypes, such as feed efficiency, have been associated with differences in composition of bacterial and archaeal communities (Myer et al. 2017; Zhou and Hernandez-Sanabria 2009). Differences in microbial communities can also affect the metabolic profile of ruminants as well (Shabat et al. 2016; Mao, Huo, and Zhu 2016; Morgavi et al. 2015). The listed functions are just a small portion of the functional capacities possessed by microbes and illustrate the importance of these microbes within the context of ruminant physiology and health.

Although the importance of the rumen microbiome is well-established, attempts to manipulate the microbiome to improve cattle production have not been met with long-term success (Henderson et al. 2015). Antibiotics, probiotics, feed additives, and other methods of disrupting the microbial ecosystem of the rumen can provide targeted, immediate, and acute alterations to the rumen microbial profile, but no significant, persistent changes have been achieved to the microbial community composition (Weimer et al. 2010; Hook et al. 2009; Ghorbani et al. 2002; Weimer 2015). These studies suggest that there is some degree of host specificity or regulation mechanism dictating the gut microbiome in ruminants. Understanding the mechanisms dictating establishment, or re-establishment, of the rumen microbiome will provide necessary information in order to effectively provide persistent, long-lasting changes that increase production and feed efficiency. Currently, much of the research has been conducted on the re-establishment of the rumen microbiome following perturbation; however, understanding the mechanisms dictating establishment (i.e., colonization) of the rumen microbiome will ultimately provide researchers and producers with novel selection criteria. The present review focuses on existing knowledge affecting microbiome establishment, host-microbiome interactions affecting community establishment and composition, as well as the physiological implications resulting from rumen microbiome activity, in an ultimate effort to identify necessary methods and advances to effectively manipulate the rumen microbiome long-term for lasting production outcomes. Figure 1 provides a brief outline of this review, as well as prospective areas of research.

Research to Shape and Manipulate the Rumen Microbiome

Research Concepts	Research Elements	Future Research Efforts
Factors Affecting MB Establishment	Diet	Early Life MB Manipulation
	Feed Additives	
	Weaning	
Host Effects on MB Composition	Genetic Regulation	Genetic Selection for Production-Specific Microbe
	Mitochondrial Genetic Effects	
Microbial Activity and Animal Physiology	Metabolomics	Multi-omics Integration
	Fermentation	

Figure 1. Overview of research presented as well as potential areas of research to address contributing factors of rumen microbiome establishment

The Average State of the Rumen Microbiome

The rumen microbiome is a diverse ecosystem, possessing many functional and phylogenetically differences. Bacteria in the rumen account for approximately half of the microbial genetic material, followed closely by protozoa, and fungi (~2%) and methanogenic Archaea (2–4%) account for the remaining microbial abundances (Nagaraja 2016). Bacteria are the most well-studied of the rumen microbiota due to their diversity, both functional and phylogenetic, and ease of analysis. Rumen bacteria have had a rich history of culture-based research, even though the majority are still non-culturable (Creevey et al. 2014), providing longer history of analysis and more information than other microbes. Robert Hungate pioneered the field of ruminant microbiology in the 1960s and characterized his findings (Hungate 1975) using culture-based methods. Since the Hungate era, new sequencing technologies have allowed expansion of Hungate's and his colleagues' research, starting first with bacteria, primarily due to the greater knowledgebase available from bacteria.

Firmicutes and *Bacteroidetes* are the dominant bacterial phyla in the rumen of cattle. *Firmicutes* are typically greatest in relative abundance in predominantly forage-based diet, whereas *Bacteroidetes* are usually more abundant in diets consisting primarily of concentrate (Fernando et al. 2010; McCann et al. 2016). *Firmicutes* and *Bacteroidetes* are then typically succeeded in abundance by Proteobacteria, Tenericutes, and Actinobacteria (Myer et al. 2015b). At the genus level, *Prevotella* are most common, potentially due to the wide range of functional capacities of species within *Prevotella* (Stevenson and Weimer 2007; Avguštin, WALLACE, and Flint 1997). Currently, there is no known “normal” rumen microbiome, though core microbiota have been found across species, diet, and geographical locations (Henderson et al. 2015). Given this, the rumen microbiome is typically examined under specific conditions, such as diet, production stage, or illness (Khafipour et al. 2009; Myer et al. 2015b; Jami, White, and Mizrahi 2014; Petri et al. 2013; Lima et al. 2015). What is normal on one diet or in one situation may not be normal in another. Therefore, discussed subsequently are some of the largely known factors affecting microbiome establishment in ruminants.

Factors Affecting Microbiome Establishment in Ruminants

Diet

Diet is the greatest known external influence on the composition of the rumen microbiome in ruminants (Henderson et al. 2015). Researchers in one study collected 742 samples of the rumen content of 32 ruminant species as well as gut content of species whose gastrointestinal systems were similar to that of a ruminant from 35 countries around the world and found that, though animal-to-animal rumen microbiome varied greatly, diet was the largest factor affecting microbial community composition (Henderson et al. 2015). Though inter-animal variation is high across most studies, diet had a significant effect on the rumen microbiota. However, these changes have been mainly conducted on bacterial communities and methanogenic archaeal communities.

During transitioning periods in which cattle are moved from a predominantly forage-based diet to a high-grain diet, large changes are seen in the relative abundances of the bacteriome. In a study conducted by Fernando et al. (2010), the transition from a hay-based diet to a high-grain diet (80%

grain) resulted in several changes to the bacteriome, including changes in the *Firmicutes*-to-*Bacteroidetes* ratio, decreased fibrolytic bacteria such as *Fibrobacteres*, and increased species from *Prevotella*, among others. These changes have also been demonstrated in other studies (Fernando et al. 2010). The ratio of *Firmicutes* to *Bacteroidetes* in particular has been linked with other phenotypic traits, namely in adipose metabolism (Jami, White, and Mizrahi 2014; Turnbaugh et al. 2009; Ley et al. 2005). Abundances of *Bacteroidetes* and *Proteobacteria* species increased as grain became a greater percentage of the diet, which may be more effective at fermenting the readily digestible carbohydrates present in grain-based diets (Fernando et al. 2010), and may be more tolerant of the lower pH that occurs when greater amounts of VFA and lactic acid are produced (Rodríguez 2003).

Certain species have been associated with grain-based diets, such as *Streptococcus bovis*. *S. bovis* is a lactate-producing bacterium that thrives at lower pH that often accompanies high-grain, rapidly fermentable diets. Enzymes produced by *S. bovis* function optimally at a pH ranging from 5 to 6, which characteristically occurs in the rumen of animals consuming high-grain diets (Russell and Hino 1985). During transition to high-grain diets, 67-fold increases in *S. bovis* have been observed (Tajima et al. 2000). Furthermore, other lactate-producing bacteria, such as *Lactobacillus* spp., also increase in abundance in the rumen on high-grain diets (Nagaraja and Titgemeyer 2007), further contributing to accumulation of lactic acid and reduction of ruminal pH.

Although some microbes tend to be diet-specific with regard to great shifts in relative abundance, others are present, regardless of external factors. Members of *Prevotella* are among the most common microbes in the rumen, regardless of diet (Henderson et al. 2015). This persistence among diets is also supported by their abundance in the rumen, as *Prevotella* account for as much as 60% of the total bacterial populations in the rumen (Stevenson and Weimer 2007). Examining this large and diverse genus of bacteria, Bekele et al. (2010) analyzed the effect of diet on the populations of ruminal *Prevotella* species using DGGE. Differences were observed between diets in targeted bacterial populations; however, unidentified species within *Prevotella* were similar between diets (Bekele, Koike, and Kobayashi 2010). This may suggest that *Prevotella*, particularly uncultured members, possess great functional diversity. Indeed, species within *Prevotella* display a wide variety of functions, including cellulolytic, amylolytic, and fibrolytic activities (Avguštin, WALLACE, and Flint 1997; Bekele, Koike, and Kobayashi 2010). This great functional diversity in the rumen warrants further interrogation of those microbiota in order to further define the multifaceted role of *Prevotella* in context of the rumen microbiome.

Diet can also influence the overall α - and β -diversity of the rumen microbiota. In a study conducted by Pitta et al. (2010), bacterial α -diversity decreased when animals were switched from a bermudagrass to a winter wheat diet, as measured by Shannon and Chao1 (Pitta et al. 2010). Principle component analyses also illustrated very distinct bacterial community composition based on diet (Pitta et al. 2010). Some of the same changes seen in shifting from one forage type to another were also some of the same trends observed in transition from forage to grain diets (Fernando et al. 2010; Tajima et al. 2000; Pitta et al. 2010; Anderson et al. 2016) as well as other forage to forage diets (Thoetkiattikul et al. 2013). In mice, humans, and other monogastric species, differences in α -diversity have been associated with divergences in host phenotype, such as

diseased states. Particularly, variation in bacterial diversity has been associated with energy utilization in humans and mice. In a study conducted by Turnbaugh and others (2009), decreased bacterial diversity of the lower gut was associated with obesity in human twins (Turnbaugh et al. 2009), a trend that has also been observed in mice (Turnbaugh et al. 2008). While the relationship between energy utilization and the diversity of rumen microbial communities is still not entirely understood, research completed in other species suggest that microbial diversity may influence feed efficiency in ruminants as well.

Methanogenic archaea account for approximately 2–4% of the total microbial genetic material (Nagaraja 2016). Methanogenic archaea are the primary cause of methane production in ruminants, and modulating their ruminal populations and/or functionality is of particular interest in the research community given that livestock contribute to 10–12% of anthropogenic greenhouse gas emissions (Smith et al. 2014). Additionally, it is estimated that methane production results in a 2–12% reduction in feed efficiency in cattle (Johnson and Johnson 1995). Given the possible negative implications of methanogenic archaea in overall ruminant production, understanding their role in the rumen microbiome and contributions to ruminant physiology is fundamental to improving feed efficiency and reducing global methane production.

Changes occur in methanogenic archaeal communities as the result of differences in diet. Zhou and colleagues conducted a study in which animals were placed on low energy (LE) or high energy (HE) diets and stratified by residual feed intake (RFI) (Zhou and Hernandez-Sanabria 2010). Several distinct differences were found between the LE and HE diets. Diversity of methanogenic species was greater in LE diet compared to the HE diet, which has been found in other studies in sheep (Wright et al. 2004). Additionally, in a separate study, bulls fed fiber-based diet had greater α -diversity of methanogenic species than those fed a starch-based diet (Popova et al. 2011). Fiber-based diets may present a wider range of substrates for methanogens, leading to greater diversity of archaeal communities. Furthermore, methane production is greater in animals on forage-based diets, which may be the result of the greater diversity of the rumen archaeal communities that occurs in predominantly forage diets. However, methane production on forage-based diets is also a function of the increased acetate/propionate ratio that is common on forage-based diets, as acetate is tightly linked to methanogenesis (Johnson and Johnson 1995; Moss, Givens, and Garnsworthy 1995; Hindrichsen et al. 2004).

Certain species, such as *Methanobrevibacter gottschalkii*, were found only in LE diet samples, whereas certain strains of *Methanobrevibacter* sp. AbM4 and *Methanobrevibacter smithii* were only observed in the HE diet samples (Zhou and Hernandez-Sanabria 2010). However, while different species were found to be associated with diet or the other, no differences were observed in total methanogens (Zhou and Hernandez-Sanabria 2010). This conflicts with a study conducted by Wallace et al. (2015), in which archaea were present in greater abundance in a medium grain diet compared to a high-grain diet (Wallace et al. 2015). Diet may not be the greatest influence of methanogenic archaeal communities because they use secondary resources, such as hydrogen, as their main source of energy. This may cause them to be more closely dictated by the populations of other microbes in the rumen, which are themselves affected by feed (Wallace et al. 2015). Thus,

archaeal communities may be indirectly affected by feed more so than directly affect by it, as is the case for bacteria and protozoa.

Protozoa and fungi are also important participants in the rumen microbial ecosystem, though fewer studies have been conducted to interrogate the relationship between their populations and the diet. Protozoal populations may follow some of the same trends as are seen in bacterial populations when animals are moved to a grain-based diet from a diet primarily made up of forage. In a study conducted by Hristov and colleagues in 2001, increased grain resulted in decreased total protozoa (Hristov et al. 2001). Several protozoal populations were lower in the high concentrate (barley) diet and several genera were present in medium concentrate but not in high concentrate, including *Eudiplodinium*, *Dasytricha*, *Diplodinium*, *Metadinium*, *Ophryoscolex*, and *Ostracodinium* (Hristov et al. 2001), which has been supported by other studies that have demonstrated that protozoal numbers peaked with diets consisting of 40 to 60% concentrate (Dehority and Orpin 1997). A study conducted by Franzolin and Dehority (1996) analyzed the long-term effects of a concentrate compared to forage-based diet. When the steers were fed a diet of 75% concentrate, total abundance of protozoa increased compared to the forage-based diet (Franzolin and Dehority 1996), but the 50% concentrate diet did not have any differences in protozoal numbers compared to either the forage-based or 75% concentrate diets. In that study, *Diplodiniinae* decreased when steers were transitioned from a 50% to a 75% concentrate diet (Franzolin and Dehority 1996), a similar trend as was found in the study conducted by Hristov et al. (Hristov et al. 2001). Although protozoal populations do change in response to diet changes, particularly in high-grain diets, they may be less flexible and resist change when compared to bacteria.

Rumen fungi are the least characterized and understood rumen microbe. The fungal populations of the rumen are superior in their ability to break down more fibrous materials that may be difficult for other microbes to break down, possibly because they are more effective at penetrating tough plant walls (Bauchop 1979; Orpin, Joblin, and Hobson 1997). Grenet and colleagues demonstrated that fungi selectively chose “stemmy forages” more frequently than other substrates and were absent entirely from grains (Grenet et al. 1989). Rumen fungi are very sensitive to pH (Orpin 1977), which can drop rapidly due to digestion of readily fermentable carbohydrates in grain. Grain particles may also be too small for the fungi to adequately attach (Grenet et al. 1989; Fonty et al. 1987). Fungi have also been found to favor older plants with thicker cell walls compared to young, more readily digestible forages, likely due to difficulty attached to the thin cell wall (Grenet et al. 1989). Overall, fungal populations seem to thrive on thicker-walled forages and survive poorly in high-grain diets.

Feed Additives

Feed additives are also used to manipulate host phenotypes as well as the rumen microbiome. Producers use feed additives to alter host phenotypes which can inadvertently alter the rumen microbial community profile. Moreover, additives can also be used to directly alter the rumen microbiota to manipulate the host phenotype. Commonly used feed additives including antimicrobials, supplements, and probiotics have been used to alter the rumen microbiota and/or

host physiology. These different feed additives function in a variety of ways to improve ruminant feed efficiency or alter host phenotypes.

Antimicrobials have been used in animal agriculture for decades. Antimicrobials can be used to decrease infections, but typically when antimicrobials are used as feed additives, they target specific microbial populations in order to decrease a defined population of microbes that may contribute to adverse effects, such as sharp decreases in pH due to ruminal acidosis. One major antimicrobial used in cattle production is monensin. Monensin is an ionophore that disrupts ion flux across microbial membranes and causes cell death (Schelling 1984). In ruminant systems, monensin, in part, is used to limit the effects of ruminal acidosis, namely decrease the negative effects of sharp decreases in pH following the introduction of a high-grain or concentrate diet with little time for microbes to adapt to the change. Monensin has also been demonstrated to improve feed efficiency and reduce methane emissions. Monensin interferes with intracellular protein transport, disrupting ion exchange across the cell membrane. When monensin is added to the ruminant diet, it disrupts production of VFA and lactate (Schelling 1984). A previous study found that steers on a backgrounding diet that were also fed monensin gained weight at the same rate as steers not fed monensin but required significantly less feed than the control (Perry, Beeson, and Mohler 1976). Another study also found that monensin decreased methanogenic archaea as well as methane production and dry matter intake (Tomkins et al. 2015). These changes, which led to improvements in host phenotype, also altered the rumen microbiome. However, while use of antimicrobials can lead to improvements in host phenotype via alterations in the rumen microbiota, these changes are often short-lived and do not produce long-lasting modifications.

Non-nutritive supplements, such as essential oils, saponins, and tannins, can be added to the diet to modify not only the rumen microbial community structure but also host phenotype, such as feed efficiency. Some oils are used as an alternative to antibiotics because some long-chain fatty acids are toxic to the rumen microbes. Many of the oils act through disruption of the cell membrane, which allows for leakage of ions across the cell membrane (Griffin et al. 1999; Sikkema, de Bont, and Poolman 1994; Ultee, Kets, and Smid 1999). Some of the non-nutritive supplements may also act as cation and proton transmembrane carriers, further disrupting the ion balance within the cell (Ultee, Bennik, and Moezelaar 2002). Additional potential modes of action are reviewed by Calsamiglia et al. (Calsamiglia et al. 2007). Non-nutritive supplements that possess antimicrobial properties may be a desirable alternative to antibiotics given the negative public perception of antibiotics and of growing global antibiotic resistance. These non-nutritive supplements are of plant-based origin and thus may provide a more “natural” method for manipulating the rumen microbiome to improve desirable phenotypes and reduce negative aspects of livestock agriculture, such as methane production.

Essential oils have been used as alternatives to antibiotics, such as ionophores, to reduce methane production; however, success in reduction of methane production has been mixed. Macheboeuf et al. (2008) performed in vitro analysis of several essential oils on rumen fermentation and methanogenesis using increasing doses of essential oils to measure dose response (Macheboeuf et al. 2008). Although essential oils reduced gas production up to 48% compared to control samples, many of the doses that resulted in decreased gas production also reduced in total VFA production,

which may negate the positive results of gas production reduction (Macheboeuf et al. 2008). However, in vivo, these results are often not observed. In a study examining the effects of supplementary oils on the microbial communities in the rumen compared to monensin, the addition of essential oils did not change methane production or archaeal communities compared to monensin but did result in decreased rumen fungal abundances (Tomkins et al. 2015), indicating that it may not provide significant enough changes to impact the rumen microbiome as well as production. Beauchemin and McGinn (2006) conducted a study to analyze the effect of essential oils on ruminal fermentation parameters and methane production (Beauchemin and McGinn 2006). Essential oils did not modify ruminal fermentation characteristics as measured by VFA concentrations, nor did essential oil supplementation affect methane production (Beauchemin and McGinn 2006). Utilization of essential oils as an alternative means of reducing methanogenesis may not be a viable option, but further analysis must be conducted.

Another supplement that has been of growing interest is the use of dried distillers grains (DDG). A previous study measured changes in bacterial communities following supplementation with different levels of DDG. The ratio of *Firmicutes* to *Bacteroidetes* changed with 25 and 50% DDG, with lower abundances of *Firmicutes* compared to *Bacteroidetes* (Callaway et al. 2010). These changes also resulted in decreased rumen pH (Callaway et al. 2010). These changes may have occurred due to the increased readily digestible starch content, which typically results in increased *Bacteroidetes*. Members of the phylum *Bacteroidetes* possess more amylolytic capabilities, whereas *Firmicutes* in the rumen possess predominantly fibrolytic abilities (Maroune and Bartos 1987). Changes to the rumen microbiome caused by supplementation are important as they can influence rate of digestion, available nutrients, and other physiological factors. However, the persistent effects of supplementation on the rumen microbiome and subsequent physiological changes are still relatively unknown.

Probiotics are a growing area of interest of late. Probiotics, or direct-fed microbials (DFM), are live microbes that are added to the diet to modulate digestion, fermentation, or the microbial community composition. Yeast, particularly *Saccharomyces cerevisiae*, as well as other fungi have been one of the most widely used and studied probiotics. Supplementation with fungi, such as *Aspergillus oryzae*, resulted in increased bacterial concentrations, particularly those associated with fibrolytic activities (Beharka, Nagaraja, and Morrill 1991). In addition to changes to the bacterial populations, supplementation with *A. oryzae* also changed physiological parameters, including increases in ruminal volatile fatty acids and the animals weaned earlier (Beharka, Nagaraja, and Morrill 1991). Studies using *S. cerevisiae* also resulted in some of the same trends (Lesmeister, Heinrichs, and Gabler 2004). However, probiotic used has yielded mixed or inconsistent results (Chaucheyras-Durand, Walker, and Bach 2008). Regardless of whether yeast use is successful or not, its use does not result in long-term changes to the rumen microbiome nor effects on efficiency. Other probiotics have also been used, such as inoculations from other ruminants. Repeated inoculation in beef heifers from bison rumen content resulted in changes to the rumen bacterial and protozoal abundances, which translated to changes of passage rate, chewing activity, and fermentation (Ribeiro et al. 2017). Bison inoculations resulted in increased α -diversity (Chao1, Shannon), as well as β -diversity (Ribeiro et al. 2017). However, in this study, effects were not measured long-term, and thus it has not been determined if repeated inoculation

would result in permanent restructuring of the rumen microbiome. As is the case with antibiotic use, probiotics seem to have potentially desired effects on the rumen microbiota with regard to production as well as host physiology; however, results have also been mixed, and extended or permanent effects of probiotic use have not been observed.

Weaning and Weaning Age

Weaning is a time of significant change for beef cattle. In most beef cattle operations, calves are kept with the dam for approximately 7 to 8 months, whereas in dairy systems, calves are typically removed from the dam within 48 h. Even prior to weaning, and regardless of diet, there is a succession of bacterial communities over time in pre-ruminant calves (Li, Connor, et al. 2012), which has been supported by other studies (Rey et al. 2014; Meale et al. 2016; Jami et al. 2013). However, at weaning, changes in microbial community composition, in part, are due to host physiological changes but also likely due to the introduction of solid feeds because diet is a large driver of microbial community composition and modulation (Henderson et al. 2015; Li, Connor, et al. 2012; Rey et al. 2014). Rey and colleagues (2014) analyzed the succession of bacteria in dairy calves. In the first several days of birth, the reticulorumen was dominated by anaerobic, lactose-consuming bacteria, primarily from the phylum Proteobacteria (Rey et al. 2014). However, once solid feed was introduced at day 4 following birth, abundances of *Bacteroidetes* increased significantly (Rey et al. 2014). At the genus level, many microbes of interest and importance in adults also changed in their relative abundance. Abundances of *Prevotella* quadrupled, rising from a mean abundance of approximately 11% to over 40% (Rey et al. 2014). *Pasteurella*, however, decreased following introduction of a solid diet and was non-detectable following 3 weeks of age (Rey et al. 2014). The shifts in bacterial abundances demonstrate the respective shift in bacterial functions in the rumen likely as a result of diet modification, rumen development, and overall fermentative environment.

Due to the importance of adequate rumen development, factors affecting the microbial success of the rumen are of interest in young ruminants. One such factor that affects the rumen microbiome is transport of the animals. Following weaning and during transport to a feedlot operation in beef calves, total amount of bacteria was not affected by transport, weaning, or changes in intake; however, protozoal populations were affected (Fluharty, Loerch, and Dehority 1994). Relative abundance of *Entodinium* increased following weaning, whereas *Diplodinium* and *Epidinium* abundances tended to decrease over time and *Dasytricha* was completely eliminated by day 7 (Fluharty, Loerch, and Dehority 1994). With regard to archaeal communities, establishment has been measured shortly after birth and has been demonstrated to stabilize soon thereafter (Fonty et al. 1987). As seen in bacterial populations, archaeal community establishment in the rumen at weaning is also affected by age and diet. For instance, one study found that methanogenic archaea abundances were greater in lambs fed forage-based diets compared to grain-based diets (Yáñez-Ruiz et al. 2010), which are trends also seen in adult animals (Zhou and Hernandez-Sanabria 2010).

Weaning age and method of weaning can also change rumen or gastrointestinal tract microbiome establishment and community structure. However, because weaning is a more labor-intensive process in the dairy industry compared to the beef industry, current understanding of the effects of

weaning on the rumen microbiome is primarily from dairy cattle, with additional information coming from small ruminant systems. Weaning in dairy calves elicited an immune response in the lower gastrointestinal tract, but adding solid feed in addition to milk replacer resulted in changes to the immune response as well as gut bacteria (Malmuthuge et al. 2013). Increased solid feed resulted in increased total amount of bacteria present in the gastrointestinal tract (Malmuthuge et al. 2013). In 2016, one study examined the effects of weaning and weaning strategies on the rumen microbiome establishment. Pre-weaned calves had greater α -diversity compared to weaned calves, but the weaning strategy did not have any effect on microbial α -diversity (Meale et al. 2016). When principle coordinate analysis (PCoA) was used, operational taxonomic units (OTU) clustered by pre- or post-weaning animals, but no differences were observed in weaning strategy (Meale et al. 2016). In addition to these changes, abundances of specific bacteria also changed from pre-weaning to weaning, including decreased *Bacteroidetes*, increased *Proteobacteria*, and increased *Firmicutes* populations (Meale et al. 2016). Moreover, weaning age also impacts microbial community establishment. Early weaning of dairy calves resulted in a rapid change of the rumen microbiome compared to calves that were gradually adapted to a post-weaning diet; however, once the microbiome OTU were established, they remained stable following weaning (Meale et al. 2017). Beyond this, not many differences were observed in the microbial community diversity metrics between early and gradually weaned calves (Meale et al. 2017). Though changes in OTU abundances of historically dominant phyla changed at different rates in early-weaned compared to gradually weaned calves, no differences were observed in *Bacteroidetes*, *Firmicutes*, or the ratio of these two once calves were fully weaned (Meale et al. 2017). Differences were observed, however, in less dominant phyla, including *Cyanobacteria*, *Spirochaetes*, *Synergistetes*, and *Verrucomicrobia*, which all decreased in early-weaned calves over time, whereas only *Elusimicrobia* and *Fibrobacteres* decreased by weaning in gradually weaned animals (Meale et al. 2017). While many similarities between these weaning ages were observed in the rumen microbiome of calves, several differences were also observed at a finer resolution, indicating that this may be an important area of research in the future to optimize the rumen microbiota for production.

