Biochemical and Microbial Biomarkers Mediating Feed Efficiency in Cattle

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Brooke Ashley Clemmons
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DEDICATION

This thesis would not have been possible without the support of my wonderful, amazing husband, Josh. Thank you for the support, from spending weekends at the farm to hearing me practice presentations. Without your support, this would have been much more difficult, and a lot less fun.

I would also like to dedicate this to my family, who always believed in me, even when I didn't believe in myself.

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ABSTRACT

Beef cattle are the primary red meat consumed in the United States and provide greater than $105 billion in retail value each year. In the beef industry, feed represents approximately 60% of the total input costs. Thus, finding novel ways of improving feed efficiency in order to reduce that cost is imperative. This research assesses several aspects of the ruminal microbiome in relation to feed efficiency in steers, including stability of the ruminal bacteriome following transition to feed, serum metabolites in steers differing in feed efficiency, as well as potential microbial and biochemical biomarkers of feed efficiency. In this study,50 Black Angus steers of 7 months of age were acclimated to the GrowSafe© feeding system and fed a step-up receiving diet before receiving a growing ration. Steers were maintained on the diet for 70d. Weekly BW was measured, serum collected, and rumen content was obtained via gastric tubing. The average RFI was calculated and steers were divided into low- (n=14) and high-RFI (n=15) groups based on 0.5 SD below and above the mean RFI, respectively. Untargeted serum metabolomics was conducted utilizing LC-MS. Genomic DNA was extracted from rumen content and the amplified V1-V3 hypervariable region of the bacterial 16S rRNA gene was sequenced for analyses. Missing values were approximated through matrix completion and data was normalized using a centered log-ratio transformation. Random Forests supervised machine learning and feature selection was performed on the bacterial compositions. Ruminal bacteria diversityfluctuated over the course of the trial, and was lower at the end of the trial compared to the beginning (P<0.05). Low-RFI steers were associated with decreased bacterial α [alpha]-(P=0.03) and β-[beta] diversity (R^2=1, P=0.001), and greater abundances of pantothenate (0.375; P=0.04) as well as reduced abundances of glucose-6-phosphate (2.13; P=0.02) and glucose-1-phosphate (2.13; P=0.03). Fold change *Flavobacteriia* abundances were greater with increased pantothenate contrasted to reduced pantothenate (5.06; P=0.04). Greater abundances of pantothenate-producing bacteria, such as *Flavobacteriia*, may result in improved nutrient utilization in low-RFI steers. Pantothenate and/or *Flavobacteriia* may serve as potentially novel biomarkers to assess or predict feed efficiency in Black Angus steers on a backgrounding diet.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Research Introduction

The global population is expected to exceed 9 billion by the year 2050, and members of the agricultural sector are charged with ensuring adequate food supply for the global population (FAO). Beef is the most consumed red meat in the United States and the beef cattle industry contributes over $100 billion in annual retail value (USDA ERS 2015). In the beef industry, feed costs represent approximately 60% of the total input cost (Keshavan, Montanari et al.). 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 ruminant has a unique gastrointestinal system in that much of the fermentation occurs in the foregut, rather than the hindgut as in monogastric species. The main fermentation chamber in the ruminant system, the rumen, is home to billions of anaerobic microbes (Hungate). The ruminal microbes, which include fungi, bacteria, archaea, and protozoa, break down low quality feedstuffs into energy precursors for the host animal (Hungate). Ruminal microbes produce volatile fatty acids (VFA) which act as glucogenic and lipogenic precursors, vitamins, and microbial crude protein (MCP), among other nutrients, which become available to the host (hungate; Russell; stewart). The ruminal microbes supply an estimated 70% of the energy required by the host, highlighting the importance of the ruminal microbiota to overall host function. Because the ruminal microbiota are the primary contributors to feed degradation, the rumen microbiome is expected to significantly contribute to feed efficiency in beef cattle.