Breed

Although breed can play a large part in physiological phenotypes of cattle, little is known about the differences in the rumen microbiota between breeds, particularly in beef cattle. Researchers conducted a study to delineate differences in the rumen bacterial and archaeal populations of beef cattle differing in sire breed and diet (Hernandez-Sanabria et al. 2013). Several differences in bacterial populations were observed in the LE diet due to sire breed, including 24 bacterial phylotypes, with four Angus-associated phylotypes (Hernandez-Sanabria et al. 2013). When steers were fed the HE diet, 37 bacterial phylotypes differed between sire breeds, with six Angus-associated phylotypes, one Charolais-associated phylotypes, and no Angus $\times$ Charolais (hybrid)-specific phylotypes (Hernandez-Sanabria et al. 2013). Although differences were observed as a result of sire breed in bacterial phylotypes, no breed-associated differences were found in methanogenic archaea phylotypes (Hernandez-Sanabria et al. 2013). This study was one of the first to analyze breed effects on the rumen microbiome, which indicated that there is possibly some host regulation or preferential selection of rumen microbiota establishment; however, these findings may have been breed-specific. Additionally, breed variation may contribute to host

regulation of the gut microbiome establishment, but individual variation in host genetic regulation may play a greater role than breed variations.

Many external and physiological factors contribute to microbiome establishment and composition in ruminants. However, data support that diet is likely the largest external dictator of the rumen microbial community structure (Henderson et al. 2015). The change from predominantly liquid to solid feed may also account for the changes in the rumen microbiota following weaning. Breed may also impact the rumen microbiome and presents an interesting future area of research. However, beyond these factors, there appears to be some degree of host regulation of microbiome establishment and composition.

Host Effects on Rumen Microbiome Establishment

The rumen microbiome is a complex ecosystem with many confounding factors that lead to its establishment. Certain traits, such as feed efficiency, have been associated with differences in bacterial communities in the rumen and lower gastrointestinal tract of cattle, providing some indication that these important host phenotypes are related to the microbiome. Although some external factors, such as diet, contribute to fluctuations in the microbiome, the question remains as to what drives changes when controlling for such factors. Some mechanisms seem to be in place from the host that dictate the microbial community composition; however, those mechanisms are still widely unknown.

Host Genetic Regulation

Many traits, such as carcass quality and milk yield, are associated with quantitative trait loci (QTL) or single nucleotide polymorphisms (SNP), typically indicating at least a moderate ability to genetically select for those traits. Attempts have been made to associate gut microbiota with genetic factors for which producers, researchers, or others can select. In one study, researchers attempted to correlate mice gut microbiomes with genetic traits. Several taxonomic groups (26) were associated with 13 QTL, with each of the four dominant bacterial phyla corresponding with a QTL (Benson et al. 2010). The QTL associated with the bacteria were distributed across eight different chromosomes, which indicated that the gut microbiome is a heritable trait (Benson et al. 2010). Similar trends have been observed in humans as well. Monozygotic twins had more similar gut bacteriomes than did dizygotic twins (Goodrich et al. 2014). Another study revealed that different taxa exhibited varying heritability (Goodrich et al. 2016). In addition, the researchers also found 37 potential SNP that may be involved in gut microbiome establishment (Goodrich et al. 2016). These studies indicate that microbiome establishment may be a complex, phenotypic trait of which can be selected.

One important production trait that has been associated with ruminant host genotype is that of methane emissions. Methane contributes approximately 2 to 12% decrease in feed efficiency in ruminants, and ruminants produce approximately 80% of global livestock emissions (Johnson and Johnson 1995). Genotypic associations have been identified between methane yield and methane production rate, and these traits were only loosely associated genetically with dry matter intake (Donoghue et al. 2016). The genetic relationship between different methane production and yield traits with little or no genetic connection to dry matter intake is ideal for selection, in which low

methane production can be selected for without negatively impacting dry matter intake (Donoghue et al. 2016). Because methane production is thought to derive primarily from methanogenic archaea in the rumen, this study indicated that the host genetics may play a strong role in selecting for or against methanogenic archaeal communities or other microbial populations (Donoghue et al. 2016). However, methane production had a strong genetic correlation with weaning weight and body weight, so selecting against methane production could result in decreased weaning weight (Donoghue et al. 2016).

While several studies have found genetic correlations between the gut microbiome and the host in mice and humans (Turnbaugh et al. 2009; Ley et al. 2005; Goodrich et al. 2016), almost no studies have been conducted to determine these same correlations in ruminants. Certain SNP have been associated with *Prevotella* abundance in the rumen of dairy cows (Gonzalez-Recio et al. 2017). In particular, *Prevotella* abundance in the rumen was associated with SNP on the DGAT1 gene, which is associated with milk fat composition (Mohammed et al. 2014; Grisart et al. 2004). Other microbial populations were also associated with SNP on other host genes associated with fatty acid or cellular metabolism, including ACSF3, AGPAT3, and STC2 (Gonzalez-Recio et al. 2017). This is the first study in cattle that has attempted to find SNP associated with the rumen microbiome and how those may be correlated with important phenotypic traits. This study presented evidence that the microbiome is indeed associated with, to some degree, the host genome, which could have large implications for the cattle industry. Additional studies should be conducted in beef cattle to determine if production parameters important to that industry are also genetically correlated with the rumen microbiome.

Mitochondrial Associations

Mitochondrial DNA (mtDNA) is an often forgotten or excluded source of genetic variation when searching for SNP-associated traits. Mitochondrial DNA is inherited solely from the dam and is known as a source of SNP. In humans, mtDNA haplogroups in stool were associated with certain bacterial taxa (Ma et al. 2014). Different haplogroups were associated with different genera in stool after further analysis, and these were not affected by gender, body mass index (BMI), or age (Ma et al. 2014). Different haplogroups were also correlated with differences in relative abundance ratios of phenotypically important taxa, such as the *Bacteroides/Prevotella* (Ma et al. 2014; Yatsunenko et al. 2012). The researchers also speculated that the gut microbiome may be impacted by the mutations identified in the mtDNA, potentially mediated by the inflammatory response to reactive oxygen species and variation in key redox pathways. Although the mechanisms relating mtDNA to the gut microbiome are not yet defined, this study provides novel and foundational information about the role that the mtDNA may play in dictating the host gut microbiome and should be considered when assessing host influence in microbiome establishment and manipulation.

Microbiome Re-Establishment Following Disturbances and Associated Parameters

In order to measure the effect of the host on microbiome re-establishment following disturbance, several studies have been conducted. However, one of the most extreme disturbances to the rumen microbiome is that of rumen content evacuation or exchange. A previous study was conducted in which the researchers performed rumen content exchanges (RCE) between Holstein cows that

were chosen based on their differences in rumen pH and total VFA concentrations. Immediately following RCE, the rumen pH and VFA most resembled that of the donated rumen content (Weimer et al. 2010). However, the rumen pH and VFA concentrations returned to their original values and concentrations within 48 h, except in one of the four animals (Weimer et al. 2010). The bacterial communities were then analyzed from each of the four animals from several time points throughout the 9 weeks of study. In the first experiment of two animals, the bacterial communities returned completely to their native bacteriome by the end of the 9 weeks. However, in the second experiment, the bacterial communities in one of the two cows did not completely return, though the communities most resembled the native bacteriome rather than the donated bacteriome (Weimer et al. 2010). In the cow that did not experience complete return of its bacterial community composition, the VFA concentrations and pH values also did not return completely, suggesting a host effect in the microbiome establishment, at least in adult animals, as well as the wide-reaching physiological impact of the rumen microbiome (Weimer et al. 2010). However, because there was not a complete re-establishment in one of the animals, this may indicate that host control over gut microbiome composition is variable, which in turn may allow for persistent manipulation of the gut microbiome.

A later study conducted used RCE to measure how microbiome re-establishment was associated with a major production parameter: milk yield. Cows used in the study were chosen based on differences in milk production and were designated low producing (LP) or high producing (HP). When LP cows received rumen content from HP cows, milk yield increased significantly (Weimer et al. 2017). Interestingly, when the bacterial communities returned to the original composition, so did the milk production (Weimer et al. 2017). Although not all cows experienced complete return of their original bacterial community composition, all bacteriomes most resembled that of the native bacteriomes than the donated bacteriomes (Weimer et al. 2017). This study provided insight as to the relationship between the host and the stability of its microbiome and the effects that changes to the rumen microbiome can have on host physiology and important production parameters.

While these studies have illustrated compelling evidence that ruminant host genetic regulation may impact microbiome establishment and community composition, and these relationships may be responsible for important production traits in both beef and dairy cattle industries, much is still unknown about the mechanisms governing these influences. The rumen and lower gastrointestinal tract microbiomes contribute significantly to the effective breakdown of feedstuffs and have been associated with feed efficiency in beef cattle (Myer et al. 2017). It may be possible in future studies to genetically associate the rumen microbiome with the host as well as other important production parameters to improve the overall efficiency and sustainability of the beef cattle industry.

Animal Physiology Resulting from Rumen Microbiome Activity

The rumen microbiome is critical for host physiology as it produces energy substrates and other nutrients that the animal needs to perform adequately. Nutrients that are generated and released in the rumen can then be absorbed across the rumen epithelium or in the lower gastrointestinal tract and typically enter the circulatory system of the ruminant. While these metabolites, nutrients, and substrates are critical to the animal, most rumen microbiome research fails to incorporate the

multitude of fermentative and physiological factors resulting from microbial activity. Unfortunately, only several products are routinely measured, such as VFA, nitrogen (several forms), and glucose. These shortcomings must be addressed in future research. Employing several additional techniques when assessing the ruminal microbiome activity can lead to better interrogation of the relationships among host physiology and the rumen microbiome, such as proteomics to assess microbial crude protein, trace mineral analyses, and transcriptomics, to name a few. Although it requires researchers to analyze great amounts of data, utilizing a more systems-based approach, in tandem with gut microbial metagenomics, and host-microbial interactions will aid in the effort to fully understand the mechanisms behind gut microbiome stability, resiliency, and establishment.

Fermentation Products

Volatile fatty acids are important by-products of microbial fermentation in the rumen as they are the main glucogenic and fat precursors, particularly propionate and acetate. Production of VFA also contributes to the rumen pH, which is a critical measurement that contributes to overall health of the rumen and, in turn, can select for or against certain microbial populations. As with the rumen microbiome, VFA production is intimately linked to diet, predominantly due to the changes in substrate, as well as rumen microbial activities. Different supplements, including corn, beet pulp, and barley, can result in varying total VFA concentrations and differences in rumen pH, without affecting dry matter intake (Carey, Caton, and Biondini 1993). Many other studies have found associations among types of feed, VFA concentrations, and pH as well (McCollum and Galyean 1985; Fulton, Klopfenstein, and Britton 1979; Brown, Ponce, and Pulikanti 2006). The relationship among the host, VFA, and the rumen microbiota has been extensively explored and previously reviewed (Bergman 1990; Van Houtert 1993; Doreau and Ferlay 1994). Although VFA production and pH have been well-studied in cattle, there is still much knowledge to gain regarding the manipulation of the microbiome and its effects on these factors or vice versa and how that will, in turn, modify energy substrate and nutrient production.

Serum Metabolome

Since their inception, metabolomic techniques have provided researchers with a multitude of information (Fontanesi 2016). The serum metabolome can provide a detailed perspective about the physiology of the animal at the time of collection, providing far more information than has ever been generated, with an average of over 100 known metabolites being identified in single sample and hundreds more unidentified (Monteiro et al. 2013). Serum metabolomics has been used widely to understand diseased states in particular (Ametaj 2015). Thus, serum metabolomics is a useful tool for researchers to be able to understand effects of a wide range of conditions on the overall host physiology.

Serum metabolomic techniques have started to be applied to ruminant systems because they can capture information about what has been absorbed from the environment (e.g., rumen) as well as endogenous production of metabolites. Animals differing in RFI also exhibited different metabolic profiles in plasma using nuclear magnetic resonance imaging (NMR) (Karisa et al. 2014). During the second period of the trial, ten metabolites were identified that differed between low and high RFI, which accounted for more than half of the differences in RFI (Karisa et al. 2014). Several

metabolites accounted for the majority of differences observed in RFI, including glutamate, citrate, acetate, and carnitine, all of which are directly or indirectly involved in intermediary metabolism (Karisa et al. 2014). This study alone provided a wealth of information with regard to phenotypic differences in beef cattle, but more studies need to be conducted to determine how these can be manipulated to optimize beef cattle production and how the microbiome relates to the serum metabolic profiles of the animals.

A recent study used LC-MS to perform untargeted metabolomics on serum from steers differing in feed efficiency (Clemmons et al. 2017). Steers with low RFI had greater serum abundances of several metabolites, including pantothenate and carnitine, which are directly involved in intermediary metabolism, particularly fat and carbohydrate metabolism (Clemmons et al. 2017). The results from this study support that of a previous study by Karisa et al. (2014) but also expanded upon the work of Karisa et al. by using LC-MS rather than NMR to analyze the serum metabolome (Karisa et al. 2014). However, the few untargeted serum metabolomic studies that exist in ruminants are still associative and have not moved beyond to provide explanations for variations in host phenotypes. Incorporating serum metabolomic techniques with other methods, including genomics, transcriptomics, and trace mineral analysis, may provide greater insight as to the relationship between the host and rumen microbiome.

Rumen Metabolome

The rumen metabolome, like the serum metabolome, can provide information for researchers about the state of the animal at the time of collection (Fontanesi 2016). Rumen fluid metabolomics has been almost exclusively applied to dairy cattle, with little research conducted to assess the use of rumen metabolomics, particularly untargeted metabolomics, in beef cattle. One study that analyzed four biofluids from dairy cows (milk, urine, rumen fluid, and serum) using NMR and gas chromatography (GC) found several metabolites present in all four fluids, while many metabolites were found solely in rumen fluid (Sun et al. 2015). Differences in metabolites were also found between biofluids from cows on different diets as well (Sun et al. 2015). This study highlighted the importance of a multi-systems approach to metabolomics and how changes in metabolites at different levels (systems) can be associated with host phenotype.

In beef cattle, protein is the main product desired, requiring effective conversion of feed to mass. Thus, understanding the relationship between the microbiome and this conversion, as well as the host physiology, will ultimately lead to the ability to optimize these various aspects of ruminant production to maximize production with minimal input. Steers differing in feed efficiency varied in their rumen fluid metabolome (Artegoitia et al. 2017). Ninety metabolites differed between high- and low-efficiency animals, with most of them involved in fatty acid and amino acid metabolism (Artegoitia et al. 2017). Differences in plasma metabolites, particularly fatty acids, were also observed between animals differing in feed efficiency (Artegoitia et al. 2017). However, concentrations of fatty acids differed between the rumen and plasma metabolomes (Artegoitia et al. 2017), indicating that some other factors are involved in the transport of metabolites from the rumen to the blood. This study was one of the first uses of untargeted metabolomic techniques to examine the beef cattle rumen metabolome in relation to host phenotypes, such as feed efficiency. This application of metabolomics has contributed to our understanding of the mechanisms

underlying divergences in phenotype of beef cattle, but much more information is needed to not only fully comprehend the contributing factors to differences in phenotype, but how those different factors can be manipulated in order to optimize production.

Summary and Future Directions

Many factors contribute to ruminant efficiency phenotypes, including the rumen microbiome, physical and genetic differences in the animal, host physiology, as well as external factors such as diet and management. The rumen microbiome is a key mediator of nutrient production in cattle, but much is still unknown about the ability to manipulate the microbiome, factors affecting its establishment, and the physiological effects on the host as a result of those manipulations. Host genetics appears to play a strong role in microbiome establishment, though this can be overcome by other factors such as diet or antibiotics, at least short-term. However, it is still unknown how all of these factors can be used and manipulated in conjunction to optimize beef cattle production, including improved feed efficiency, reduced methane emissions, and other efficiency metrics.

The ruminant metabolome has revealed associations between different metabolites and important production-related phenotypes in beef cattle, particularly metabolites associated with intermediary metabolism. However, few studies have attempted to relate the rumen fluid metabolome with the serum metabolome, in order to further define and understand the relationship between rumen dynamics and the animal's endogenous metabolism. Those studies that have been conducted have primarily been conducted in dairy cattle or other species, as is the case with host-microbiome interactions (Mao, Huo, and Zhu 2016; Morgavi et al. 2015; Zhao et al. 2014). To advance this field of research in cattle, these types of systems approaches must be undertaken. Further, these studies should also mirror that of other fields, in which multinational groups perform analyses to interrogate many aspects of the same experiment to add additional layers of robustness and analytical overlap once systemic methods of data analysis are attempted.

Beyond the relationship between the rumen and serum metabolome, no studies have performed metabolomic analyses of rumen and serum in conjunction with the beef cattle rumen microbiome, particularly following a rumen disturbance (such as RCE). In other environments and disciplines, such as environmental toxicology or cancer biology, “-omics” technologies have provided deeper information as to the effects microbes have on their environment because metabolomic techniques can analyze over 100 metabolites at once, compared to traditional assay techniques in which only one or few molecules can be measured at a time (Aardema and MacGregor 2003; Desai, Pathak, and Madamwar 2010; Davis and Hord 2005). In addition, many of the metabolites that have been found using untargeted metabolomics in animals of varying phenotypes, such as differences in RFI, have been those known to be involved in intermediary metabolism and may help explain differences observed in the physiology of the animal (Artegoitia et al. 2017). Applying multiple -omics techniques as well as across multiple physiological systems (e.g., blood, rumen fluid, urine) will provide more details regarding the critical relationships between the host and its microbiome. A more detailed and inclusive approach will provide more in-depth associations among the various factors contributing to divergences in feed efficiency, such as ssNMR, epigenetics, and other “-omics” techniques, in conjunction with microbiomics, and would yield a systemic data set amenable for concerted multi-omics approach. Such approaches will allow researchers to make

connections that have not been previously not been available to researchers. Utilizing the entirety of these tools in cattle to acquire a comprehensive picture of host-microbe interactions will ultimately lead to a better understanding of these complex relationships, enable to researchers to better elicit production outcomes through persistent microbiome manipulation, and provide better selection criteria to improve livestock systems.

CHAPTER II
RUMEN FLUID METABOLOMICS OF BEEF STEERS DIFFERING IN
FEED EFFICIENCY

A version of this chapter was published by Brooke A. Clemmons, Joshua B. Powers, Shawn R. Campagna, Taylor B. Seay, Mallory M. Embree, and Phillip R. Myer:

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Brooke A. Clemmons performed research, analyzed data, and wrote the paper.

Joshua B. Powers contributed analytical tools and analyzed data.

Shawn R. Campagna contributed analytical tools and analyzed data.

Taylor B. Seay analyzed data and wrote segments of the paper.

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Abstract

Introduction

Beef is the most consumed red meat in the United States, and the US is the largest producer and consumer of beef cattle globally. Feed is one of the largest input costs for the beef cattle industry, accounting for 40–60% of the total input costs. Identifying methods for improving feed efficiency in beef cattle herds could result in decreased cost to both producers and consumers, as well as increased animal protein available for global consumption.

Methods

In this study, rumen fluid was collected from low- ($n = 14$) and high-RFI ($n = 15$) steers. Rumen fluid was filtered through a $0.22\ \mu\text{M}$ syringe filter, extracted using 0.1% formic acid in acetonitrile:water:methanol (2:2:1) and injected into the Dionex UltiMate 3000 UHPLC system with an Exactive Plus Orbitrap MS. Peaks were identified using MAVEN and analyzed using MetaboAnalyst 4.0 and SAS. Significance was determined using an $\alpha \leq 0.05$.

Results

Eight metabolites were greater in low-RFI steers compared to high-RFI steers, including 3,4-dihydroxyphenylacetate, 4-pyridoxate, citraconate, hypoxanthine, succinate/methylmalonate, thymine, uracil, and xylose ($P \leq 0.05$). These metabolites were predominantly involved in amino acid and lipid metabolism.

Conclusions

Rumen fluid metabolomes differ in steers of varying feed efficiencies. These metabolites may be used as biomarkers of feed efficiency, and may provide insight as to factors contributing to differences in feed efficiency that may be exploited to improve feed efficiency in beef cattle herds.

Introduction

The United States is both the largest producer and consumer of beef cattle globally (ERS 2019). In the beef cattle industry, approximately 40–60% of the total input cost comes from feed

(Montaño-Bermudez, Nielsen, and Deutscher 1990). Identifying and incorporating methods for improving feed efficiency would not only potentially result in increased global protein availability, but would also result in decreased cost to producers and consumers. In order to select for more feed-efficient animals, these animals must first be identified, preferably through rapid, inexpensive methods. Untargeted metabolomics may provide a method for quickly and readily identifying more feed-efficient animals.

Untargeted metabolomics analyses aim to detect, quantify and subsequently identify as many metabolites within a given sample as possible (Fontanesi 2016). These metabolites can be both of endogenous and xenobiotic origin, with many of the xenobiotic metabolites derived from plants or microbiota (Fontanesi 2016). The metabolites measured are, by definition, intermediates or products of metabolism. Of particular interest with regards to feed efficiency are those metabolic processes involved in carbohydrate, fat, and protein metabolism (Nafikov and Beitz 2007). Alterations in intermediary metabolism partially contribute to divergences in feed efficiency (Ferrell and Jenkins 1984). Variations in those metabolites involved in intermediary metabolism could be suggestive of more or less efficient nutrient utilization, leading to more or less feed-efficient animals, respectively.

Untargeted metabolomics, using ultrahigh-pressure liquid chromatography (UHPLC-MS), has been applied to serum from beef cattle, and differences in metabolite abundances were observed in steers varying in feed efficiency, as measured by differences in residual feed intake (RFI) (Clemmons et al. 2017). Clemmons and others found that some of the metabolites that differed in steers divergent in feed efficiency were those vital to intermediary metabolism, such as pantothenate and carnitine (Clemmons et al. 2017). The observed differences suggested variances in host metabolism, as serum metabolites are predictive of feed efficiency phenotypes in cattle (Clemmons, Martino, Powers, et al. 2019). Further studies performing untargeted and targeted metabolomic techniques have also found differences in rumen fluid and blood metabolites in cattle differing in feed efficiency (Artegoitia et al. 2017; Novais et al. 2019). However, the relationship between blood and rumen fluid metabolomes is not well-defined, and the blood metabolome may not be reflective of rumen function. Differences in the rumen fluid metabolome that are not reflected in the blood may still contribute to variation in feed efficiency.

Although untargeted metabolomics can provide a wealth of information, few studies have applied these techniques to examine the relationship between feed efficiency in beef cattle and the rumen fluid metabolome. The objective of this study was to use untargeted metabolomic techniques to identify metabolites in rumen fluid of beef steers that are associated with variation in feed efficiency phenotypes in beef steers.

Methods

Animal handling and sample collection

This study was approved and carried out in accordance with the recommendations of the University of Tennessee, Knoxville Institutional Animal Care and Use Committee.

The study utilized fifty purebred Angus steers from the University of Tennessee Institute of Agriculture Plateau Research and Education Center in Crossville, TN. Steers were 7 months of age and weighed 264 ± 2.7 kg at the start of the trial (Clemmons et al. 2017). Two weeks post-weaning, steers were transitioned for 10 days to a corn silage-based growing ration (11.57% crude protein and 76.93% total digestible nutrients on a dry matter basis) with 28 mg monensin/kg using the GrowSafe System© (GrowSafe Systems Ltd. Airdrie, Canada), which continually monitors feed intake, before beginning a 70 days feed efficiency trial. The steers remained on the diet and in the GrowSafe System© for the duration of the trial. Weekly, body weights were measured using a chute scale following the adaptation period for the duration of the 70 days feed efficiency trial. Feed intake was continually monitored following the adaptation period for 70 days. Residual feed intake was calculated as the difference between actual and expected dry matter intake (Koch et al. 1963). Following the end of the trial, steers were ranked based on RFI, and high- ($n = 15$) and low- ($n = 14$) RFI was chosen as those with 0.5 standard deviations above and below the mean, respectively. Rumen fluid was collected on the final day of the ten-week study via esophageal tubing and placed on ice for approximately two hours prior to processing. The animal handling and sample collection are further described by Clemmons et al. (Clemmons et al. 2017).

For metabolomics analysis, a 2 mL aliquot of rumen fluid was transferred to sterile 2 mL microcentrifuge tubes and centrifuged for 15 min at $6000 \times g$ at 4°C . The supernatant was aspirated using a 3 mL syringe with a 25G needle and filtered through a $0.22\ \mu\text{m}$ pyrogen-free syringe filter (MidSci, St. Louis, MO, USA) into a 2 mL microvial. Filtered samples were stored at -80°C until further processing. The rumen fluid metabolome was extracted and analyzed similarly to Clemmons et. al. (Clemmons et al. 2017). Briefly, 50 μL of rumen fluid were extracted using 0.1% formic acid in acetonitrile:water:methanol (2:2:1) as previously described (Rabinowitz and Kimball 2007). A Synergy Hydro-RP column ($100 \times 2\ \text{mm}$, $2.5\ \mu\text{m}$ particle size) was used to separate metabolites, with 10 μL of sample injected into the Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific, Waltham, MA). An Exactive™ Plus Orbitrap MS (Thermo Fisher Scientific, Waltham, MA) was used to introduce samples via electrospray ionization, using an established method (Kamphorst et al. 2011; Lu et al. 2010).

Data analysis

The RAW files generated by Xcalibur MS software (Thermo Electron Corp. Waltham, MA) were converted to the.mzML format using the tool MS convert, a part of the suite of utilities in ProteoWizard software (Chambers et al. 2012). MAVEN (Metabolomic Analysis and Visualization Engine for LC–MS Data), a software package (Clasquin, Melamud, and Rabinowitz 2012), that automatically performs non-linear retention time correction and calculates peak areas across samples, was used to pick known peaks. In MAVEN, a preliminary mass error of ± 10 ppm and retention time window of 5 min were used to detect peak areas across samples. Metabolites were annotated using expanded methods by Rabinowitz and others (Lu et al. 2010) based on retention time-accurate m/z pairs taken from MS1 spectra. The annotation parameters have been verified previously with pure standards as part of establishing the method. For a metabolite to be annotated as a known compound, the eluted peak had to be found within 2 min of the expected retention time, and the metabolite mass had to be within ± 5 ppm of the expected value. Data values obtained from MAVEN were pre-processed and analyzed using Metaboanalyst 4.0 (Chong et al.

2018). Metabolites were median-centered, log-transformed, and pareto-scaled using Metaboanalyst 4.0. Metabolites differing significantly ($P < 0.05$) between low- and high-RFI steers were identified using student's *t*-test. Multiple testing was addressed by setting a false discovery rate of 5%, using the method of Benjamini and Hochberg (Benjamini and Hochberg 1995). Orthogonal partial least squares discriminant analysis (O-PLS-DA) was used to visualize separation between the two groups of steers based on serum metabolite profiles. The metrics R^2Y and Q^2 were used to evaluate the fit and prediction power, respectively, of the O-PLSDA model. Validity of model estimates was evaluated using permutation testing (Westerhuis et al. 2008). Sample labels were randomly assigned 1000 times, and new models were fitted and estimates of R^2Y and Q^2 were calculated for each random permutation. Values of R^2Y and Q^2 from the original model were compared to the distribution of values from the permutations to calculate empirical *p*-values for R^2Y and Q^2 (Online Resource 1). A principal component analysis was also used to visualize low- and high-RFI metabolomes and a variable importance in projection (VIP) plot with the top 25 variables. Pathway analyses to determine significant pathways impacted by RFI were analyzed in Metaboanalyst 4.0 using a global test with relative-betweenness centrality topology analysis with a reference library of *Escherichia coli* K-12 MG1655 (Kanehisa et al. 2013). Normality was assessed using the PROC UNIVARIATE procedure on raw, untransformed data in SAS 9.4 (SAS Institute, Cary, NC, USA). Normal distribution was considered based on a Shapiro–Wilk score of ≥ 0.85 and visual analysis of histogram and plots of residuals. Normally distributed metabolites were analyzed using one-way ANOVA in SAS with Tukey's HSD post-hoc test, whereas non-normally distributed variables were analyzed using Wilcoxon Rank Sum and Kruskal Wallis H test. Significance was set using $\alpha \leq 0.05$.

Results

A total of 88 known metabolites were identified. Eight metabolite abundances differed between low- and high-RFI steers based on either one-way ANOVA or Kruskal Wallis H test (Table 1). Orthogonal PLS-DA was used to visualize the discrimination between low- and high-RFI steers among rumen fluid samples (Figure 2). The O-PLS-DA illustrated moderate overlap or similarity among animals differing in feed efficiency; however, two distinct metabolomes were present based on RFI (Figure 2). A heatmap was also used for visualization of distribution of metabolites between high- and low-RFI steers (Figure 3). The heatmap displays differences in the abundance of the top 25 metabolites of individual steers, with clustering by RFI (Figure 3). Metabolite abundances by low- and high-RFI groups are presented in Online Resource 2. A PCA visualizing the low- and high-RFI rumen fluid metabolomes are also presented (Figure 4). The VIP scores are presented based on the top 25 variables of importance are presented in Figure 5. Several metabolic pathways were impacted as a result of RFI (Table 2).

Discussion

Feed efficiency is an important factor in the beef industry as consumers and producers realize the environmental and economic cost of producing animal proteins (Myer et al. 2019). The ruminal microbiota contribute significantly to feed efficiency and animal production by converting feed into energy and nutrients for the host animal, which are subsequently released in the rumen and available for absorption in both the rumen and lower gastrointestinal tract. The

Table 1. Significant metabolites differing between high- and low-RFI.

Metabolite	High-RFI^c	Low-RFI^c	P-value^a
3,4-dihydroxyphenylacetate (DOPAC)*	$1.96 \times 10^7 \pm 1.18 \times 10^6$	$3.85 \times 10^7 \pm 2.58 \times 10^6$	0.04
4-pyridoxate	$4.79 \times 10^8 \pm 5.93 \times 10^7$	$6.71 \times 10^8 \pm 4.62 \times 10^7$	0.02
Citraconate ^b	$1.53 \times 10^9 \pm 3.48 \times 10^8$	$8.42 \times 10^8 \pm 4.23 \times 10^8$	0.05
Hypoxanthine	$1.22 \times 10^9 \pm 1.21 \times 10^8$	$1.63 \times 10^9 \pm 1.09 \times 10^8$	0.02
Succinate/Methylmalonate	$3.44 \times 10^9 \pm 5.82 \times 10^8$	$6.35 \times 10^9 \pm 8.25 \times 10^8$	0.01
Thymine	$4.68 \times 10^8 \pm 6.15 \times 10^7$	$8.41 \times 10^8 \pm 1.39 \times 10^8$	0.02
Uracil	$1.04 \times 10^9 \pm 1.14 \times 10^8$	$1.51 \times 10^9 \pm 1.51 \times 10^8$	0.02
Xylose	$1.40 \times 10^8 \pm 1.42 \times 10^7$	$1.95 \times 10^8 \pm 1.67 \times 10^7$	0.02

^aSignificance determined at $P \leq 0.05$

^bAnalyzed using Kruskal Wallis H test

^cValues are measured as mean $\pm$ SEM of area under the peak

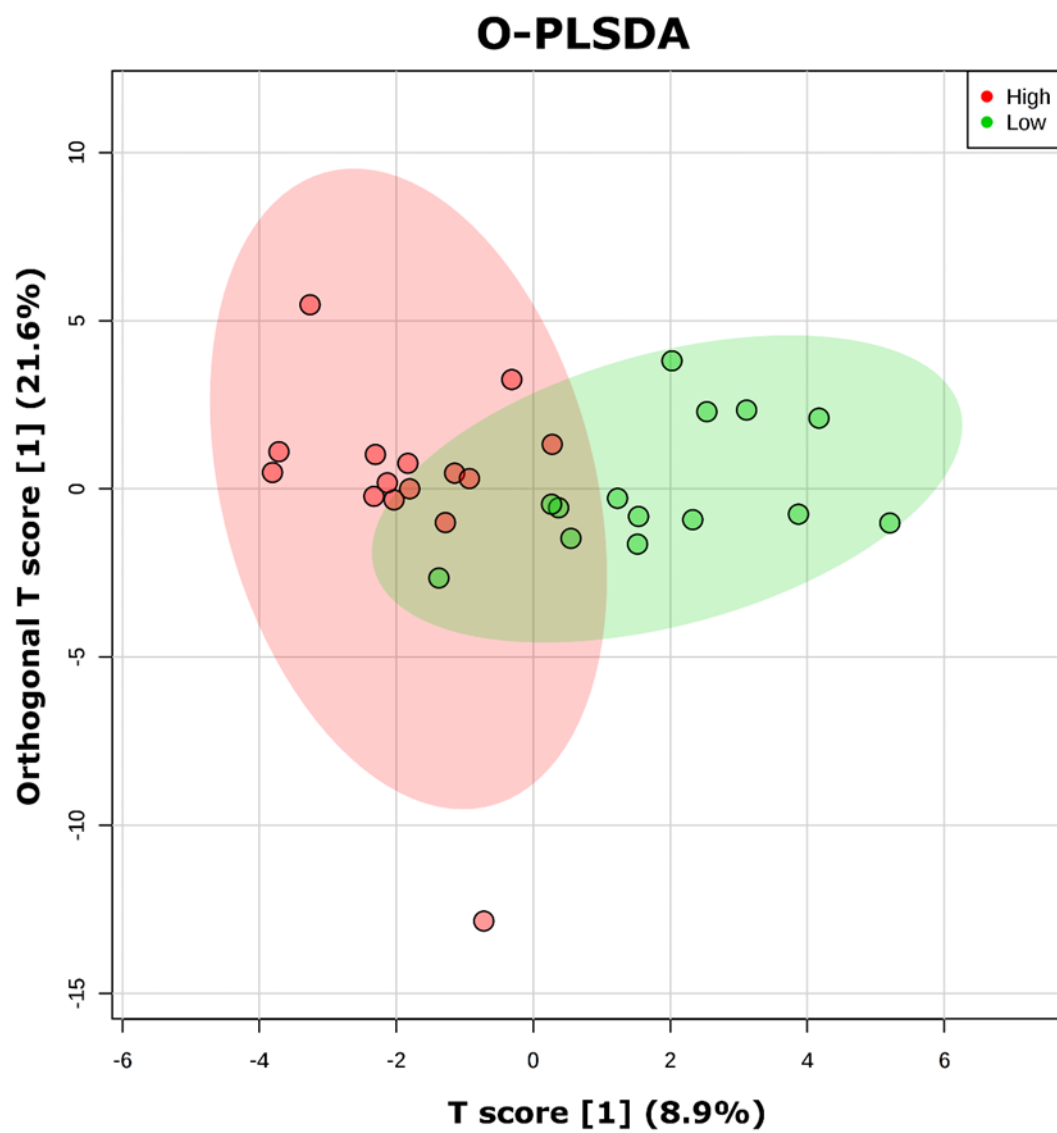


Figure 2. Orthogonal partial least squares discriminant analysis of rumen fluid metabolites differing between high- and low- RFI steers. Ellipse represents 95% confidence interval

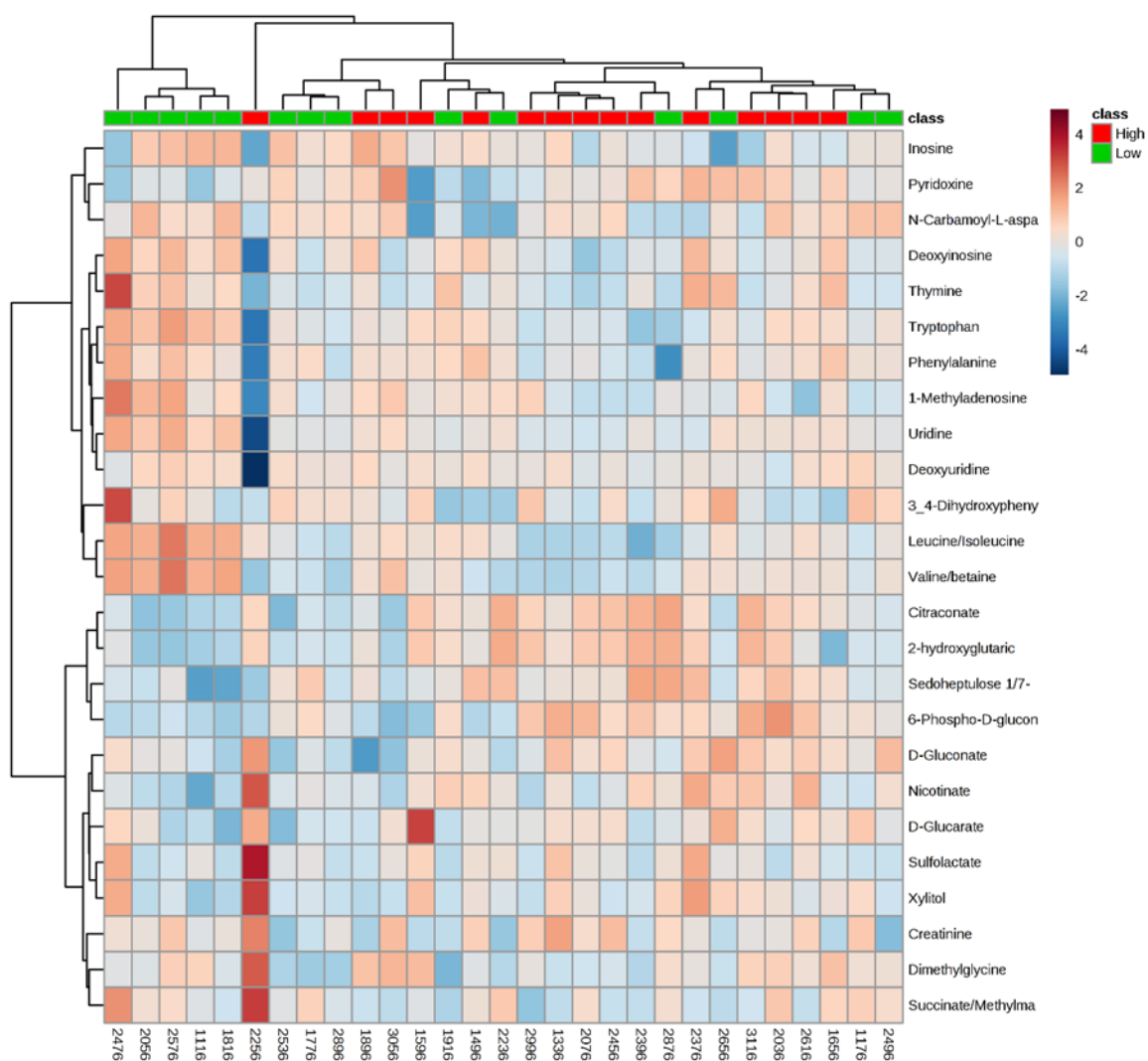


Figure 3. Heatmap of the top 25 metabolites differing between high- and low-RFI steers

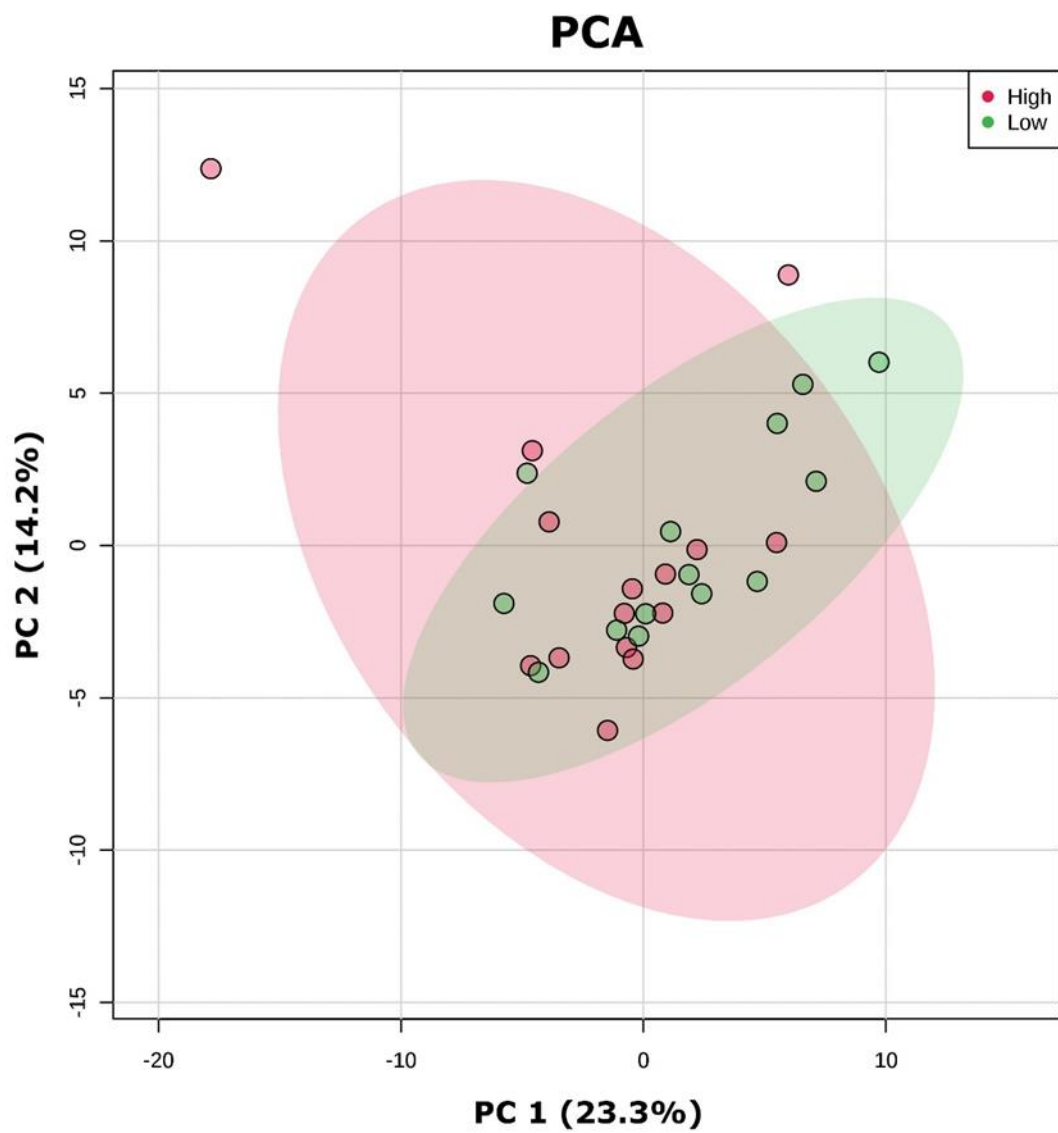


Figure 4. Principal component analysis of rumen metabolomes of high- and low-RFI steers. Ellipse represents 95% confidence interval

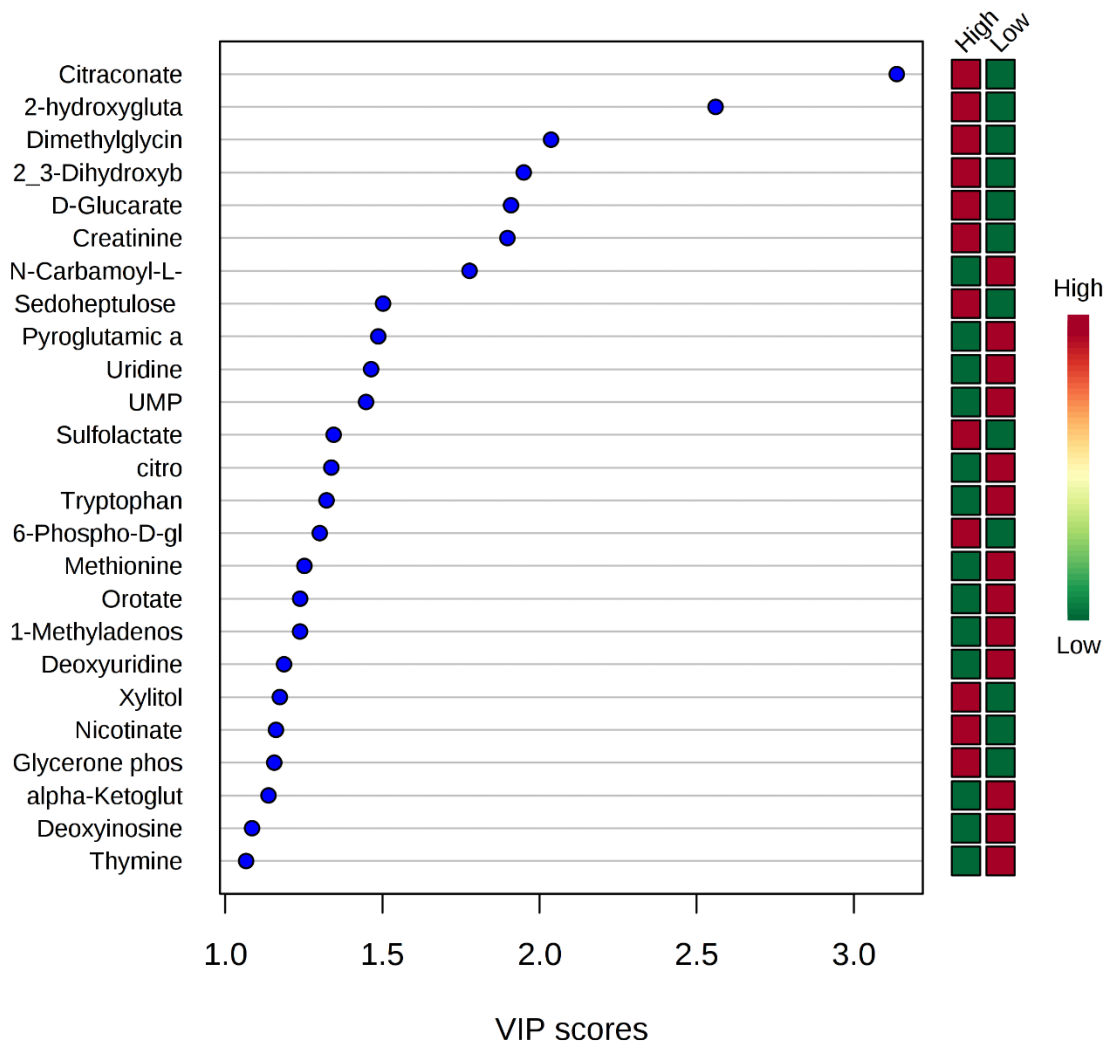


Figure 5. Variable importance analysis of top 25 metabolites between high- and low-RFI steers

Table 2. Metabolic pathway analysis of pathways that differed significant by low- and high-RFI steers

Metabolic Pathway	Impact	P-value^a
C ₅ -Branched dibasic acid metabolism	0.50	0.01
Valine, leucine and isoleucine biosynthesis	0.11	0.01
Ascorbate and aldarate metabolism	0.01	0.05

^aSignificance determined at $P \leq 0.05$

metabolites produced and released by the rumen microbiome have the potential to influence host phenotypes, and thus are of particular interest for understanding contributing factors to variation in feed efficiency phenotypes.

Succinate/methylmalonate was greater in the rumen fluid of low-RFI compared to high-RFI animals in this study. Succinate is a precursor of propionate, whose primary fate is towards gluconeogenesis in ruminants (Van Gylswyk 1995; Yost et al. 1977). In the rumen, bacteria such as *Fibrobacter succinogenes* produce succinate, which is then converted to propionate by bacteria such as *Selenomonas ruminantium* (Ushida, Miyazaki, and Kawashima 1985; Weimer 1998). Succinate is converted to propionate through a multi-enzymatic process with the assistance of coenzyme A (CoA), in which CoA becomes bound to succinate, isomerized to methylmalonyl-CoA, converted to propionyl-CoA, which eventually produces propionate (Wirth et al. 2018). Propionate can then be absorbed through the rumen epithelium and used in the liver for gluconeogenesis. The greater abundance of succinate in the low-RFI (more efficient) steers may indicate more glucogenic precursors are available to the host through a rumen microbiome that may produce these glucogenic precursors more efficiently or effectively. Indeed, in a study conducted by Myer and colleagues, more efficient steers had greater abundances of succinate- and propionate-producing bacteria in the rumen, including *Succiniclasticum* spp. (Myer et al. 2015b). Additionally, a study conducted by Clemmons et. al. found that steers with low-RFI had greater abundances of serum pantothenate, a precursor of CoA (Clemmons et al. 2017), which was found to be predictive of feed efficiency in steers (Clemmons, Martino, Powers, et al. 2019). This could potentially suggest that not only are the precursors available with the greater abundance of succinate/methylmalonate in this study, but more efficient steers could be utilizing these energy precursors more effectively since CoA is used in the conversion of succinate to propionate.

Succinate is also an intermediate of γ -aminobutyric acid (GABA) metabolism. Succinate is converted from succinate semialdehyde to succinate by succinate semialdehyde dehydrogenase (Blasi et al. 2002), and the succinate can then be converted back to GABA via the citric acid cycle as conversion to 2-oxoglutarate (Berg, Tymoczko, and Stryer 2002). The 2-oxoglutarate can then be converted back to GABA (Berg, Tymoczko, and Stryer 2002). The metabolite GABA is a potent neurotransmitter. In cattle, GABA is a significant mediator of feed intake and weight gain (Fan et al. 2015). Fan and colleagues (2015) performed a genome-wide association study (GWAS) across 807 Simmental cattle to determine single-nucleotide polymorphisms (SNP) associated with production-relevant traits related to feed intake and weight gain (Fan et al. 2015). The authors found that the GABAergic pathways were associated with both feed intake and weight gain according to nominal P-values (Fan et al. 2015). The authors further describe the effect of providing exogenous GABA in other species, including the observed feed intake and weight gain in pigs (Fan et al. 2007) and dairy cattle (Wang et al. 2013). The greater abundance of succinate in this study could be a possible indication of greater GABA metabolism in cattle from any succinate that may have escaped ruminal fermentation, leading to differences in weight gain. Although further analyses are warranted to confirm these speculations, succinate could be a promising biomarker of feed efficiency from the rumen of beef cattle.

Several nucleic acids or metabolites associated with nucleic acid metabolism differed between low- and high-RFI groups. Uracil, thymine, and hypoxanthine were all greater in low-RFI steers compared to high-RFI steers. Hypoxanthine is a purine derivative, while uracil and thymine are nucleic acid bases found in RNA and DNA, respectively. The greater abundance of these nucleic acids may be indicative of higher turnover of microbial or plant genetic materials. The amount of purine metabolism derivatives found in urine are positively correlated with the amount of microbial nucleic acids that enter the small intestine (McAllan et al. 1982; Thanh and Ørskov 2006), and greater amounts of nucleic acids in the rumen can be indicative of greater availability of microbial crude protein (MCP) (Leng and Nolan 1984). Studies conducted by McAllan and Smith analyzed degradation of DNA and RNA in rumen fluid in vitro (McAllan and Smith 1973). They found that, while many components of DNA and RNA are completely or mostly degraded, hypoxanthine, uracil, and thymine resisted degradation after four hours of incubation (McAllan and Smith 1973). Of these three nucleic acids or nucleic acid derivatives, uracil was most resistant to degradation, with approximately 60% of the original uracil still present after the four hour incubation, followed by hypoxanthine (45% remaining), and thymine (30% remaining) (McAllan and Smith 1973). Given that these nucleic acids or derivatives are most resistant to degradation by the rumen microbiota, they may be indicators of genetic material turnover in the rumen. Because these were also found in greater abundances in the rumen of more feed-efficient steers, this may be indicative of more rapid turnover or increased production of microbial crude protein, especially given that these animals were on the same diets.

Pyrodixate, or pyridoxic acid, was also greater in low-RFI animals than in high-RFI animals. Pyridoxic acid is a byproduct of vitamin B₆ catabolism, and provides an estimate of the vitamin B₆ oxidation that occurs in humans (Linkswiler and Reynolds 1950). Vitamin B₆ is required for many biological reactions, including those involved in amino acid, carbohydrate, and fatty acid metabolism (Leklem and Machlin 1991; McCormick 2006; Merrill and Burnham 1990). Except in production stages requiring extensive nutrient requirements, such as early lactation in dairy cattle, the rumen microbiota and diet provide sufficient B vitamins to meet the need of the animal (Bechdel et al. 1928). Although B vitamins, including vitamin B₆, have demonstrated vital and wide-ranging functions in metabolism, there is little understanding or information about the use of vitamin B₆ supplementation to improve cattle production (Weiss and Ferreira 2006). Given that a vitamin B₆ metabolism byproduct was greater in the rumen of more feed-efficient animals, it is possible that vitamin B₆ metabolism is greater in the rumen of cattle. This may permit greater protein and muscle accretion, leading to a more efficient animal. Additionally, 4-pyridoxiate can be converted downstream to succinate semialdehyde by members of Proteobacteria (Blasi et al. 2002), one of the most abundant phyla in beef cattle rumen. Succinate semialdehyde can be irreversibly converted to succinate (Chambliss and Gibson 1992; Ryzlak and Pietruszko 1988), which was found to also be greater in the rumen of low-RFI beef cattle in this study. This could provide further support of the impact of vitamin B₆ turnover. However, this is speculative, and more research would be needed to support this concept.

Although other metabolites differed between low- and high-RFI steers, including citraconate (Krishnamachar, Subramanian, and Rao 1964), DOPAC (Gnegy 2012), and xylose (Pearson and Baldwin 1981), their biological roles are not well-known or understood. Citraconate is an

intermediate of citric acid metabolism and xylose is a plant monosaccharide, which both may have contributed to carbohydrate metabolism, whereas DOPAC is a byproduct of dopamine metabolism (Gnegy 2012). Indeed, the C₅-branched dibasic acid metabolism was impacted as a result of high- and low-RFI, a pathway in which citraconate is involved (Kanehisa et al. 2013). Although the role of these three metabolites is not well-defined currently, they represent potential biomarkers of feed efficiency in beef cattle, or may represent future metabolites of interest to study in relation to feed efficiency in cattle.

The steers in this study were fed monensin, an ionophore that alters the rumen microbiome and is routinely used in the United States to improve feed efficiency and reduce bloat (Goodrich et al. 1984). Monensin has a demonstrated effect on the rumen microbial populations, microbial function, and the rumen fluid metabolome (Ogunade et al. 2018; Ogunade and Schweickart 2018). Although monensin alters both feed efficiency and the microbial populations and metabolism, all steers in this study received monensin and RFI separation was still observed. The rumen fluid metabolomes also differed as a result of RFI differences. The differences in rumen fluid metabolomes could be the result of several different factors, such as possible variation in the rumen microbiome and microbial metabolism as well as differences in host metabolism. Steers that differ in RFI and other measurements of feed efficiency have a demonstrated variation in the rumen microbiome (Myer et al. 2015b). Additionally, microbial differences by steers differing in feed efficiency were observed in animals also consuming monensin (Myer et al. 2015b). Thus, potential differences in the rumen microbiome as a result of feed efficiency could alter the rumen metabolome. However, further studies analyzing the rumen metabolome in cattle differing in RFI not consuming monensin should be conducted in order to determine biomarkers of feed efficiency when cattle do not have access to the ionophore.

The differences in the rumen fluid metabolites as a result of feed efficiency could also be due to variation in host metabolism. Feed efficiency is a moderately heritable trait, suggesting that divergences in feed efficiency in beef cattle are the result of some host physiology (Arthur, Renand, and Krauss 2001). Indeed, Kong et. al. analyzed the transcriptome of the rumen epithelium in high- and low-RFI steers using RNA-Seq (Kong et al. 2016). Kong and colleagues found that 122 genes were differentially expressed between high- and low-RFI steers, with increased expression of genes related to energy utilization via glycolysis and the tricarboxylic acid cycle, as well as an increased number of mitochondrial gene copy number (Kong et al. 2016). This potentially relates to some of the metabolites observed in this study that are intermediates of these metabolic processes. Citraconate and succinate are both intermediates of the tricarboxylic acid cycle, and were in greater abundance in the rumen of low-RFI cattle. Additionally, Kong and colleagues demonstrated that there was enrichment of genes regulating protein turnover (Kong et al. 2016). The greater abundance of nucleic acids observed in the rumen fluid low-RFI steers in the present study may be related to this, as greater protein and/or genetic material turnover could result in greater abundances of nucleic acids being released into the rumen. Further studies utilizing both transcriptomics and metabolomics or other pathway analyses could corroborate this hypothesis.

Although several metabolites differed in steers divergent in feed efficiency, the heatmap illustrated some overlap of steers from both phenotypes. There may be several possible reasons for this. First, all steers were on the same diet and maintained under the same conditions. Given this, it stands to reason that the byproducts of catabolism of the feedstuffs would be similar across all animals, regardless of phenotype. Eight of the 88 known metabolites observed in this study differed between low- and high-RFI steers, thus the remaining 80 metabolites could have contributed to the overlap of feed efficiency phenotypes observed in the heatmap. Additionally, one of the greatest sources of variation in ruminant microbiomes or rumen environment besides diet is individual animal variation (Henderson et al. 2015). The rumen metabolome as a whole may not be highly correlated or indicative of feed efficiency given that the host and its diet contribute to the rumen fluid metabolome. Although the study was conducted for a duration long enough to observe stabilization of the rumen microbiome (Clemmons, Martino, Schneider, et al. 2019), as well as enough experimental units to observe differences between low- and high-RFI, having additional animals or conducting the study for a longer length of time may have allowed us to discern greater separation in the rumen metabolomes of cattle differing in feed efficiency.

In this study, several metabolites differed in the rumen fluid of steers differing in feed efficiency. Most of these metabolites are involved in genetic material recycling and protein metabolism, in addition to others that are involved in carbohydrate and lipid metabolism. Not only could the identified metabolites be potential biomarkers of feed efficiency, but they could also provide insight into the metabolic, microbial, and physical differences that result in variation of feed efficiency in cattle. In the future, additional studies should be conducted to expand upon these results, such as analyzing the rumen fluid metabolome in female cattle at different production stages. Additionally, the roles of the individual metabolites identified in this study could be examined in the greater context of feed efficiency in cattle, as well as investigating the relationship among beef cattle feed efficiency, the rumen microbiome, and the rumen fluid and serum metabolomes to gain a comprehensive insight into the impacts of microbial rumen function on host metabolism and feed efficiency.

CHAPTER III
RUMINAL PROTOZOAL POPULATIONS OF ANGUS STEERS
DIFFERING IN FEED EFFICIENCY

Abstract

In the beef cattle industry, feeding those animals can account for as much as 60% of the total cost of production; thus, determining methods for improving feed efficiency could result in millions of dollars returned to the United States beef industry. The rumen microbiome contributes significantly to the feed utilization by the host animal, however most studies focus on the bacterial and archaeal communities because of the genetic and functional diversity those communities. This study focused on the protozoal communities because they account for approximately 50% of the biomass and participate in regulation of bacterial populations and nitrogen cycling via engulfment of bacteria. In this study, 50 weaned steers of seven months of age weighing approximately 260 kg were chosen for the feed efficiency study. The steers were adapted to a growing ration and the GrowSafe for two weeks prior to a 70-day feed efficiency trial. Body weights were collected via chute scale on the first day of the feed efficiency trial, every seven days throughout the trial, and the final day of the trial. On day 70 of the trial, approximately 50 mL of rumen content was collected via oro-gastric tubing and frozen at -80°C. Body weight and feed intake was used to calculate residual feed intake (RFI), and steers were categorized as high- (n=14) or low- (n=10) RFI based on ± 0.5 standard deviations about the mean RFI. Microbial DNA was extracted using a modified repeated bead beating plus column method, the 18S gene was amplified to target eukaryotic populations, and sequencing using the Illumina MiSeq. Protozoal sequences were cleaned and taxonomy assigned using QIIME 1.9 and analyzed using QIIME and SAS 9.4 with significance determined at $\alpha \leq 0.05$. The only taxa that differed after false discovery rate correction were unassigned taxa ($P=0.03$), with greater abundance of unassigned taxa in high-RFI steers. Differences were observed between low- and high-RFI steers in protozoal community phylogenetic diversity, including weighted PERMANOVA ($P=0.04$), Faith's Phylogenetic Diversity ($P=0.03$), and observed OTU ($P=0.03$). The unassigned taxa and differences in phylogenetic diversity of protozoal communities may contribute to divergences observed in feed efficiency phenotypes in beef steers.

Introduction

The rumen microbiome is key to effective feed digestion and utilization by the host animal, ultimately affecting its nutritional status. In cattle, the rumen microbiota are estimated to produce approximately 70% of the total energy substrates absorbed and used by the animal. Much of the research conducted to understand the relationship between the ruminant host and the rumen microbiome has focused on bacterial communities. The bacteria are arguably the most diverse microbial kingdom in the rumen and are the most abundant microbes. Coupled with many critical functions in support of ruminant nutrition and health, such as proteolytic and fibrolytic activities and vitamin production, is it apparent as to the intensive bacterial research in the cattle rumen conducted to date (Hernandez-Sanabria et al. 2012; McCann et al. 2014; Myer et al. 2015b). Nevertheless, it cannot be overlooked that rumen protozoa represent approximately equal volume of microbial bio-mass (Williams and Coleman 1992). Researchers using photomicrograph and electron microscopic techniques have demonstrated that protozoa actively participate in the rumen ecosystem and exhibit behaviors different than that of bacteria, such as starch hoarding (Williams and Coleman 1992). Protozoa participate in multiple forms of relationships with other microbes, including commensal and mutualistic relationships, and can also act as predatory species

(Belanche et al. 2012). However, much is still to be elucidated as to their role in ruminal fermentation, and host metabolism and nutrition.

Next-generation sequencing (NGS) technology is currently and abundantly used to study rumen microbial community composition in cattle (Karnati et al. 2003; Kittelmann and Janssen 2011; Myer et al. 2015a). Different hypervariable regions of the 18S rRNA gene can be used to delineate protozoal populations more effectively than methods relying on microscopy, which can be difficult and time consuming (Dehority 1993, 2003). Although individual rumen protozoal species have been studied via microscopy and through target sequencing of hypervariable regions of the eukaryotic 18S rRNA gene, little is currently understood about the roles of protozoa within the context of their ecological niche or their functional role in the context of host physiology (Williams and Coleman 1992). All the more, few have provided insights as to the diversity of ciliate protozoa in ruminant species and their function in weight gain, intake, and feed efficiency.

Given the functional importance of ciliate protozoa in the rumen environment, as some have cellulolytic and amylolytic capabilities (Gijzen et al. 1988; Béra-Maillet et al. 2005), and intensively interact with the entirety of the microbiome (Newbold et al. 2015), it is critical to not only categorize the diversity and phylogeny of rumen ciliate protozoa, but also determine their association with nutrition and feed efficiency in beef cattle. To address such deficiencies, we investigated the ciliate protozoal community in the rumen from steers differing in feed efficiency, using deep 18S ribosomal RNA gene (18S rDNA)-based community profiling. We hypothesize that variation in the protozoal populations within the rumen could contribute to variation in feed efficiency.

Materials and Methods

This study was approved and carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee at the University of Tennessee, Knoxville.

Animals, Experimental Design, and Rumen Content Sampling

Fifty purebred Black Angus steers from the University of Tennessee Institute of Agriculture Plateau Research and Education Center (Lamprecht and Williams) in Crossville, TN were utilized in the study, and were 7 months of age and weighed 264 ± 2.7 kg at the start of the trial. Two weeks after weaning, steers were acclimated in the GrowSafe feeding system (GrowSafe Systems Ltd., Airdrie, Canada) for 10 d prior to measuring feed intake. Steers were fed a step-up receiving diet for approximately 14 d before receiving a growing ration (11.57% crude protein and 76.93% total digestible nutrients on a dry matter basis) with 28 mg monensin/kg DM. After the acclimation period, body weight (Krysl and Hess) was measured at 7 d intervals and feed intake was continuously measured using the GrowSafe© system for a 60 d feed efficiency trial. As a measure of feed efficiency, at the end of the feeding period, steers were ranked by residual feed intake (RFI) based on performance and feed intake measured from d 0 to d 60 (Koch et al.). The average value of RFI (actual dry matter intake (DMI) vs. expected DMI) and standard deviation (SD) were calculated for individual animals and were divided into two groups of low ($n = 10$), and high ($n = 14$) RFI. High RFI was defined as $\text{RFI} \geq 0.5 \text{ SD}$ above the mean; and Low RFI was $\text{RFI} \leq -0.5 \text{ SD}$ below the mean. At the end of the trial, approximately 50mL of rumen content were sampled

via esophageal tubing, which has been shown to be an effective sampling method for rumen bacterial community studies, especially on higher grain diets (Manzano, Kononoff, and Fernando 2016).

DNA Extraction, Amplification and Sequencing

Microbial DNA in the rumen content was extracted using the repeated bead beating plus column method (Yu and Morrison 2004) and modified similar to that of Myer and colleagues (Myer et al. 2016). Metagenomic DNA concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

Amplicon library preparation was performed by PCR amplification encompassing variable regions V3 and V4 and rumen ciliate signature regions 1 and 2 of the 18S rRNA gene, using the modified primer set P-SSU-316F (5'-adaptor/index/GCTTTCGWTGGTAGTGTATT) and GIC758R (5' adaptor/index/ CAACTGTCTCTATKAAYCG)(Ishaq and Wright 2014), including adapters sequences and custom indices, as well as AccuPrime Taq high fidelity DNA Polymerase (Life Technologies, Carlsbad, CA). The following conditions were used for PCR amplification: initial denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 2 min, and a final extension step at 72°C for 7 min. Amplicons were purified using AmPure XP bead purification (Agencourt AMPure, Beckman Coulter, Danvers, MA) and all libraries were quantified by the PicoGreen ds- DNA quantitation kit (Invitrogen Corp., Carlsbad, CA) and by real-time PCR on the LightCycler 480 system (Roche Diagnostics, Mannheim, Germany). The amplicon libraries were sequenced using the v3 2×300 kit and the Illumina MiSeq sequencing platform (Illumina, Inc., San Diego, CA).

Sequence Analysis

Protozoal 18S amplicon sequence reads were processed using the Quantitative Insights Into Microbial Ecology (QIIME) bioinformatics pipeline, version 1.9.1 (Caporaso et al. 2010). Adapters/index sequences were trimmed, and sequences were removed if they resulted in an average quality score < Q30. Chimeric sequences were identified and filtered using usearch61 (Edgar et al. 2011). Operational taxonomic unit (OTU) picking was completed utilizing the cleaned sequences and were clustered with a pairwise identity threshold of 97%, and further assigned to taxonomy using the Silva reference database, 128 release (Quast et al. 2012).

Statistical Analysis

Protozoal OTU were analyzed in SAS 9.4 (SAS Institute, Cary, NC) and QIIME. Alpha diversity metrics, including Good's coverage, chao1, Faith's Phylogenetic Diversity, Observed OTU, Shannon's Diversity Index, and Simpson's Evenness E, as well as genus-level taxa were evaluated for normal distribution in SAS 9.4 using the PROC UNIVARIATE procedure. Normality was determined based on a Shapiro-Wilk statistic ≥ 0.85 and visualization of histograms. Variables that followed a normal distribution were analyzed using a one-way analysis of variance (ANOVA). Those variables not following a normal distribution were first transformed by either log or ranked to achieve normality, and then analyzed using a one-way ANOVA. Multiple test correction was applied using false discovery rate (FDR) corrected P-values. Beta diversity was assessed using analysis of similarity (ANOSIM) and permutational multivariate analysis of variance

(PERMANOVA). Relationships between protozoal taxa and VFA were analyzed using Spearman correlation with the PROC CORR procedure in SAS 9.4. Statistical significance was determined using $\alpha \leq 0.05$.

Results

A total of 13,793,949 sequences remained for subsequent analyses after quality and chimeric sequence filtering. After clustering at 97%, a total of 7,237 OTU were identified across all samples. Two alpha diversity metrics, observed OTU ($P = 0.03$) and Faith's Phylogenetic diversity ($P = 0.03$), differed by RFI (Table 3). Beta diversity was visualized using principal coordinates analyses (PCoA) based on unweighted (Figure 6A) and weighted (Figure 6B) UniFrac distances. Beta diversity was further analyzed using ANOSIM and PERMANOVA, and divergence was observed by RFI in weighted analyses (Table 4). The sole taxon that differed by RFI following FDR correction was unassigned, though there were large numerical differences in other genera by RFI (Table 5). No VFA differed by RFI (Table 6), although several significant correlations were observed between protozoal taxa and VFA (Table 7).

Discussion

Globally, beef consumption is rising, as beef provides an excellent source of animal protein (Smith, Gotoh, and Greenwood 2018). The United States is one of the largest exporters of beef, exporting more than one million tons of beef annually (ERS 2019; Smith, Gotoh, and Greenwood 2018). In the United States beef industry, feed costs account for upwards of 70% of the total input costs of production (ERS 2019, 2015; Montaña-Bermudez, Nielsen, and Deutscher 1990); thus, identifying methods for improving feed efficiency would decrease production costs as well as allow for greater amounts of animal protein available to for global consumption. The rumen microbiome appears to contribute to variation in feed efficiency phenotypes; however, typically, studies focus on the bacteria and archaea because they are most functionally and genetically diverse, or contribute to methane production, respectively. Much less is understood about the relationship between the rumen protozoa and feed efficiency phenotypes in cattle even though they account for upwards of 50% of the biomass of the rumen (Coleman and Sandford 1979); therefore, this study examined the relationship between the rumen protozoal communities and feed efficiency in beef cattle.

Interestingly, measures of both alpha and beta diversity differed by RFI in this study. The metrics that differed were those related to phylogenetic diversity, including Faith's Phylogenetic Diversity, number of observed OTU, and weighted PERMANOVA, with greater phylogenetic diversity observed in low-RFI steers. These results are antithetical to those regularly observed in bacterial communities, in which bacterial diversity does not typically differ by RFI (Myer et al. 2015b; McCann et al. 2014). However, greater ecosystem diversity on a macro scale tends to result in greater adaptability and resilience of an ecosystem (Elmqvist et al. 2003); in particular, functional diversity leads to a more productive ecosystem (Díaz and Cabido 2001). In the rumen, greater phylogenetic diversity of protozoa is reflective of greater functional diversity given the variation in substrate utilization and metabolism (Newbold et al. 2015; Jouany 1996). Increased protozoal diversity may increase substrate availability to other microbiota as well as the host, and may reduce any negative effects of nitrogen turnover by protozoal communities (Newbold et al. 2015).

Table 3. Alpha-diversity metrics between high- and low-RFI steers.

Metabolite	High-RFI^a	Low-RFI^a	P-value^b	FDR^{b,c}
Good's Coverage	0.99 (0.00)	0.99 (0.00)	0.03	0.07
Observed OTU	297.93 (1.79)	306.60 (2.71)	0.01	0.03
Faith's Phylogenetic Diversity	142.11 (0.44)	144.42 (0.74)	<0.001	0.03
chao1	310.11 (2.64)	315.01 (1.08)	0.15	0.22
Shannon's Diversity Index	1.86 (0.12)	1.77 (0.21)	0.69	0.69
Simpson's Evenness E	0.0074 (0.00)	0.0079 (0.00)	0.67	0.69

^aMean (SEM)

^bSignificance determined at $P \leq 0.05$

^cFalse discovery rate corrected P value

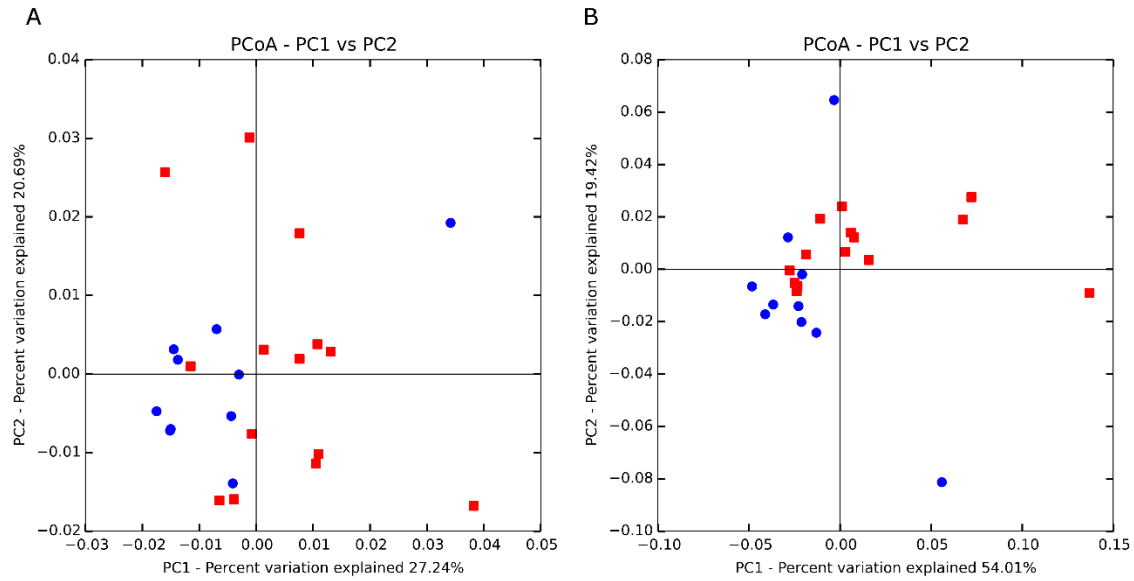


Figure 6. Principal coordinates analysis based on unweighted (A) and weighted (B) UniFrac distances of different levels of residual feed intake based on 9999 permutations. Low-RFI = blue circle, High-RFI = red square

Table 4. Beta-diversity metrics between low- and high-RFI steers

Metric¹	Test Statistic	P-value
PERMANOVA ² – weighted	2.76 ⁴	0.04
PERMANOVA ² – unweighted	0.80 ⁴	0.58
ANOSIM ³ – weighted	0.11 ⁵	0.07
ANOSIM ³ – unweighted	-0.06 ⁵	0.85

¹Metrics based on weighted or unweighted UniFrac distances with 9999 permutations

²Permutational multivariate analysis of variance

³Analysis of similarity

⁴Test statistic is pseudo-F

⁵Test statistic is R

Table 5. Genus-level taxon differences between high- and low-RFI steers.

Metabolite	High-RFI^a	Low-RFI^a	P-value^b	FDR^{b,c}
<i>Diplodinium</i>	0.12 (0.06)	0.04 (0.03)	0.05 ^d	0.15 ^d
<i>Entodinium</i>	0.67 (0.09)	0.81 (0.07)	0.13 ^d	0.19 ^d
<i>Isotricha</i>	0.06 (0.06)	0.05 (0.05)	0.28 ^d	0.33 ^d
<i>Ophryoscolex</i>	0.08 (0.06)	0.02 (0.02)	0.13 ^d	0.19 ^d
<i>Trichostomatia</i>	0.02 (0.01)	0.05 (0.03)	0.74 ^e	0.74 ^e
Unassigned	0.05 (0.00)	0.03 (0.00)	<0.001 ^d	0.03 ^d

^aMean (SEM)

^bSignificance determined at $P \leq 0.05$

^cFalse discovery rate corrected P value

^dBased on ranked data

^eBased on log-transformed data

Table 6. Volatile fatty acids by high- and low-RFI steers

VFA^a	High-RFI^b	Low-RFI^b	P-value^c
Total ^d	26.35 (2.39)	31.49 (2.38)	0.15
Acetate	63.88 (1.37)	63.09 (1.33)	0.69
Propionate	8.62 (0.89)	7.47 (0.98)	0.39
Isobutyrate	0.37 (0.04)	0.42 (0.04)	0.33
Butyrate	25.43 (1.22)	26.01 (1.69)	0.78
Valerate	1.69 (0.42)	3.00 (0.88)	0.16

^aVolatile fatty acids (VFA) expressed as mM × 100mM¹, except where otherwise noted

^bMean (SEM)

^cSignificance determined at $P \leq 0.05$

^dConcentration in mM

Table 7. Correlations between rumen protozoal genera and volatile fatty acids

Taxon	Metabolite	ρ^2	P-value^{a,b}
<i>Trichostomatia</i>	Propionate	-0.41	0.05
<i>Ophryoscolex</i>	Isobutyrate	-0.44	0.03
<i>Ophryoscolex</i>	Butyrate	0.43	0.03
<i>Diplodinium</i>	Butyrate	0.55	<0.01
<i>Entodinium</i>	Butyrate	-0.55	<0.01

^aSignificance determined at $P \leq 0.05$

^bBased on Spearman correlation

The only taxa that differed by RFI in this study were the unassigned taxa, a trend also observed in bacterial communities (McCann et al. 2014). There were more unassigned taxa in high-RFI steers compared to low-RFI steers. These unassigned taxa may provide great insight as to divergences in feed efficiency phenotypes in ruminants, particularly given that there is increasing evidence that rare or low abundance taxa may drive important phenomena in other ecosystems. For instance, Aanderud and others examined rare taxa in various ecosystems before and after a soil re-wetting event (Aanderud et al. 2015). The authors found that, following the re-wetting events, formerly rare taxa often became dominant, sometimes accounting for up to 60% of relative abundance of bacteria (Aanderud et al. 2015). Additionally, those bacterial community changes were associated with drastic alterations in gas production, with up to 20-fold increases in CO₂ production and a 150% reduction in methane production (Aanderud et al. 2015). The same impacts of rare microbial taxa have been observed in other organisms and ecosystems (Shade et al. 2014; Gobet et al. 2012; Jousset et al. 2017). These rare taxa may significantly alter host phenotypes, and further research could divulge deeper understanding of mechanisms driving divergences in feed efficiency phenotypes in cattle.

Although not statistically significant after false discovery rate correction, there were three times as many *Diplodinium* in the high-RFI steers compared to low-RFI steers. *Diplodinium* possess cellulolytic capabilities that are not portrayed by all ruminal ciliates and appear to perform better in the rumen of animals consuming greater proportions of forage in the diet. Eun examined the effect of increasing concentrate in the diet on protozoal populations in sheep, with concentrate ranging from 10-70% of the diet. Eun observed that *Diplodinium* increased in sheep fed diets consisting of up to 40% concentrate; however, at concentration inclusion rates of 50% or greater, abundances of *Diplodinium* decreased (Eun 1990). The steers in the present study were fed a primarily corn-silage based growing ration, and the greater proportion of readily-digestible feedstuffs may not have been an optimal diet for the *Diplodinium*. This may lead to a less efficient ruminal microbiome, resulting in overall decreased feed efficiency in the ruminant host.

Furthermore, protozoa are responsible for supplying amino acids to the host ruminant. The protozoal fractions have been known to supply greater amounts of isoleucine, leucine, phenylalanine, and lysine (Weller 1957). This is particularly interesting given that a previous study found that valine, leucine, and isoleucine biosynthesis pathways in the rumen differed by steers divergent in feed efficiency (Clemmons et al. 2020). Members of both *Diplodinium* and *Entodinium* are known to feed on bacteria in the rumen; however, they appear to have different preferences in bacterial species as a food source (Gutierrez and Davis 1959). The difference in preference for various bacteria may contribute to differences observed in bacterial taxa, as well as amino acids available to the host. The differences in nutrient availability may contribute to divergences in feed efficiency phenotypes in host ruminants.

In this study, there were no differences observed in abundance of *Entodinium*, though there were numerically greater abundances of *Entodinium* in low-RFI steers compared to high-RFI steers. Members of *Entodinium* have previously been thought to contribute to system inefficiency because of their role in nitrogen cycling. Belanche and others examined the biological activity of several protozoal communities from cattle *in vitro* (Belanche et al. 2012). The authors found that

Entodinium spp. were primarily responsible for CP breakdown, as well as approximately 70% of bacterial breakdown (Belanche et al. 2012). The *Entodinium* preferentially engulf and use bacteria as their primary source of protein and, in turn, release free amino acids and ammonia into their environment (Belanche et al. 2012; Wallace and McPherson 1987; Newbold et al. 1997). Thus, *Entodinium* may contribute to regulation of bacterial populations as well as availability of free amino acids for the host. This could prove an interesting area of future research.

However, protozoa do have a demonstrated negative impact on amino acid and protein availability to the host, primarily due to bacterial engulfment. When the protozoa engulf the bacteria, they typically degrade the microbial crude protein. The free amino acids are may then be rapidly degraded by aminogenic bacteria to ammonia (Williams and Coleman 1992). This is evident by the substantially lower amount of ammonia present in defaunated ruminants, with a greater than 25% reduction in ammonia production in protozoa-absent rumen environments (Newbold et al. 2015; Belanche, de la Fuente, and Newbold 2015). Ammonia production is also affected by protozoal populations, with holotrichs consuming fewer bacteria and different populations of bacteria than entodiniomorphs (Belanche et al. 2012).

Protozoa fulfill a distinctive niche in the rumen of most domesticated ruminants. Ruminal ciliate protozoa possess two unique abilities: the first is the ability to hoard fats and starches, and the second is the ability to engulf bacteria. When protozoa engulf and retain starches and fats, they are able to slowly digest and release catabolites of that process, including VFAs (Karnati et al. 2009). A meta-analysis conducted by Newbold and others examined the effects of protozoal communities on the rumen environment and production. The authors found that VFA production increased in faunated compared to defaunated ruminants, potentially as the result of the exogenous fat degradation (Karnati et al. 2009; Newbold et al. 2015). Protozoal defaunation also resulted in a greater than 20% average reduction in butyrate production, as well as a 3% increase in acetate, with no changes in propionate production (Newbold et al. 2015). Furthermore, the delayed release of VFA from the storage of starches and fats provides a steadier supply of gluconeogenic precursors which are, in turn, available to both the host and other rumen microbiota.

This study was one of few studies to examine the relationship between feed efficiency and ruminal protozoal communities. Ruminal protozoal communities are interesting and vital members of the rumen microbiome, accounting for approximately 50% of the microbial biomass and contributing to feed efficiency in several ways, including regulating bacterial populations through engulfment as well as release of several vital metabolites. The present research provides preliminary insight as to the relationship between the ruminal protozoa and feed efficiency in beef cattle; additional research is needed to further understand the relationship between the protozoal communities and feed efficiency in ruminants.

CHAPTER IV
MICROBIAL AND METABOLOMIC BIOMARKERS OF FEED
EFFICIENCY IN ANGUS HEIFERS

Abstract

By the year 2050, the agricultural sector is charged with producing upwards of 50% more food to feed an expected global population of over ten billion people. In the beef cattle industry, this means 30 cow-calf cycles to improve feed efficiency. Given that feed efficiency is a moderately heritable trait, developing methods to identify more or less feed efficient animals could lead to improved breeding management decisions. Therefore, this study determined potential microbial and metabolic biomarkers of feed efficiency in Angus heifers. Seventeen two-year-old, previously cannulated Angus heifers weighing approximately 560 kg were chosen for the trial. The heifers were provided a 14-day adaptation period to the GrowSafe system and the diet, followed by a 70-day feed efficiency trial. Residual feed intake was calculated based on ± 0.25 standard deviations about the mean RFI, as well as analyzed via regression based on RFI as a continuous variable. On day 70 of the trial, approximately 50mL of rumen content and 10mL of blood collected and used for microbial and metabolomic analyses. The bacterial and archaeal populations were examined by targeting the V4 region of the 16S gene and sequenced using the Illumina platform. Microbial data was quality filtered and OTU picked using QIIME 1.9 and analyzed using a one-way ANOVA in SAS 9.4. Metabolites were extracted and determined using a Dionex UltiMate 3000 UPLC system. Metabolite peaks were identified in MAVEN and analyzed using MetaboAnalyst 4.0. No microbial taxa differed after false discovery rate correction, but seven did differ ($P \leq 0.05$) prior to correction. No differences were observed in alpha diversity metrics, although unweighted PERMANOVA significant. When examining variation by biofluid, 143 metabolites differed after false discovery rate correction on t -test ($P \leq 0.5$). The metabolites that differed were those primarily involved in nutrient signaling and microbial crude protein availability and utilization in both the rumen fluid and serum. These data suggest variation in availability of nutrients, primarily amino acids, as well as a relationship among microbiota, metabolome, and host feed efficiency phenotypes in heifers that serve as future biomarkers of feed efficiency.

Introduction

Beef cattle are the primary agricultural commodity in Tennessee, and account for a multi-billion dollar industry worldwide (ERS 2019). The greatest input costs for the beef industry are feed costs, accounting for 40-70% of total cost of production (Herd, Archer, and Arthur 2003; Montañó-Bermudez, Nielsen, and Deutscher 1990). Given this, finding methods to reduce input costs through improved feed efficiencies stands to provide millions of dollars in savings to industry stakeholders. This is particularly important in heifers and cows, given that they are the members primarily responsible for propagation of genotypes and phenotypes, along with bulls. In order to select for more efficient animals, methods must be identified in order to detect these animals. Several methods may be used in order to do this, including microbiomics and metabolomics.

One of the primary contributors to variation in feed efficiency is the rumen microbiome. The rumen microbiota provide the majority of energy precursors required by the host animal as well as many of the required nutrients (Young 1977; Hungate 1966). Several studies have been conducted to examine the relationship between the rumen microbiome and feed efficiency in beef cattle. Myer and colleagues found several bacterial populations that were associated with variation in feed efficiency in beef steers, including *Prevotella*, the predominant genus within the rumen (Myer et al. 2015b). Other studies have supported these data in other beef cattle production classes,

including in bulls (McCann et al. 2014; McGovern et al. 2018) and steers (Shabat et al. 2016), as well as in dairy cattle and other ruminants (Jewell et al. 2015; Pitta et al. 2016; Jami, White, and Mizrahi 2014; Ellison et al. 2017). However, few, if any, studies have examined the rumen microbiome and feed efficiency in female beef cattle, including heifers and cows. Given that these cattle contribute to propagation of genetics in the herd, understanding feed efficiency in female beef cattle is imperative for improving feed efficiency in the whole herd.

Although the rumen microbiome is strongly associated with feed efficiency in cattle and other ruminants, the rumen microbiome is not readily available to producers and those without access to research equipment; thus, identifying readily accessible and inexpensive methods for examining the rumen microbiome as well as host physiology that contribute to variations in feed efficiency phenotypes are necessary. Measuring metabolites in the blood and how those are associated with the rumen microbiome and metabolites could serve as a method of identifying and selecting more feed efficient cattle (Fontanesi 2016). Further understanding of the relationships among the rumen microbiome and metabolome, blood metabolome, and cattle feed efficiency is expected to lead to improved selection criteria and overall herd feed efficiency. To date, most studies conducted to understand variation in feed efficiency phenotypes has thus far been conducted in beef steers and bulls. Therefore, this study was conducted to establish the relationship among the metabolome, rumen microbiome, and feed efficiency in female cattle. The hypothesis was that there would be differences in the rumen microbiome and metabolomes of heifers that differed in feed efficiency, eventually leading to the objective to determine potential biomarkers of feed efficiency in heifers.

Materials and Methods

Experimental design and sample collections

This study was carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee at the University of Tennessee, Knoxville. The protocol was approved by the University of Tennessee, Knoxville Institutional Animal Care and Use Committee.

In this study, 17 previously cannulated Angus heifers of approximately two years of age weighing 563 ± 12 kg were chosen for a feed efficiency trial. The heifers were provided with a two-week adaptation period to the diet and the GrowSafe System[®] prior to the 70-day feed efficiency trial. Body weight was taken on days -1, 0, 35, 70, and 71 of the feed efficiency trial, with day 0 denoting the first day of the feed efficiency trial and used to estimate average daily gain (ADG). Average daily feed intake (ADFI) and ADG were used to determine residual feed intake (RFI) as a measurement of feed efficiency (Koch et al. 1963). On day 70 of the feed efficiency trial, rumen content was collected via orogastric tubing in a 50mL conical tube and pH was measured using a portable pH meter (Denver Instruments, Arvada, CO, United States). After pH was measured, two mL aliquots of rumen fluid were removed and processed for metabolomic analysis. Rumen content was immediately frozen and stored at -80°C . Approximately nine mL of blood was collected via coccygeal venipuncture into serum separator tubes (Corvac, Kendall Health Care, St. Louis, MO). Blood samples were centrifuged at $2,000 \times g$ and 4°C for 20 minutes. Serum was transferred to 5mL plastic culture tubes and a 100 μL aliquot was removed for untargeted metabolomic analysis.

Serum samples were then frozen at -80°C. After determining ADFI and ADG, one heifer had a negative ADG and was thus eliminated from subsequent analyses.

DNA Extraction, Sequencing, and Analysis

Microbial DNA was extracted from rumen content using methods described by Yu and Morrison (2004). Briefly, approximately 0.2 g of rumen content was transferred to a lysis tube and underwent a modified repeated bead beating plus column method with an isopropanol nucleic acid precipitation. Microbial DNA was purified using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) and quantified using a DeNovix DS-11+ (DeNovix Inc., Wilmington, DE, USA). Bacterial and archaeal DNA was amplified using a two-step polymerase chain reaction (PCR). The V4 region of the 16S hypervariable gene was targeted using primers described by Parada (2016) and Apprill (2015) under the following conditions for the first step of PCR: initial denaturation step of 95°C for five minutes; then 25 cycles of denaturation for 30 s at 95°C, annealing for 60 s at 55°C, and elongation for 90 s at 72°C; followed by a final elongation step at 72°C for ten minutes. Gel electrophoresis was used to ensure correctly-sized DNA was amplified, then DNA was purified using AMPure XP beads (Beckman Coulter, Inc., Brea, CA, USA) per manufacturer protocol. Unique indices (Nextera, Illumina Inc., San Diego, CA, USA) were added to the purified amplified bacterial and archaeal 16S DNA via PCR using the following conditions: initial denaturation at 95°C for five min followed by eight cycles with a denaturation step of 30 s at 95°C, annealing at 55°C for 30 s, and an elongation step at 72°C for 30 s, followed by a final elongation step at 72°C for 10 min. Amplified, barcoded DNA was again purified using AMPure XP beads per manufacturer protocol. The amplicons were quantified and quality-checked using spectrophotometry with a DeNovix DS-11+ and a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Bacterial and archaeal amplicons were then sequenced on the Illumina MiSeq (Illumina Inc., San Diego, CA, USA) using a v2, 2×250 kit per manufacturer protocol at the University of Tennessee Genomics Core.

Microbial Sequence Processing

Bacterial and archaeal sequences were processed using the Galaxy server (Afgan et al. 2018) and Quantitative Insights Into Microbial Ecology v.1.9 (Caporaso et al. 2010). First, Illumina adapters were trimmed using TrimGalore! (Krueger 2015). Trimmed sequences were then quality filtered to 90% sequences at $Q \geq 30$. Chimeric sequences were identified and filtered from sequences using usearch61 (Edgar 2010) and then operational taxonomic units (OTUs) were identified using uclust (Edgar 2010). Operational taxonomic units identified as Cyanobacteria were filtered from the OTU table, and then each sample was subsampled to 25,000 sequences based on the lowest number of sequences in a single sample. In order to be retained in the dataset, OTUs had to be observed in at least two samples.

Metabolomics Processing and Analysis

For each heifer, metabolites were extracted from serum and filtered rumen fluid (100µL) at the University of Tennessee, Knoxville Biological and Small Molecule Mass Spectrometry Core. Metabolites were extracted using protocol previously described (Clemmons et al. 2017; Kamphorst et al. 2011). Briefly, metabolites were extracted using 0.1% formic acid in acetonitrile:water:methanol (2:2:1). Metabolites were separated using a Synergy Hydro-RP

column (100 × 2 mm, 2.5 µm particle size) at 25°C and constant flow rate of 0.200 mL/min. Mobile phases of A: 97:3 H₂O:MeOH with 11 mM tributylamine and 15 mM acetic acid and B: MeOH with a gradient consisted of the following: 0.0 min, 0% B; 2.5 min 0% B; 5.0 min, 20% B; 7.5 min, 20% B; 13 min, 55% B; 15.5 min, 95% B; 18.5 min, 95% B; 19 min, 0% B, and 25 min, 0% B. The sample (10 µL) was introduced into the Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific, Waltham, MA) and electrospray ionization was used to introduce the samples into an Exactive Plus Orbitrap MS (Thermo Fisher Scientific, Waltham, MA) using methods previously described (Kamphorst et al. 2011; Lu et al. 2010).

The .raw files from Xcalibur (Thermo Fisher Scientific, Waltham, MA) were converted to the mzML format using ProteoWizard (Chambers et al. 2012) and imported into (Metabolomic Analysis and Visualization Engine for LC–MS Data (MAVEN) (Clasquin, Melamud, and Rabinowitz 2012). Peaks were picked in MAVEN with a preliminary mass error of ±20 ppm and a retention time window of five minutes. The final metabolite annotations were based on MS1 spectra that have been previously verified using pure standards. For final metabolite annotation, the eluted peak of the metabolite would be identified ±2 minutes of anticipated retention time as well as ±5 ppm of expected mass. Metabolites were then identified in MAVEN and the Quan Browser function of the Xcalibur MS Software (Thermo Electron Corp., Waltham, MA) was used to integrate peak areas. The identified metabolites were then analyzed using MetaboAnalyst 4.0 (Chong et al. 2018). The peak intensities were median-normalized, log-transformed, and range-scaled prior to analysis in MetaboAnalyst 4.0.

Statistical Analyses

Bacteria and Archaea. Two different methods of analyzing the relationship between RFI and microbiota were employed. First, heifers were classified as high- or low-RFI based on ±0.25 standard deviations about the mean RFI, which resulted in 14 total heifers (high n=6; low n=8). Using this model, alpha-diversity metrics, including chao1, Faith's Phylogenetic Diversity, Good's coverage, Shannon's Diversity Index, and Simpson's Evenness E, as well as phylum- and genus-level taxa data were first analyzed for normal distribution using the PROC UNIVARIATE procedure in SAS 9. Data were considered to follow a normal distribution or approximately normal based on visual histogram and a Shapiro-Wilk statistic ≥ 0.90 . Those following a normal distribution were analyzed using a one-way ANOVA in SAS 9.4. Those data not following a normal distribution were first transformed using log, square root, or ranked transformations in SAS 9.4. The transformation that resulted in the greatest Shapiro-Wilk statistic was selected for use in subsequent analysis, then analyzed using a one-way ANOVA. P-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) correction (Benjamini and Hochberg 1995). Beta-diversity was determined using analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) based on unweighted and weighted UniFrac distances. The second model, using RFI as a continuous variable, was analyzed using the PROC REG procedure in SAS 9.4 with a stepwise variable selection using the normally distributed variables, including those that were previously transformed. Linear discriminant analysis effect size (LeFSe) was used to identify potential microbial biomarkers of RFI in Galaxy using the tools developed by Segata and others (Segata et al. 2011). Significance was determined at $P \leq 0.05$.

Metabolomics. As with bacterial and archaeal data, relationships between RFI and metabolites were analyzed using two different models of RFI with RFI as both a discrete and continuous variable. Differences in metabolites by high- and low-RFI were analyzed using a *t*-test for rumen fluid and serum metabolomes analyzed individually in MetaboAnalyst 4.0 using Benjamini-Hochberg FDR corrected P-values. Principal components analyses (PCA) and orthogonal partial least squares discriminant analyses (O-PLS-DA) were used to visualize differences in biofluid metabolites by high- and low-RFI based on 2,000 permutations. Supervised random forests were used to generate variable importance in projection (VIP) plots in MetaboAnalyst 4.0. In order to determine relationships between rumen metabolites and microbiota, Spearman correlation analyses were performed using the PROC CORR procedure in SAS. In order to analyze the relationship between the metabolites and RFI as a continuous variable, the metabolomic data was first transformed to achieve a normal distribution using the rank transformation in SAS 9.4, and then analyzed using the PROC REG procedure in SAS 9.4 with a stepwise variable selection. Significance was determined at $P \leq 0.05$. Additionally, the relationship between the rumen and serum metabolomes were visualized using PCA and O-PLS-DA, with the metabolites contributing most to differences by biofluid were determined using VIP plots.

Results

Microbiome

A total of 917,433 quality filtered sequences were generated, with 20,919 total OTU across all samples. All significant taxa are presented in Table 8. No alpha-diversity metrics differed by RFI (Table 9). The unweighted PERMANOVA did differ by high- and low-RFI, although no other beta-diversity metrics differed (Table 10). Principal coordinates analyses were used to visualize beta-diversity by RFI based on unweighted (Figure 7A) and weighted (Figure 7B) UniFrac distances. Bacteria identified as significant by LeFSe are presented in Figure 8. Taxa associated with RFI based on regression are presented in Table 11.

Global metabolites

A total of 143 known metabolites were identified. Of the 143 known metabolites, 124 differed as a result of biofluid. A PCA (Figure 9A) and O-PLS-DA (Figure 9B) provide visual representations of differences by differences by biofluid. The top 25 metabolites that differ by biofluid as well as the VIP scores that drove the differences in biofluids are presented in Figures 10 and 11, respectively.

Rumen metabolome

No metabolites differed by *t*-test; however, 21 metabolites had > 2-fold difference between high- and low-RFI heifers (Table 12). Although no differences in low- and high-RFI metabolomes in the PCA (Figure 12A) were observed, the O-PLS-DA (Figure 12B) illustrates two distinct metabolomes by RFI. A heatmap representing the top 25 differences in the rumen fluid metabolomes by RFI are presented in Figure 13A, and the top ten metabolites driving those differences are presented in a VIP score plot (Figure 14A). In the rumen, the only pathway that

Table 8. Significant bacteria and archaea that differ by low- and high-RFI heifers

Taxa Level	Taxon	High-RFI ¹	Low-RFI ¹	P-value	FDR ²
Family	<i>Lachnospiraceae (Other)</i>	2.94×10^{-3} (7.07×10^{-4})	7.82×10^{-3} (1.71×10^{-3})	0.04	0.98
Family	<i>Desulfobulbaceae</i> ⁵	0.00	2.61×10^{-5} (1.11×10^{-5})	0.05	0.98
Family	<i>Neisseriaceae</i> ³	3.37×10^{-5} (2.65×10^{-5})	1.09×10^{-4} (3.20×10^{-5})	0.02	0.98
Genus	<i>Shuttleworthia</i> ⁴	1.35×10^{-4} (2.48×10^{-5})	5.89×10^{-4} (1.58×10^{-4})	0.02	0.98
Genus	<i>Corynebacterium</i>	3.49×10^{-4} (4.89×10^{-5})	1.53×10^{-4} (5.14×10^{-5})	0.02	0.98
Genus	<i>p-75-a5</i>	2.75×10^{-4} (7.19×10^{-5})	1.11×10^{-4} (3.16×10^{-5})	0.04	0.98
Genus	<i>L7A-E11</i>	2.15×10^{-4} (8.59×10^{-5})	1.49×10^{-5} (7.28×10^{-6})	<0.001	0.98

¹Mean (SEM) based on raw abundances

²Benjamini-Hochberg false discovery rate corrected P-values

³Based on log-transformed data

⁴Based on square root-transformed data

⁵Based on ranked data

Table 9. Alpha-diversity metrics by low- and high-RFI heifers

Metric	High-RFI¹	Low-RFI¹	P-value
Good's Coverage	0.95 (0.00)	0.95 (0.00)	0.74
Observed OTU	2366.83 (148.53)	2195.63 (87.38)	0.31
Chao1	4658.78 (315.16)	4536.07 (252.66)	0.76
Faith's Phylogenetic Diversity	121.40 (5.39)	113.03 (3.28)	0.19
Shannon's Diversity Index	8.01 (0.31)	7.80 (0.16)	0.52
Simpson's Evenness E	0.02 (0.01)	0.02 (0.00)	0.64

¹Mean (SEM)

Table 10. Beta-diversity metrics between low- and high-RFI heifers

Metric¹	Test Statistic	P-value
PERMANOVA ² – weighted	1.30 ⁴	0.22
PERMANOVA ² – unweighted	1.17 ⁴	0.03
ANOSIM ³ – weighted	0.08 ⁵	0.22
ANOSIM ³ – unweighted	0.16 ⁵	0.09

¹Metrics based on weighted or unweighted UniFrac distances with 9999 permutations

²Permutational multivariate analysis of variance

³Analysis of similarity

⁴Test statistic is pseudo-F

⁵Test statistic is R

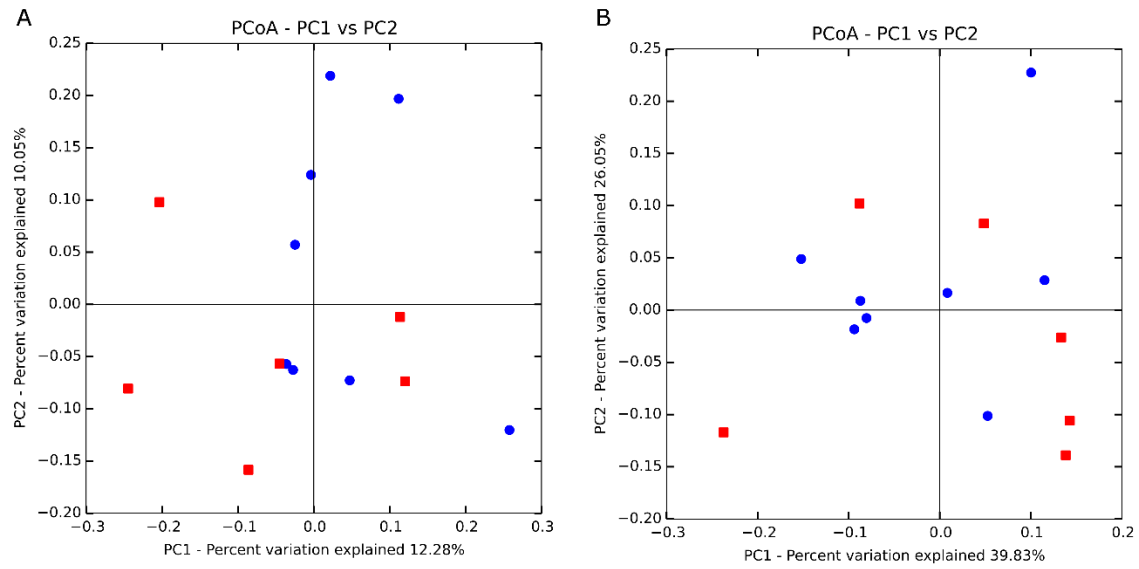


Figure 7. Principal coordinates analysis based on unweighted (A) and weighted (B) UniFrac distances of different levels of residual feed intake 16S analyses based on 9999 permutations. Low-RFI = blue circle (n=8), High-RFI = red square (n=6)

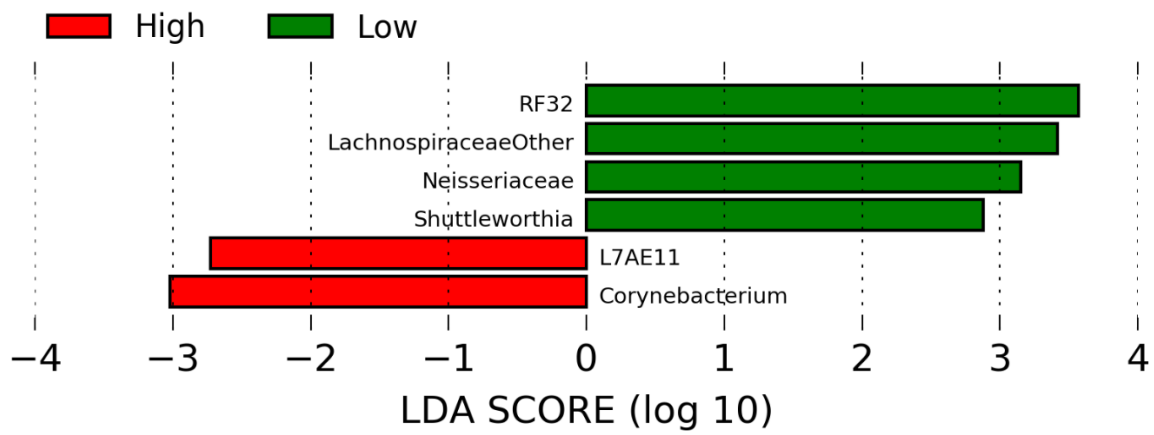


Figure 8. Linear discriminant analysis effect size of bacteria that are associated with low-RFI (green) or high-RFI (red) phenotypes

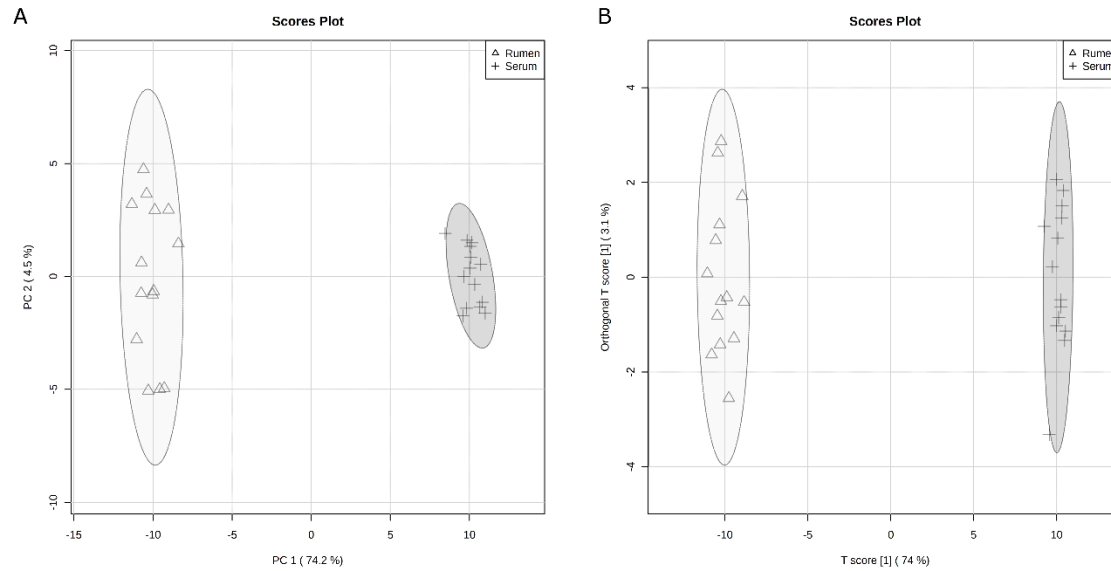


Figure 9. Principal component analysis (A) and orthogonal partial least squares discriminant analysis (B) of biofluid based on 2000 permutations. The ellipses represent 95% confidence interval. Triangle = rumen fluid, plus sign = serum

Table 11. Taxa associated with RFI based on regression

Taxon	β^1	Model R²	P-value²
<i>SJA88</i>	-6.24×10^{-1} (2.63×10^{-7})	0.44	0.004
<i>Mollicutes</i>	-3.11×10^{-1} (1.54×10^{-7})	0.75	0.001
<i>Lachnospiraceae (Other)</i>	-2.19×10^2 (1.53×10^{-4})	0.88	0.004
<i>Agrobacterium</i>	1.95×10^{-1} (1.46×10^{-7})	0.92	0.03
LD1	1.01×10^{-1} (1.99×10^{-7})	0.97	0.005
<i>Brevibacillus</i>	-7.32×10^{-2} (1.53×10^{-7})	0.98	0.008
<i>Microbacterium</i>	1.59×10^{-1} (5.46×10^{-6})	0.99	0.002
<i>Coriobacteriaceae (Other)</i>	7.99×10^{-2} (4.15×10^{-7})	0.99	0.004
<i>Legionella</i>	-3.17×10^{-2} (1.42×10^{-7})	0.99	0.001
<i>Sphingobium</i>	1.13×10^{-2} (2.06×10^{-7})	1.00	0.007
<i>Caulobacteraceae</i>	-3.01×10^2 (8.47×10^{-3})	1.00	0.001
<i>Rhodobacteraceae (Other)</i>	-1.71×10^{-3} (2.34×10^{-7})	1.00	0.004
<i>Syntrophomonas</i>	3.99×10^{-4} (2.17×10^{-7})	1.00	0.005
<i>Leuconostocaceae</i>	1.69×10^{-2} (8.67×10^{-5})	1.00	0.003

¹Beta value (SE)²Significance determined at $P \leq 0.05$

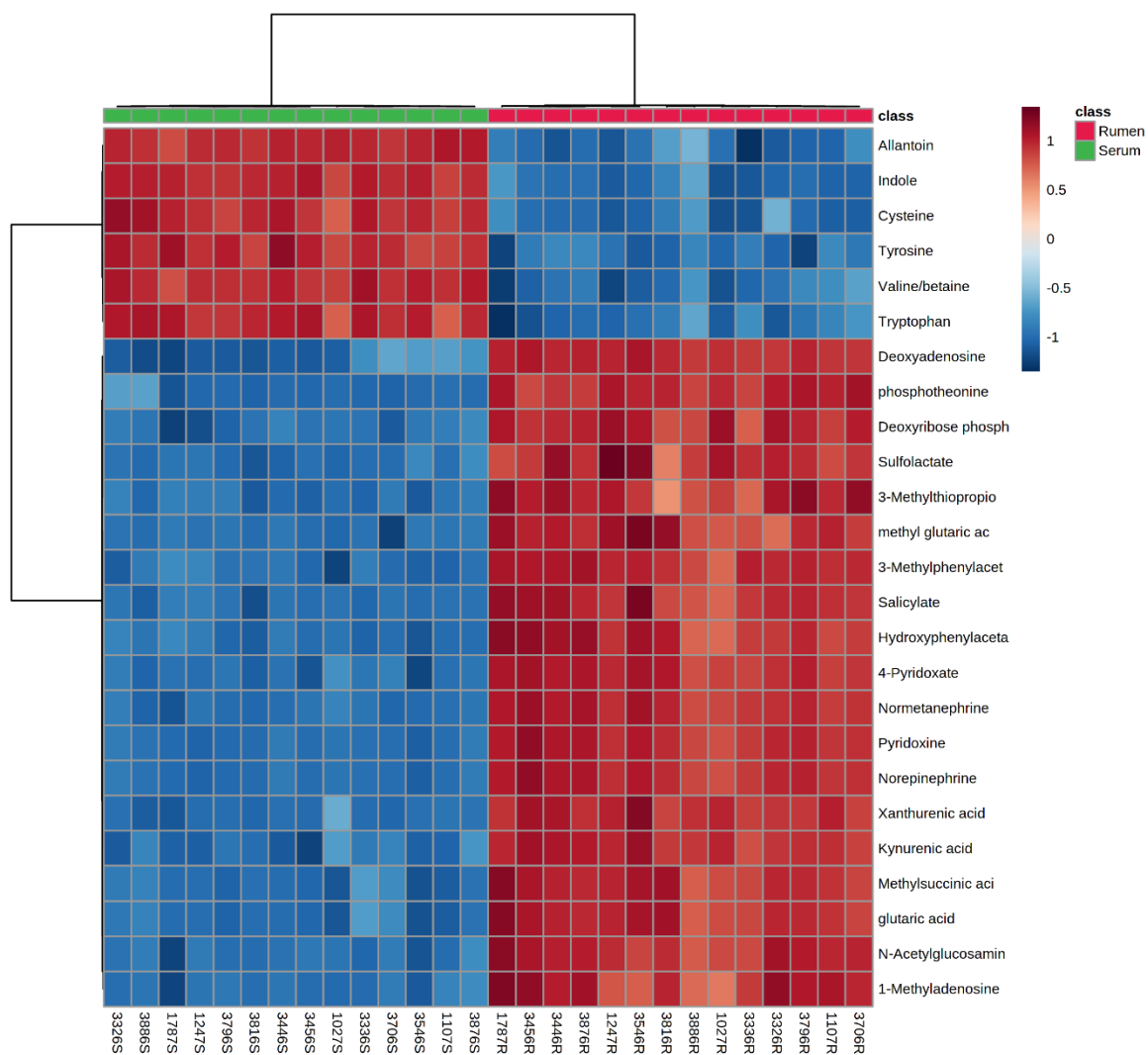


Figure 10. Heatmap of top 25 metabolites that differed as a result of biofluid

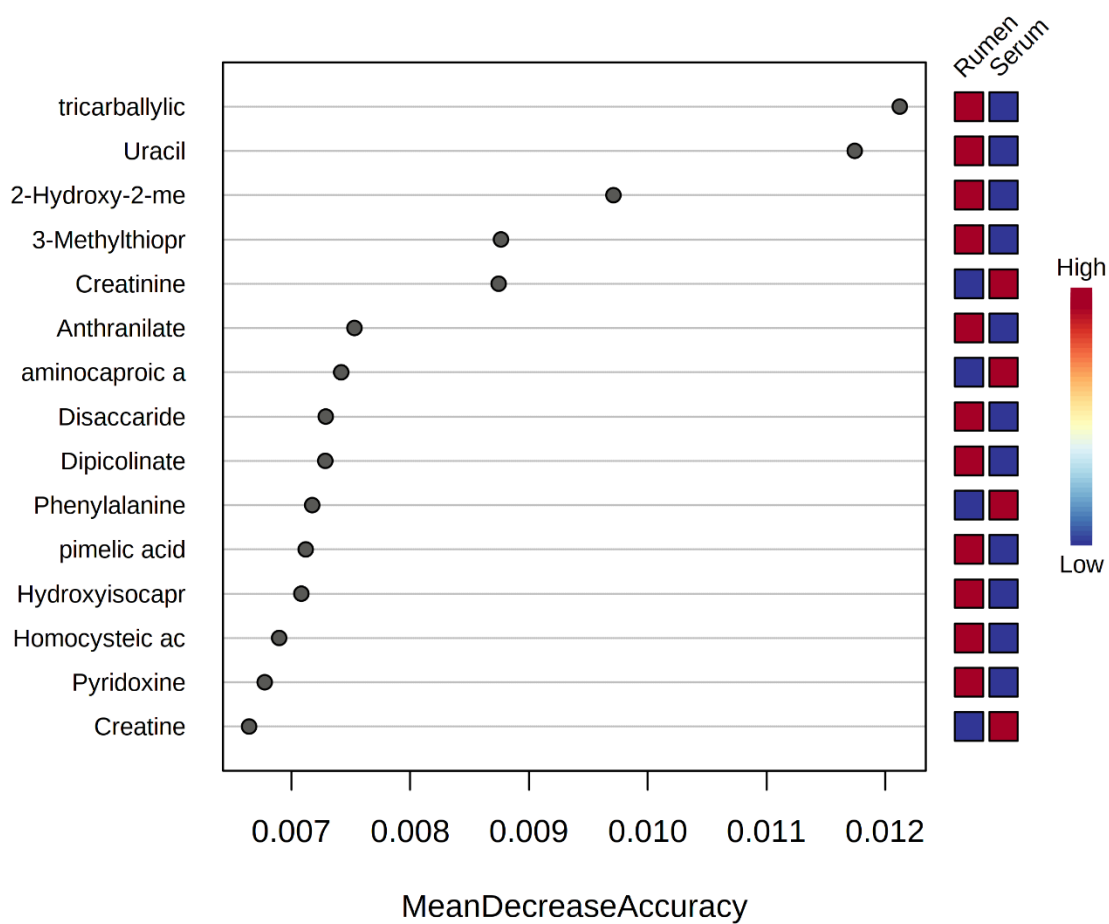


Figure 11. Variable importance in projection plot describing metabolites that are the 15 greatest contributors to differences observed in metabolomes that differ by biofluid

Table 12. Rumen fluid metabolomes that had ≥ 2 -fold difference between low- and high-RFI heifers

Metabolite	High-RFI¹	Low-RFI¹	Fold change
UDP-N-acetylglucosamine	8.44×10^2 (8.44×10^2)	3.23×10^5 (1.83×10^5)	0.0032488
NAD	1.18×10^2 (1.18×10^2)	1.84×10^4 (1.09×10^4)	0.01375
Taurine	2.22×10^3 (1.54×10^3)	1.35×10^5 (1.01×10^5)	0.020692
Cholate	4.83×10^5 (1.36×10^5)	7.08×10^6 (2.82×10^6)	0.075272
Creatine	1.89×10^3 (6.28×10^2)	3.77×10^4 (2.75×10^4)	0.076414
Taurodeoxycholate	n.d.	5.48×10^5 (3.36×10^5)	0.081299
Glycodeoxycholate	n.d.	4.29×10^5 (3.10×10^5)	0.12439
Cystathionine	1.08×10^4 (5.76×10^3)	2.03×10^3 (8.88×10^2)	8.004
Arginine	9.39×10^3 (3.90×10^3)	1.07×10^5 (6.57×10^4)	0.13458
IMP	6.48×10^3 (2.24×10^3)	4.42×10^4 (3.51×10^4)	0.14821
UMP	4.93×10^4 (1.15×10^4)	2.59×10^5 (1.08×10^5)	0.19245
UDP	n.d.	7.78×10^2 (6.71×10^2)	0.195
phosphorylethanolamine	3.88×10^2 (1.76×10^2)	3.68×10^3 (1.96×10^3)	0.20153
cAMP	4.04×10^5 (3.79×10^5)	1.25×10^6 (9.49×10^5)	0.24847
Asparagine	4.18×10^4 (7.51×10^3)	1.53×10^5 (6.18×10^4)	0.32909
Octulose biphosphate	5.12×10^5 (1.67×10^5)	1.89×10^5 (7.19×10^4)	2.6493
Creatinine	6.67×10^4 (9.29×10^3)	7.68×10^4 (3.23×10^4)	0.39336
Homocysteine	1.59×10^2 (1.59×10^2)	9.04×10^2 (7.49×10^2)	0.42019
Cystine	2.22×10^4 (7.15×10^3)	1.01×10^4 (3.22×10^3)	2.1765
FAD	2.81×10^3 (2.81×10^3)	1.04×10^3 (6.76×10^2)	2.1052
3-Phosphoglycerate	9.84×10^5 (1.77×10^5)	2.10×10^6 (3.99×10^5)	0.49568

¹Mean (SEM)

n.d. = not detected

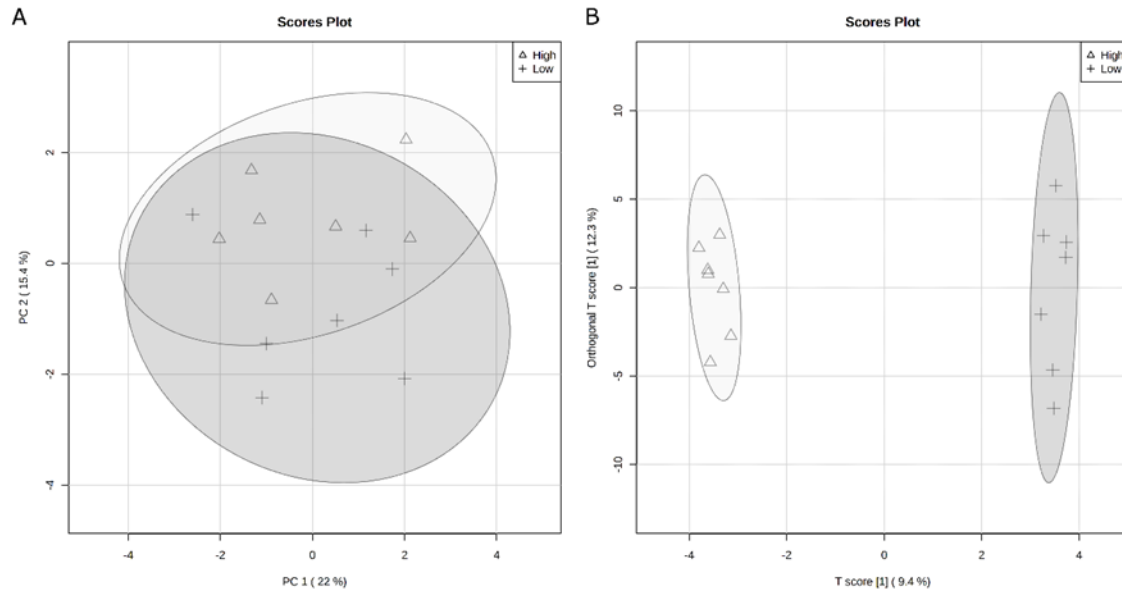


Figure 12. Principal components analysis (A) and orthogonal partial least squares discriminant analysis (B) comparing the rumen fluid metabolomes of low- and high-RFI heifers. Ellipses represent 95% confidence intervals, with low-RFI represented by the plus sign and high-RFI represented by the triangle.

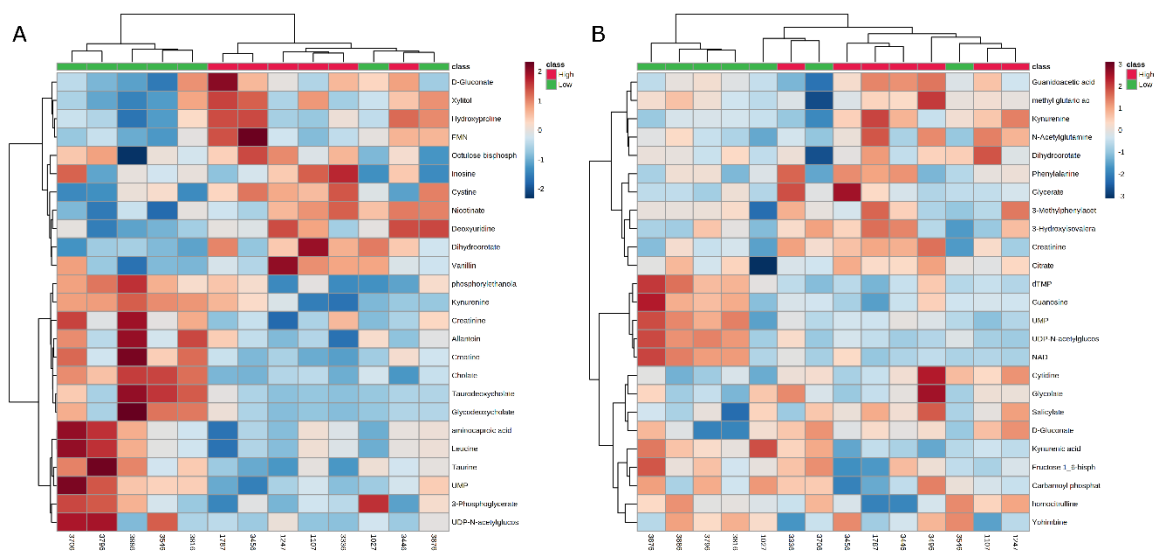


Figure 13. Heatmap of top 25 metabolites that differed by low- and high-RFI in rumen fluid (A) and serum (B).

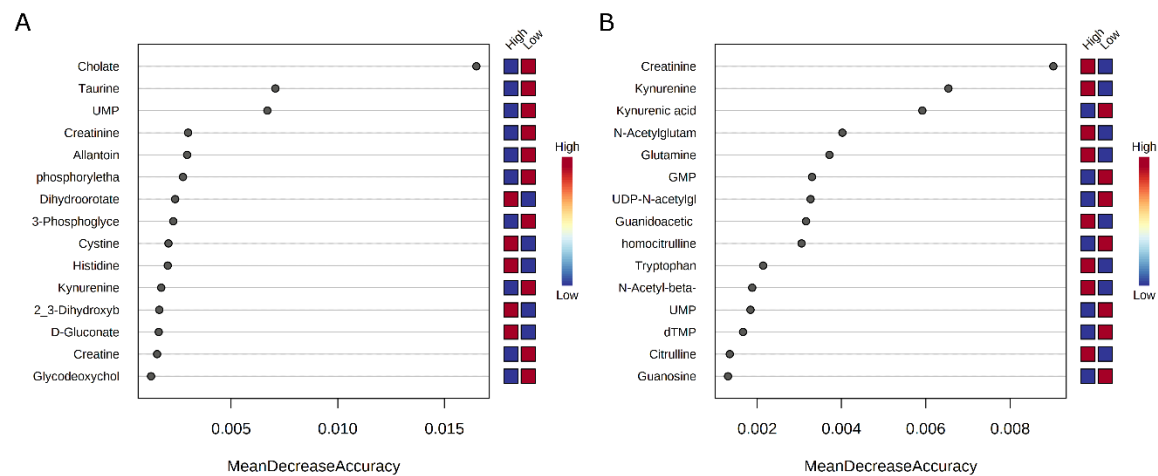


Figure 14. Variable importance in project plots for the top 15 metabolites contributing to differences in metabolomes by low- and high-RFI in rumen fluid (A) and serum (B).

was impacted as a result in differences in RFI phenotype was primary bile acid synthesis ($P = 0.02$, impact = 0.04). Rumen fluid metabolites that were associated with RFI based on regression are presented in Table 13.

Serum metabolome

No differences were determined by t-test between low- and high-RFI serum metabolites but 15 serum metabolites had > 2-fold difference between high- and low-RFI heifers (Table 14). No separation was observable in PCA (Figure 15A), but separation was apparent in O-PLS-DA (Figure 15B). A heatmap with the top 25 serum metabolites that differ by RFI are provided in Figure 12B. The top ten metabolites that are driving differences in metabolomes by RFI are presented in a VIP score plot in Figure 13B. Serum metabolites that were associated with RFI based on regression are presented in Table 15.

Rumen microbiome and metabolome

Relationships between the rumen microbiome and rumen metabolome were examined by performing correlation analyses of significant bacteria and archaea by RFI phenotype with rumen metabolites that differed ≥ 2 -fold by low- and high-RFI heifers. Significant metabolites are presented in Table 16.

Discussion

Beef cattle is one of the largest sectors of the United States' agricultural sector, and the United States is the largest exporter of beef globally (ERS 2019). Given that the beef cattle industry serves as a multi-billion-dollar industry around the world, identifying methods for improving efficiencies and production-relevant traits could save millions or billions of dollars for producers and consumers. These changes could also result in a more environmentally friendly and sustainable beef cattle industry, resulting in fewer required resources while optimizing outputs. Giving the feed costs typically account for the largest input costs for beef production, improving feed efficiency in environmentally and economically sustainable manners could increase global animal protein supply while decreasing resource needs (ERS 2019, 2015). Several factors can be predictive of or contribute to differences in feed efficiency phenotypes, including the rumen microbiome, pH, and metabolome, as well as metabolites that circulate throughout the body. These different factors could be used as biomarkers of efficiency in cattle. Additionally, much of the work that has been conducted to interrogate these biomarkers in beef cattle have been conducted in steers, with little working focusing on the female cattle at different production stages.

Rumen Microbiome

No metric of alpha microbial diversity differed by feed efficiency phenotype in the heifers in this study. This is not unusual, given that many studies have failed to identify differences in rumen microbial diversity in cattle varying in feed efficiency. A study conducted by Myer and colleagues in 2015 examined the rumen bacterial communities in the rumen of steers differing in feed efficiency using a cartesian quadrant model of feed efficiency with high- or low-ADG and high- or low-ADFI (Myer et al. 2015b). In that study, no significant differences were observed among feed efficiency contemporary groups in alpha nor beta diversity (Myer et al. 2015b). This has also been supported by other studies (McCann et al. 2014; Jewell et al. 2015). In the rumen microbiome,

Table 13. Rumen metabolites associated with RFI based on regression

Metabolite	β^1	Model R ²	P-value ²
Taurine	$-9.77 \times 10^{-1} (1.24 \times 10^{-6})$	0.51	0.003
Glucose phosphate	$6.14 \times 10^{-1} (1.41 \times 10^{-6})$	0.73	0.008
Homocitrulline	$-5.05 \times 10^{-1} (1.84 \times 10^{-6})$	0.92	<0.001
FAD	$3.09 \times 10^{-1} (3.95 \times 10^{-6})$	0.95	0.02
Cysteate	$3.84 \times 10^{-1} (1.49 \times 10^{-6})$	0.98	0.003
UDP	$-2.92 \times 10^{-1} (2.20 \times 10^{-6})$	0.99	0.006
Xanthurenic acid	$-1.05 \times 10^{-1} (3.82 \times 10^{-6})$	0.99	0.009
C-methyl-D-erythritol phosphate	$6.79 \times 10^{-2} (1.65 \times 10^{-6})$	0.99	0.002
cAMP	$-1.46 \times 10^{-2} (1.27 \times 10^{-6})$	0.99	0.02
myo-Inositol	$-2.44 \times 10^{-2} (2.89 \times 10^{-6})$	1.00	0.004
Norepinephrine	$-1.04 \times 10^{-2} (4.69 \times 10^{-6})$	1.00	0.003
Orotate	$-2.03 \times 10^{-3} (1.88 \times 10^{-6})$	1.00	0.003
Asparagine	$-1.50 \times 10^{-4} (1.68 \times 10^{-6})$	1.00	0.007

¹Beta value (SEM)

Table 14. Serum metabolites that differed by ≥ 2 -fold between high- and low-RFI heifers.

Metabolite	High-RFI ¹	Low-RFI ¹	Fold change
IMP	2.75×10^3 (1.31×10^3)	6.12×10^5 (4.99×10^5)	0.0050715
UDP-N-acetylglucosamine	1.52×10^3 (1.03×10^3)	1.09×10^5 (6.45×10^4)	0.013976
NAD	2.24×10^2 (1.64×10^2)	1.19×10^4 (7.07×10^3)	0.023292
UMP	3.69×10^4 (7.16×10^3)	3.87×10^5 (1.67×10^5)	0.094823
GMP	2.17×10^4 (5.49×10^3)	1.38×10^5 (6.40×10^4)	0.1514
dTMP	2.46×10^4 (2.09×10^3)	9.26×10^4 (3.17×10^4)	0.29886
N-Acetylglucosamine 1/6-phosphate	2.06×10^4 (1.93×10^4)	4.35×10^3 (3.02×10^3)	2.8714
IDP	2.41×10^5 (1.26×10^5)	5.65×10^5 (2.83×10^5)	0.35639
Guanosine	4.20×10^4 (3.29×10^3)	1.29×10^5 (4.69×10^4)	0.36182
Fructose 1,6-bisphosphate	1.23×10^5 (4.62×10^4)	3.55×10^5 (1.46×10^5)	0.37308
cAMP	9.52×10^2 (3.60×10^2)	2.66×10^3 (2.35×10^3)	0.38656
Kynurenic acid	6.59×10^4 (1.34×10^4)	1.32×10^5 (1.89×10^4)	0.47088
Glutathione	6.30×10^3 (2.24×10^3)	4.05×10^3 (1.88×10^3)	2.1148

¹Mean (SEM)

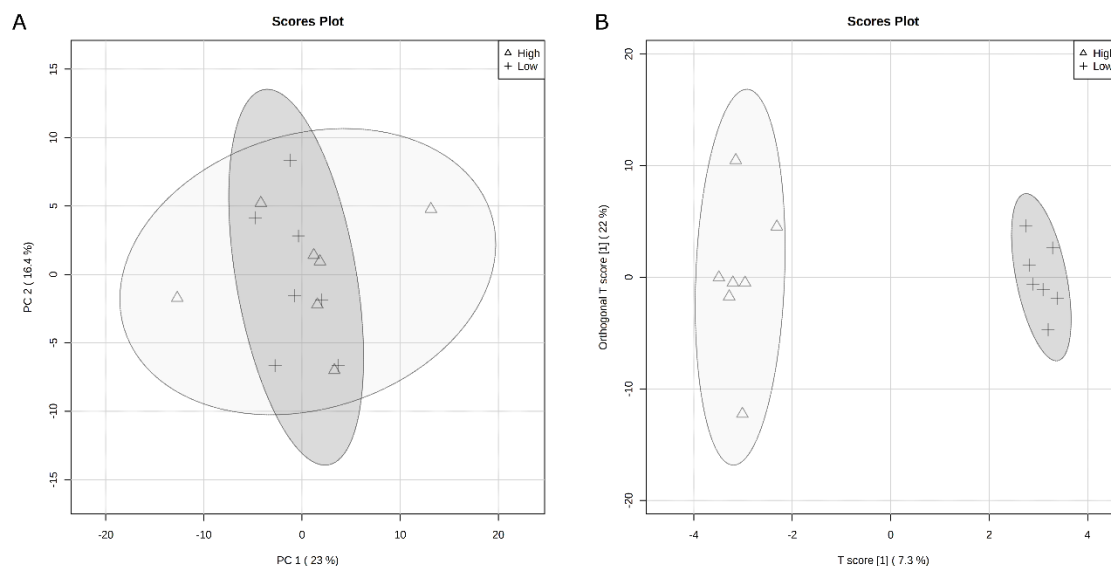


Figure 15. Principal components analysis (A) and orthogonal partial least squares discriminant analysis (B) of serum metabolites that differ by low-RFI (plus sign) and high-RFI (triangle). Ellipses represent a 95% confidence interval.

Table 15. Serum metabolites associated with RFI based on regression

Metabolite	β^1	Model R^2	P-value ²
Glutamine	1.08 (1.96×10^{-5})	0.49	0.003
UDP-N-acetylglucosamine	-3.04×10^{-1} (7.06×10^{-6})	0.69	0.01
Sucralose	5.79×10^{-1} (1.79×10^{-5})	0.81	0.02
4-Pyridoxine	-3.74×10^{-1} (1.52×10^{-5})	0.90	0.007
3-Sulfolactate	-2.77×10^{-1} (6.41×10^{-6})	0.95	0.02
Yohimbine	-1.29×10^{-1} (1.22×10^{-5})	0.97	0.05
Glucose phosphate	-3.53×10^{-1} (8.08×10^{-6})	0.99	0.01
Uracil	2.97×10^{-1} (1.47×10^{-5})	0.99	0.04
2-Oxo-4-methylthiobutanoate	-1.17×10^{-1} (1.09×10^{-5})	0.99	0.01
Methyl glutaric acid	-1.07×10^{-1} (1.34×10^{-5})	0.99	0.02
Oxoisovalerate	5.42×10^{-2} (8.97×10^{-6})	1.00	0.002
Hydroxyproline	-1.43×10^{-2} (1.76×10^{-5})	1.00	0.006
Glutathione	2.52×10^{-3} (9.92×10^{-6})	1.00	0.008
Leucine	1.20×10^{-3} (2.35×10^{-5})	1.00	0.01

¹Beta value (SEM)²Significant determined at $P \leq 0.05$

Table 16. Correlations of rumen fluid metabolites and microbiota.

Taxon	Metabolite	ρ^1	P-value²
p-75-a5	2-Oxoisovalerate	-0.54	0.04
	3-Phosphoglycerate	-0.53	0.04
	Creatinine	-0.73	<0.01
	Cytidine	-0.61	0.01
	Glutamine	-0.74	<0.01
	IMP	-0.63	0.01
	N-acetylmethionine	-0.64	0.01
	Pimelic acid	-0.56	0.03
	Valine	-0.52	0.05
Lachnospiraceae (Other)	2-Oxoisovalerate	0.71	<0.01
	3-Phosphoglycerate	0.55	0.03
	Arginine	0.55	0.03
	Creatinine	0.52	0.05
	Cysteine	0.59	0.02
	Cytidine	0.56	0.03
	Glutamine	0.59	0.02
	Phosphorylethanolamine	0.56	0.03
	Taurine	0.69	<0.01
	UMP	0.52	0.05
Corynebacterium	2-Oxoisovalerate	-0.66	<0.01
	3-Phosphoglycerate	-0.53	0.04
	Citrate	-0.55	0.03
	Phosphorylethanolamine	-0.60	0.02
	Succinate/Methylmalonate	-0.54	0.04
	Taurine	-0.74	<0.01
Neisseriaceae	Creatine	0.71	<0.01
	Cystine	-0.78	<0.001
	Dihydroorotate	-0.56	0.03
	FMN	-0.53	0.04
	Glyceroxycholesterol	0.53	0.04
	Hydroxyproline	-0.53	0.04
	Nicotinate	-0.58	0.02
	Phosphorylethanolamine	0.55	0.03
	Taurodeoxycholate	0.52	0.05
	UMP	0.59	0.02
	Xylose	-0.56	0.03
Shuttleworthia	2,3-Dihydroxybenzoate	-0.57	0.03
	2-Oxo-4-methylthiobutanoate	0.53	0.04
	2-Oxoisovalerate	0.59	0.02
	Asparagine	0.58	0.02
	Taurine	0.66	<0.01
	UDP	0.59	0.02
Desulfobulbaceae	2-Dehydro-D-gluconate	-0.57	0.03
	Cystine	-0.55	0.04

Table 16. (continued)

	Deoxyuridine	-0.56	0.03
	FMN	-0.64	<0.01
	Histidine	-0.55	0.04
	Hydroxyproline	-0.60	0.02
	Methionine	-0.62	0.01
	Methionine sulfoxide	-0.53	0.04
	N-carbamoyl-L-aspartate	-0.53	0.04
	Nicotinate	-0.59	0.02
	Tyrosine	-0.62	0.01
	Uracil	-0.53	0.04
	Xylose	-0.56	0.03
L7A-E11	Succinate/Methylmalonate	-0.72	<0.01
	Taurine	-0.59	0.02
	UDP-N-acetylglucosamine	-0.76	<0.01
	UMP	-0.66	<0.01

¹Spearman's rank correlation test statistic²Significance determined at $P < 0.05$

variation in diversity may not contribute as much to divergences in ruminant feed efficiency phenotypes as individual taxa. Indeed, it is now thought that there are keystone species that drive differences more than global differences in microbiota (Jousset et al. 2017; Banerjee, Schlaeppi, and van der Heijden 2018; Li et al. 2019). Thus, the lack of observed disparities in alpha and most beta diversity metrics between high- and low-RFI heifers in this study was unsurprising.

Lachnospiraceae was present in greater relative abundance in low-RFI compared to high-RFI, as well as predictive of RFI based on multiple regression. *Lachnospiraceae* is one of the most commonly found taxa in mammalian gut microbiomes (Biddle et al. 2013; Meehan and Beiko 2014; Kittelmann et al. 2013). The family *Lachnospiraceae* as well as taxa within *Lachnospiraceae* have been previously been found associated with feed efficiency in cattle (Myer et al. 2015b; McGovern et al. 2018; Shabat et al. 2016; Jewell et al. 2015). *Lachnospiraceae* has also been suggested as a keystone taxon in humans (Ashman and Krishnamurthy 2019; Goodrich et al. 2014) and may serve this role in cattle as well (Li et al. 2019). Furthermore, Li and others (2019) identified several beef cattle single nucleotide polymorphisms that were associated with unclassified members of *Lachnospiraceae*, indicating a possibility to select for this more feed-efficient microbiome.

Lachnospiraceae are butyrate-producing bacteria, and members of *Lachnospiraceae* possess genes for both butyrate-producing pathways, including butyrate kinase and butyryl-CoA:acetate CoA-transferase (BCoAT). *Shuttleworthia*, a genus within *Lachnospiraceae*, possesses the ability to synthesize butyrate through the butyrate kinase (Meehan and Beiko 2014). *Shuttleworthia* were also present in greater relative abundance in the low-RFI heifers in this study. Butyrate production by *Lachnospiraceae* may reduce methanogenesis in a similar manner to *Succinivibrionaceae* in that it reduces substrate availability for methane production (Shabat et al. 2016). Besides serving as a gluconeogenic precursor in ruminants, butyrate also has health-related functions. In humans and mice, butyrate is the main source of energy for colonic cells (Donohoe et al. 2011; Binder 2010). Beyond this, butyrate and butyrate-producing bacteria are thought to have anticarcinogenic properties, antimicrobial properties, and anti-inflammatory properties, among others (Canani et al. 2011; Liu et al. 2018). In ruminants, butyrate is imperative for ruminal development in pre-weaned animals, in part because it serves as the primary energy source in rumen epithelial cells (Sander et al. 1959; Baldwin and Jesse 1992). In beef cattle, greater concentrations of butyrate were found in the rumen of low-RFI steers compared to high-RFI steers (Guan et al. 2008). Additionally, as has been observed in humans and mice, butyrate has the ability to alter the rumen microbiome (Li, Wu, et al. 2012) as well as alter host gene transcription (Baldwin et al. 2012). Butyrate-producers, and butyrate, likely contribute to variation in beef cattle feed efficiency phenotypes by altering the rumen microbiome and host physiology.

Corynebacterium was observed in greater relative abundance in less feed efficient heifers in this study. Many species within *Corynebacterium* are known causative agents of diseases in ruminants. For example, *Corynebacterium pseudotuberculosis* causes caseous lymphadenitis, a contagious disease that causes abscesses to form in the skin and on the organs of sheep and goats (Williamson 2001). Additionally, *Corynebacterium pyogenes* has been found associated with liver abscesses in feedlot cattle, particularly in relation with other pathogenic bacteria (Brown et al. 1973). Although

some members of *Corynebacterium* are found in humans and animals with no known pathogenicity (Moore et al. 2010), more research is needed to understand the dynamics among *Corynebacterium*, other rumen microbiota, and host production-relevant phenotypes. Further understanding of these dynamics may elucidate currently unknown contributions of the rumen microbiome to host feed efficiency phenotypes.

Additionally, *Coriobacteriaceae* (other) were positively associated with RFI in heifers. Members of *Coriobacteriaceae* have been found associated with feed efficiency in other studies. Freetly and others examined the relationship between more or less ADG with similar ADFI throughout the gastrointestinal tract of steers (Freetly et al. 2020). The authors found that most members of *Coriobacteriaceae* were reduced in steers with greater ADG (thus greater feed efficiency). *Coriobacteriaceae* are able to metabolize bile acids and other cholesterol-derived metabolites. A study conducted by Just examined the effect of *Coriobacteriaceae* on bile acids and host metabolism in mice (Just 2017). The author found that increased abundances of *Coriobacteriaceae* resulted in increased white adipose tissue (Just 2017). It has been hypothesized that *Coriobacteriaceae* function to convert some energy products towards adipose tissue deposition that would otherwise be put towards protein production (Freetly et al. 2020). Given that protein production is the primary goal of growing beef cattle for consumption as well as weighs more than fat tissues, microbiota that increase fat tissues over protein production may result in decreased feed efficiency (Freetly et al. 2020).

Metabolome

Other recent work has applied untargeted and targeted metabolomics to identify potential biomarkers of feed efficiency in cattle and attempted to relate the rumen fluid and serum metabolomes. Artegoitia and colleagues analyzed the rumen fluid metabolomes using ultra performance liquid chromatography quadrupole time-of-flight tandem mass spectrophotometry (UPLC-qTOF-MS/MS) in steers of similar feed intake but differing average daily gain to represent high and low feed efficiency (Artegoitia et al. 2017). After correcting for false discovery rate, Artegoitia and others found 33 metabolites that differed by high or low average daily gain, including fatty acids such as pentadecanoic acid, linoleic acid, and alpha-linolenic acid. The authors then attempted to identify if those same fatty acids could be measured from the plasma that would reflect the differences observed in the rumen fluid using a targeted approach. The plasma concentrations of the measured fatty acids that varied by average daily gain the rumen fluid did not differ based on a false discovery rate correct P-value in steers by high and low average daily gain. Although other studies have found that rumen fluid metabolomes differ in beef steers varying in feed efficiency using other models of feed efficiency (Clemmons et al. 2020; Novais et al. 2019), these studies have failed to directly connect the rumen fluid to serum metabolome.

Although the rumen fluid and serum metabolomes may independently differ by feed efficiency using different models of feed efficiency, the metabolomes of the rumen fluid and blood appear to be distinctly separate. Previous research has attempted to find similarities in the rumen metabolome and blood metabolome, which would be useful for early identification of rumen environment abnormalities given that blood is much more readily accessible than the rumen content. However, the blood metabolome does not appear to be a direct representative of the rumen

content metabolome and thus does not accurately reflect the rumen environment. Using blood metabolites to attempt to directly measure the rumen metabolites is not a viable method; however, other tools may be useful in attempting to assess the rumen environment or to differentiate animals of varying-production relative phenotypes.

With regards to global differences across biofluids, biofluid appeared to be more influential in dictating variation in the metabolomes. In both the PCA and PLS-DA, there was very clear separation in metabolomes by biofluid, in that serum and rumen fluid were very distinct. After grouping by biofluid, there was significant overlap in metabolomes by RFI classification with much greater variability in low-RFI metabolomes than high-RFI. This is not unsurprising, given that previous research has shown that various ruminant biofluids possess distinct metabolomes (Sun et al. 2015). Furthermore, specific analyses examining the relationship between the rumen fluid and serum metabolomes demonstrate very separate metabolomes (Clemmons et al. 2018). This may suggest that one cannot be substituted for the other (i.e. metabolites in the rumen fluid cannot be directly measured from the blood) but there could still be strong relationships between the blood and rumen fluid metabolomes; thus, it is important to understand that relationship between them.

One metabolite that was present in greater abundance in low-RFI heifers in both the rumen fluid and serum that has significant biological relevance is nicotinamide adenine dinucleotide (NAD⁺). This molecule is one of the most widely utilized co-substrates and used in many different cellular reactions (Goody and Henry 2018). In humans, decreased NAD⁺ is associated with metabolic diseases and decreased mitochondrial function (Nikiforov, Kulikova, and Ziegler 2015). Skeletal muscle cells are particularly reliant on NAD⁺ concentrations, which is important in beef cattle production given that the product is primarily skeletal muscle. Nicotinamide adenine dinucleotide is thought to participate in nutrient sensing in growing animals which contributes to muscle growth. A study conducted by Wilson, Ross, and Harris examined the effects of caloric restrictions during pregnancy and lactation on rat muscle development and found that secondary myogenesis was negatively affected, resulting in poor secondary myotube formation (Wilson, Ross, and Harris 1988). This is thought to potentially be due to inadequate glucose and NAD⁺, but the exact mechanisms governing this are not well understood (Goody and Henry 2018). In growing beef cattle, greater availability of NAD⁺ from the rumen that enters the blood may provide greater muscle accretion capacity.

Although no metabolites differed significantly between low- and high-RFI heifers in the serum based on a *t*-test, several metabolites had ≥ 2 -fold difference between the two feed efficiency phenotypes. Of these, most of them were associated with purine and pyrimidine metabolism, including inosine monophosphate (IMP), guanine monophosphate (GMP), guanosine, cyclic adenosine monophosphate (cAMP), uridine monophosphate (UMP), deoxythymidine monophosphate (dTMP), and inosine diphosphate (IDP), all of which were present in greater abundance in both the rumen fluid and serum of the low-RFI heifers than the high-RFI heifers. This collection of results also mirrors the rumen fluid metabolome in this study. Purine and pyrimidine intermediates have previously been used to extrapolate microbial protein synthesis in ruminants, though typically purine and pyrimidine derivatives excreted in the urine have been used

(Chen, Ørskov, and Hovell 1990). Belenguer and others examined the relationship between urine purine derivatives and flow of purines through the duodenum of goats (Belenguer et al. 2002). The authors found a linear response of excreted purine derivatives in the urine and measured purines in the duodenum (Belenguer et al. 2002). In ruminants, most nucleic acids and nucleic acid precursors, including purines and pyrimidines, are derived from microbes (Belenguer et al. 2002; Chen and Gomes 1992; Broderick and Merchen 1992). These can be used to estimate microbial crude protein supply available to the animal (Broderick and Merchen 1992). The greater availability of these purine and pyrimidine intermediates in the serum of more feed efficient heifers likely signals greater protein- and muscle-building capacity, leading to more feed efficient animals. Furthermore, because these same trends in these metabolites were observed in the rumen of the more efficient heifers, the rumen microbiome in the low-RFI heifers may be supplying greater amounts of microbial crude protein available to the host.

Rumen

In this study, the rumen fluid metabolomes of low- and high-RFI heifers were different based on an O-PLS-DA, but not the PCA. It is not uncommon for O-PLS-DA and PCA to differ because O-PLS-DA consider more information than is taken into account in PCA, and PCA is weaker when within-group variation is not significantly lower than between-group variation (Worley and Powers 2013). These results were interesting because similar studies conducted in beef cattle have demonstrated different results. For instance, a study conducted by Clemmons and others in 2020 examined the differences in the rumen metabolome in beef steers differing in feed efficiency as measured by RFI (Clemmons et al. 2020). The authors did not observe divergences in the rumen fluid metabolomes by high- or low-RFI steers as illustrated in the PCA as well as the O-PLS-DA, although differences were observed in individual metabolites (Clemmons et al. 2020). However, other studies have found similar results to those presented in the current study, including a study conducted by Artegoitia et al. (2017). In that study, the authors did not observe differences in the PCA between more and less feed efficient steers, but did observe differences in the PLS-DA (Artegoitia et al. 2017). The differences in rumen fluid metabolomes provides further evidence for variation in rumen microbial activities in cattle divergent in feed efficiency phenotypes.

In the rumen, one metabolite that differed by ≥ 2 -fold difference by low- and high-RFI was cholate, which was found in greater abundance in the rumen of low-RFI heifers than high-RFI heifers. Additionally, byproducts of cholate metabolism were observed in greater abundances in the rumen of low-RFI heifers, including cystathionine, taurine, taurodeoxycholate, and deoxycholate, all of which require microbial enzymes to be catabolized from cholate (Kaddurah-Daouk et al. 2011). Additionally, these cholate byproducts have all been previously measured in the rumen (Sheriha et al. 1968). Immig conducted a study analyzing the effect of exogenous porcine bile acids on ruminal fermentation and methanogenesis *in vitro* with rumen fluid from a fistulated cow (Immig 1998). Immig found that increasing bile acids resulted in decreased methanogenesis, which shunted H_2 towards propionate production (Immig 1998). Indeed, bile acids have a demonstrated effect on the reduction of methanogenesis in the lower gastrointestinal tract of humans and other monogastric species (Florin and Jabbar 1994; Florin and Woods 1995; Maczulak, Wolin, and Miller 1989). Methanogenesis in ruminants has been hypothesized to result in a 2-12% decrease in feed efficiency due to carbon sources being used for methane production rather than volatile

fatty acid production (Johnson and Johnson 1995). Methane is released as a waste product, whereas volatile fatty acids serve as gluconeogenic precursors and provide approximately 70% of the energy precursors in ruminants (Seymour, Campbell, and Johnson 2005; Yost et al. 1977). Additionally, recent research in humans and mice has shown that bile acids also have important signaling functions in glucose and lipid metabolism (Taoka et al. 2016); however, these have not yet been demonstrated in ruminants and could serve as a future area of research.

Homocysteine and its precursor, 3-phosphoglycerate, were present in greater quantities in the rumen of low-RFI heifers compared to high-RFI heifers in this study. Homocysteine is a non-proteinogenic amino acid that is an intermediate in methionine synthesis (Faeh, Chiolo, and Paccaud 2006) and has been previously suggested to serve as a biomarker for more efficient cattle (Clemmons et al. 2017). Methionine is important because it is often the rate-limiting amino acid in protein accretion for cattle (Titgemeyer and Merchen 1990; Seymour 2016), and supplementation of rumen-protected methionine can result in increased gains. A study conducted by Klemesrud, Klopfenstein, and Lewis examined the effect of increasing supplementation of rumen-protected methionine on growth in steers. The authors found that providing additional rumen-protected methionine in the diet of steers at 2.9 g/d resulted in increased gain (Klemesrud, Klopfenstein, and Lewis 2000), and supplementation of methionine has been supported in research of other ruminants (Schwab 1996; Nelson, Lehninger, and Cox 2008; Seymour 2016).

Increased homocysteine can improve methionine production in ruminants. A study conducted by Amos et al. (1974) determined the effect of rumen-protected methionine and homocysteine on concentrations of plasma methionine in wethers. Supplementation of homocysteine, both orally and through intra-abomasal infusion, resulted in increased plasma methionine concentrations (Amos et al. 1974). The only oral supplement that resulted in increased plasma methionine was homocysteine, suggesting the ability for the rumen microbiome to produce additional methionine that is available to the host (Amos et al. 1974). Furthermore, rumen bacteria and protozoa have the demonstrated ability to generate methionine from homocysteine (Or-Rashid, Onodera, and Wadud 2001), supporting the possibility that increased homocysteine and homocysteine precursors could contribute to variation in host feed efficiency phenotypes.

Serum

In this study, as was observed in the rumen fluid metabolome, no differences were observed in the serum metabolome between low- and high-RFI heifers in the PCA. However, the O-PLS-DA did illustrate distinct serum metabolomes by high- and low-RFI heifers, though the separation in metabolomes was not very apparent. A study conducted by Clemmons and colleagues in 2017 found similar results to those observed in this study. In that study, the authors examined the serum metabolomes in high- and low-RFI steers, and observed distinctly separate serum metabolomes in steers divergent in feed efficiency (Clemmons et al. 2017). However, unlike that study, no differences were found in individual metabolites in the present study. As mentioned previously, this is likely due to the lack of power to detect differences; additional studies should be conducted with larger cohorts of heifers to achieve sufficient power to detect differences by feed efficiency phenotype.

Another set of serum metabolites that differed by ≥ 2 -fold were those involved in the hexosamine signaling pathway, including UDP-N-acetylglucosamine (greater in low-RFI heifers), fructose 1,6-bisphosphate (greater in low-RFI heifers), and N-acetylglucosamine 1/6-phosphate (greater in high-RFI heifers). The hexosamine signaling pathway involves posttranslational modification of proteins and provides nutrient signaling (Buse 2006; Love and Hanover 2005). Briefly, fructose-6-phosphate is converted to glucosamine-6-phosphate, which eventually becomes UDP-N-acetylglucosamine (UDP-GlcNAc) (Love and Hanover 2005). The metabolite UDP-GlcNAc is then thought to serve as a mediator of protein posttranslational modification (Love and Hanover 2005). Additionally, UDP-GlcNAc may be involved in many other cellular and tissue remodeling, including transcription, apoptosis, and cell proliferation, which is important for protein accretion and tissue repair (Love and Hanover 2005). Furthermore, UDP-GlcNAc is sensitive to glucose concentrations (Love and Hanover 2005), particularly important in ruminants as gluconeogenesis is the primary source of glucose (Young 1977). In the hexosamine pathway, N-acetylglucosamine-6-phosphate is supposed to be rapidly converted to UDP-GlcNAc; however, in the present study, greater levels of N-acetylglucosamine-6-phosphate were measured in the serum of high-RFI heifers. This, combined with the lower abundances of UDP-GlcNAc in the serum of the high-RFI heifers compared to the low-RFI heifers, suggest that there may be some dysfunction of the enzyme acetyltransferase Emeg32, which is responsible for this conversion (Boehmelt, Fialka, et al. 2000; Boehmelt, Wakeham, et al. 2000). Indeed, a study conducted by Boehmelt and others examined the effect of inactivated Emeg32 in mouse embryos (Boehmelt, Wakeham, et al. 2000). In that study, the authors found that inactivation of Emeg32 resulted in decreased proliferation and growth (Boehmelt, Wakeham, et al. 2000). Although this and the rest of the hexosamine signaling pathway needs much further exploration, particularly in ruminants, before serving as an explanatory variable for divergences in feed efficiency phenotypes, the data provided in this study suggest that interruptions or insufficiencies in the hexosamine signaling pathway may contribute to variation in feed efficiency phenotypes in cattle.

Relationships among the rumen microbiome, metabolites, and host physiology

A relationship observed among feed efficiency, the rumen microbiome, and rumen metabolome was one involving asparagine and *Shuttleworthia*. A positive correlation between asparagine concentrations and *Shuttleworthia* was detected. Furthermore, there was greater abundance of *Shuttleworthia* as well as asparagine in the rumen of low-RFI heifers. Asparagine, though not considered an essential amino acid, is required for proper brain development in mammals as well as several other biological functions (Ruzzo et al. 2013) and can become essential in specific situations (Pavlova et al. 2018). In the rumen, asparagine can be rapidly degraded by the rumen microbiota, although it has been demonstrated to be degraded at different rates dependent upon the type of microbes. Warner examined the rate of degradation of asparagine, glutamine, nicotinamide and formamide from different sheep at various timepoints (Warner 1964). Warner observed that asparagine was primarily degraded by bacterial populations, but rates of degradation were affected by the time or animal from which the samples came, possibly suggesting that the different bacterial populations may result in diversity in rates of asparagine degradation (Warner 1964). The results from Warner coupled with the results described in this paragraph from the present experiment illustrate that there are likely differences in asparagine and other amino acid degradation in the rumen as a result of divergent microbial populations. This is of particular interest and importance

for ruminant production because microbial consumption of amino acids results in fewer amino acids available to the host ruminant for muscle growth, neurotransmission and development, and other biological activities as amino acids are catabolized to ammonia which is excreted in the urine as urea (Pathak 2008).

Furthermore, there were relationships between the bile acid products and bacterial taxa that differed by RFI phenotype. Those microbiota that were present in greater abundance in high-RFI heifers, including *Corynebacterium* and *L7A-E11*, were both negative correlated with taurine, a component for generation of secondary bile acids. The microbiota found in greater abundance in low-RFI heifers were positively correlated with metabolites associated with secondary bile acid formation include *Lachnospiraceae*, *Neisseriaceae*, and *Shuttleworthia*. These bacteria were positively correlated with taurine, glycodeoxycholate, and taurodeoxycholate. The dynamic between bile acids and gut microbiota is interesting, and could account for some of the variation observed in feed efficiency phenotypes in cattle. Bile acids are known regulators of gut microbial populations in the human gut as well as host metabolism (Ridlon et al. 2014). Interestingly, microbially-conjugated bile acids (such as glycodeoxycholate and taurodeoxycholate) rapidly activate insulin regulation pathways via G-protein coupled receptors (GPCR) as well as activate gluconeogenic pathways via FOXO1, CEBP α , and HNF4 α (Yamagata et al. 2004) in vitro and in monogastric species. Although these specific genes do not appear to be up- or down-regulated in cattle divergent in feed efficiency, feed efficiency is associated with differential expression in genes related to energy metabolism (Chen et al. 2011; Tizioto et al. 2015; Shabat et al. 2016). *Lachnospiraceae*, in particular, is known to produce the microbial-derived secondary bile acids (Weingarden et al. 2014; Devlin and Fischbach 2015). *Lachnospiraceae*, as well as the other bacteria found to be associated with differences in RFI and the secondary bile acids, could be altering host gene expression related to metabolism. The relationship between *Lachnospiraceae* and RFI in the heifers, as well as with the bile acids, may contribute to variation in feed efficiency phenotypes.

A substantial amount of money each year is spent in development of rumen bypass proteins, also referred to as rumen undegradable protein. One method of reducing protein degradation by the rumen microbiota is by heat treating the feed or protein supplementation. In doing this, either sugars or other amino acids link to the protein, which are better at resisting enzymatic activities leading to degradation (Kung Jr and Rode 1996). One amino acid that is ubiquitous and stable that can be used for this is asparagine (Kung Jr and Rode 1996). The increased abundances of asparagine in low-RFI heifers in addition to the relationship with the bacterial populations in the low-RFI heifers may be indicative of a more effective microbiome and metabolome such that low-RFI heifers have more access to amino acids that may bypass the rumen and be available for absorption in the lower gastrointestinal tract. In addition to incorporation into proteins, asparagine is a glucogenic amino acid, serving as a glucose precursor, increasing blood glucose supply (Overton et al. 1999). Increased diversity of glucogenic substrates could allow the animal to be more resilient and potentially greater feed efficiency.

Creatinine was observed in greater abundances in the rumen fluid in low-RFI heifers and was negatively correlated with *p-75-a5* as well as positively correlated with *Lachnospiraceae* (*Other*).

Creatinine is regularly used as a measure of MCP that is supplied to the ruminant animal via measurements in the urine (Verbic et al. 1990; Chen, Ørskov, and Hovell 1990; Chen et al. 1990). Urine creatinine concentrations are linearly correlated with MCP as well as ruminally-infused protein, and thus are an indirect marker of availability of protein in the rumen and protein degradation in the rumen (Chen, Ørskov, and Hovell 1990; Chen et al. 1990; Verbic et al. 1990). In the rumen, proteins can be degraded to creatinine by rumen microbiota and then creatinine is further degraded to other metabolites, a byproduct of which is ammonia (Caspi et al. 2006). In the present study, the relationship among feed efficiency, *p-75-a5*, and creatinine may be indicative of a rumen microbiome that may be more conducive to optimal feed efficiency because there were increased relative abundances of *p-75-a5* in high-RFI, and *p-75-a5* had a negative relationship with creatinine abundances in the rumen. Additionally, there were greater amounts of creatinine observed in the rumen fluid of low-RFI as well as a positive correlation with *Lachnospiraceae*, a family that was present in greater abundance in low-RFI heifers. Given that creatinine concentrations can be indicative of MCP production, it may be possible that the negatively-correlated relationship between *p-75-a5* and creatinine in the rumen of high-RFI heifers coupled with the increased abundance of creatinine as well as lower abundance of *p-75-a5* in low-RFI heifers may indicate that the order *p-75-a5* has a negative effect on protein availability to the ruminants. This, coupled with the positive relationship between low-RFI, *Lachnospiraceae*, and creatinine may indicate greater MCP available to the host animal. The exact mechanisms underlying this hypothesis have yet to be elucidated, though it may be one of a couple mechanisms: the first, that *p-75-a5* or other microbiota may degrade proteins into waste products such as ammonia that do not support the growth of the host animal; or second, to simply represent or interact with other microbiota to produce less protein (rather than to degrade protein), resulting in less protein available to the host animal.

In this study, several microbial taxa of interest were correlated with 2-oxoisovalerate, including those with negative correlations (*p-75-a5* and *Corynebacterium*) and those with positive correlations (*Lachnospiraceae [Other]* and *Shuttleworthia*). Interestingly, those microbes that were negatively correlated with 2-oxoisovalerate were also those present in greater abundances in high-RFI heifers, and those positively correlated with 2-oxoisovalerate were observed in greater abundance in the low-RFI heifers. The metabolite 2-oxoisovalerate is involved in several vital metabolic reactions, including valine, leucine, and isoleucine biosynthesis, as well as pantothenate and coenzyme A biosynthesis. Primarily, however, it is involved in branched chain amino acid (BCAA) metabolism via the branch chain α -keto acid dehydrogenase (BCKDH) complex as a product of transamination of valine (Brosnan and Brosnan 2006). The production of α -keto acids, such as 2-oxoisovalerate, results in allosteric inhibition of the BCKDH kinase (Brosnan and Brosnan 2006). The inhibition of the BCKDH kinase prevents activation of the BCKDH, resulting in conserved BCAA (Brosnan and Brosnan 2006). Branched chain amino acids are important for muscle growth, particularly in growing cattle. A study conducted by Li and others examined the effects of BCAA supplementation during nursing on the ADG of growing dairy cattle from three to 28 days after birth (Li et al. 2005). The authors observed that the steers that received the treatment during the first four weeks of life had sustained greater ADG than the control group starting at two months of age through slaughter at 18 months of age (Li et al. 2005). The treatment group had, on average, a 14.8% greater final body weight at slaughter than did the control group

(Li et al. 2005). The greater availability of BCAA to the growing animal may provide greater muscle growth potential (Li et al. 2005). Some bacteria possess the ability to degrade BCAA into byproducts, including 2-oxoisovalerate, which can subsequently be absorbed by the ruminant (Early et al. 1984). The greater production and absorption of the 2-oxoisovalerate could potentially result in some decreased transamination of BCAA, allowing for greater muscle development in low-RFI heifers. This is further supported by some of the other results observed in this study, including the negative correlation of *p-75-a5* with valine, and the positive correlation of *Shuttleworthia* with 2-oxo-4-methylthiobutanoate, an additional intermediate of BCKDH.

Another metabolic pathway that is associated with RFI as well as specific microbiota in the rumen is that involved in amino acid metabolism of serine and the homocysteine cycle. In the production of serine, 3-phosphoglycerate is converted through a series of enzymatic processes to serine. Serine can, in turn, be converted into glycine, cysteine, and homocysteine. Additionally, serine is a precursor for sphingolipids. In the present study, 3-phosphoglycerate was negatively correlated with *p-75-a5* and *Corynebacterium*, both of which are present in greater abundances in high-RFI heifers. *Lachnospiraceae (Other)*, found in greater abundance in low-RFI heifers, was positively correlated with 3-phosphoglycerate, as well as cysteine and phosphorylethanolamine, a sphingolipid precursor. Rumen bacteria possess the ability to break down cysteine and serine into smaller components (Chalupa 1976), which may make the 3-phosphoglycerate available to the host animal for use, as cattle possess the ability to produce serine and cysteine from 3-phosphoglycerate (Wu 2014). Indeed, Shabat et. al. found that amino acid/protein metabolism was upregulated in the rumen of high-RFI cattle, indicating that less feed efficient cattle possess rumen microbiota that may decrease availability of amino acids or amino acid precursors available to the animal (Shabat et al. 2016).

In addition to availability for incorporation into proteins, cysteine as well as BCAA may play important roles in immunity of cattle. In 2010, Miller and colleagues examined the role of cysteine in eliciting immune response with increased parasitic and non-parasitic infection (Miller et al. 2000). The authors found that cysteine infusion treatment resulted in greater peripheral eosinophilia as well as globular leukocyte count, and also increased parasitic antibody response (Miller et al. 2000). These data have also been supported in non-ruminant animals as well (Takahashi, Ohta, and Akiba 1997; Konashi, Takahashi, and Akiba 2000). However, previous research has attempted to identify associations between RFI and immune traits in cattle, but these studies have yielded conflicting results (Kelly et al. 2017; Herd et al. 2019). The differences in immunity by feed efficiency phenotypes may be promising, but requires further research.

Another relationship observed in this study was that of glutathione, cystine, and various bacteria with RFI, in which glutathione and cystine were positively associated with RFI as well as bacteria present in greater abundance in high-RFI heifers and the opposite trend observed with bacteria present in low-RFI heifers. Glutathione and cystine are vital antioxidants that reduce the presence of destructive reactive oxygen species (ROS). Low abundances of glutathione and cystine have been implicated in increased oxidative stress, which is interesting given that greater amounts of glutathione and cystine were observed in the high-RFI heifers in this study. However, previous studies have demonstrated that antioxidant species may decrease during the depletion of ROS as

well as release of glutathione into media during loss of intracellular glutathione *in vitro* (Yu and Long 2016). Kizil and others examined the response of plasma markers of oxidative stress in response to subclinical and clinical mastitis infection (Kizil et al. 2007). The authors found that plasma glutathione levels increased with severity of mastitis infection, indicating increased oxidative stress during mastitis (Kizil et al. 2007). The increased levels of glutathione and cystine in the serum of high-RFI heifers in this study, as well as the negative correlation of various bacteria with cystine in the rumen of low-RFI heifers suggest a potential increased oxidative stress in high-RFI; however, the underlying cause of this is yet unknown but warrants further investigation.

Feed efficiency is an incredibly complex phenotypic trait, can be measured using several different methods, and changes based on time of year, production stage, and diet, among others. In ruminants, this is further compounded by the fact that the animal relies substantially on the gut microbiome, primarily the rumen microbiome, as the primary source of energy precursors, vitamins, and other nutrients (Hungate 1975; Young 1977). Physiologically, feed efficiency, including RFI, is associated with differences in energy partitioning and methane production (Nkrumah et al. 2006). Researchers have also demonstrated that beef cattle with decreased feed efficiency have altered liver function, the primary site of gluconeogenesis. Alexandre and others in 2015 examined the liver transcriptome in 16 beef steers differing in feed efficiency using a residual intake and body weight gain (RIG) method. The authors observed that in the low feed efficient group, the cattle had greater fat deposition and as well as increased transcripts for inflammatory response, the result of which was corroborated using histological methods (Alexandre et al. 2015). Additionally, the low feed efficient steers demonstrated upregulation of genes associated with saturated fatty acid synthesis and processes involving lipid metabolism (Alexandre et al. 2015). The results have been further supported using other methods in beef cattle in other studies (Richardson and Herd 2004; Connor et al. 2010; Abo-Ismael et al. 2013; Clemmons, Voy, and Myer 2019; Archer et al. 1999). However, one significant factor that is largely ignored in most studies is the effect of sex on physiological differences and feed efficiency. The majority of beef cattle studies regarding feed efficiency are conducted in steers and occasionally bulls, but very few, if any, focus solely on female cattle or examine sex as a factor affecting feed efficiency.

As previously mentioned, sex, and the related hormonal differences, may impact the gut microbiome. In ruminants, differences in the rumen microbiome as a result of sex have been rarely studied; however, sex and hormones affecting the gut microbiome have been examined in ruminants. A study conducted by Org and others examined the effect of sex, gonadectomized and hormone treated mice on the gut microbiome and related metabolites. Within each strain studied, differences between the gut bacteria of male and female mice were observed (Org et al. 2016). Interestingly, several taxa potentially associated with low-RFI heifers in the present study were observed in greater abundance in male mice than in female mice, such as *Roseburia* and *Lachnospiraceae* (dependent upon strain) (Org et al. 2016). Furthermore, in gonadectomized mice, the effect of sex hormones became more apparent. In female mice, gonadectomy was associated with differences in gut bacteria, sometimes accounting for almost 20% variation in relative abundance of specific taxa (Org et al. 2016). There were also differences in bile acids between intact and gonadectomized mice, including cholate (Org et al. 2016), which is of particular interest

given that cholate was the sole metabolite that differed in the present study in the rumen fluid between low- and high-RFI heifers. In previous research, variation in bile acids have been demonstrated to alter gut microbiota in monogastric species, which could alter nutrient absorption (Islam et al. 2011; Li and Chiang 2015).

Conclusion

The agriculture industry is tasked with producing 50% more food in the next 30 years (Alexandratos and Bruinsma 2012). In the beef cattle industry, this is only 30 cow-calf cycles to generate more animal protein in environmentally and economically sustainable manners. Feed efficiency, or the conversion of feedstuffs into animal protein, could resolve, in part, the growing global food needs. Improving feed efficiency could result in millions of saved dollars by reduced input costs and decreased environmental resource cost. In order to do this, tools must be developed to quickly, efficiently, and cost-effectively assess feed efficiency to inform selection decisions. This study found several potential biomarkers of feed efficiency in both low and high feed efficient cattle. Additionally, this particularly important as it was conducted in heifers, and the data could be used to inform breeding management decisions.

CONCLUSION

The objectives of this dissertation included identification of prospective metabolomic and microbial biomarkers of feed efficiency in both Angus steers and heifers, which were achieved. In both steers and heifers, rumen fluid metabolites may serve as biomarkers of feed efficiency, as well as provide insight as to the mechanisms contributing to divergences in feed efficiency phenotypes in beef cattle. In particular, nucleic acid and amino acid turnover differences were observed between cattle divergent in feed efficiency, with greater evidence of more nucleic acid and amino acid production in more efficient compared to less efficient beef cattle.

Because the relationship between feed efficiency and the rumen bacterial communities has been well-studied in beef steers, the protozoal communities were analyzed in this dissertation. Protozoal communities account for approximately half of the biomass of the rumen, and possess unique abilities such as bacterial engulfment and starch hoarding. In this dissertation, the only protozoal taxa that differed between low- and high-RFI steers were unassigned taxa. These unassigned taxa may lead to inefficient cycling of nutrients or could contribute to differences in bacterial communities via selection engulfment of certain bacterial populations. The unassigned taxa warrant further investigation in future research.

The research in this dissertation also examined potential serum metabolite and rumen microbial biomarkers of feed efficiency in Angus heifers. With regards to feed efficiency in beef cattle, both cows and heifers have been largely excluded from analyses. This is particularly compelling given that heifers and cows contribute a significant portion of genetic variety in the herd and breeding management decisions are frequently made from a female perspective. In this study, several candidate metabolites were identified, although the sole metabolite that differed by low- and high-RFI heifers was rumen cholate. This was potentially a result of lower statistical power and a greater number of animals may have permitted detection of more differences in metabolites by feed efficiency phenotype. Additionally, no differences were observed in bacterial or archaeal populations after false discovery rate correction; however, this was also likely due to insufficient power. Future studies should use a greater number of animals to ensure adequate statistical power to detect differences in microbial populations. Furthermore, female cattle at different production stages should be used to examine the relationship among female cattle, feed efficiency phenotypes, and the rumen microbiome as this relationship could inform breeding management decisions.

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VITA

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