Several previously studies have analyzed the effects of feed efficiency on the ruminant gut microbiome, as well as physiological changes that occur because of differences in feed efficiency. A study conducted by Myer et. al. in 2015 examined the bacterial communities in steers that differed in feed efficiency (Myer et. al. 2015). The authors found differences in relative abundances of bacterial taxa among steers with divergent feed efficiency phenotypes (Myer et. al. 2015), suggesting that the rumen microbiota contribute to variance of feed efficiency phenotypes in cattle. Additional studies have observed similar trends, with identification of bacterial taxa that vary among cattle of divergent feed efficiencies, including those on different diets and physiological conditions (McCann et al 2014; Fernando et al 2010). Although in several studies differences in ruminal microbiota among cattle differing in feed efficiency, these studies have failed to move beyond correlation to causation to identify host physiological factors that are modified as a result of differences in the ruminal microbiota.

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 spectrometery (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. However, few studies have utilized untargeted metabolomics tools and technologies to investigate biochemical differences that occur as a result of feed efficiency phenotypes in cattle, particularly in beef cattle, with the exception of Artegoitia and colleagues (2017). Artegoitia et. al. performed untargeted rumen fluid metabolomics on the ruminal fluid of steers differing in feed efficiency (Artegoitia et al 2017). The authors found 33 metabolites that differed in the ruminal fluid among steers that had divergent feed efficiency phenotypes, and subsequently performed targeted metabolomics on the plasma of those steers (Artegoitia et al 2017). Differences in the abundances of several plasma fatty acids were observed among feed efficiency groups, suggesting that it may be possible to identify biomarkers of feed efficiency in cattle (Artegoitia et al 2017). Although that study was preliminary, it was the first in beef cattle to apply untargeted metabolomic techniques to addressing differences in feed efficiency phenotypes in beef cattle, and provides further evidence of physiological changes that occur as a result of differences in feed efficiency in cattle.

To date in beef cattle, most studies regarding feed efficiency have been singularly dimensional, only emphasizing one aspect of host feed efficiency, without integrating multiple components of host physiology and the ruminal microbiome using a variety of tools and technologies. The central objective of this thesis was to identify potential microbial and biochemical biomarkers of feed efficiency in Black Angus steers by incorporating novel –omics technologies such as metagenomics and metabolomics, machine learning, and traditional livestock nutritional measurements, such as body weight, rumen pH, and feed intake. This objective was achieved in three aims. The first aim examined the temporal stability of the rumen bacteriome over time following transition to a growing diet. The second aim identified serum metabolites that differed in steers of divergent feed efficiency phenotypes. The third and final aim determined several biomarkers associated with differences in feed efficiency phenotypes, including serum biochemical and ruminal microbial biomarkers.

CHAPTER I
LITERATURE REVIEW

Abstract

Beef is the third most consumed animal protein in the United States and is a popular source of protein on a global scale. In beef cattle production, feed accounts for 50 to 70% of the total cost of production. Improving feed efficiency in beef cattle would reduce feed input cost and increase animal protein supply. However, the mechanisms dictating feed efficiency in beef cattle are still not entirely known. Traditional methods of measuring feed efficiency have relied on targeting one metabolite known to be associated with macronutrients or tissue accumulation, such as urea nitrogen for protein status and non-esterified fatty acids for energy status. Although these types of measurements have been used for decades in livestock production, recent studies have revealed that urea nitrogen, glucose, and non-esterified fatty acids are poor predictors of RFI. More recent studies utilizing current technologies and techniques such as microbiomics and metabolomics, have been able to interrogate physiological and microbial differences associated with divergences in feed efficiency. Variation observed in feed efficiency phenotypes can be partially explained by differences in the rumen microbiome as well as the metabolome, but few studies have integrated these techniques to account for all dissimilarities in feed efficiency in beef cattle.

Introduction

Beef cattle are the primary red meat consumed in the United States and provide greater than $100 billion in retail value (ERS 2015). The rumen microbiome of beef cattle 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 et al. 2005). Spanning several kingdoms, including fungi, protozoa, archaea, and bacteria, the rumen microbial ecosystem fulfills several functional niches, including proteolytic, fibrolytic and lipolytic functions. Following the degradation of forages or concentrates, a several metabolites are produced and released, such as volatile fatty acids (VFA), biohydrogenated lipids, and to name a few. These microbes also provide microbial crude protein (MCP), an important source of protein in the ruminant. Given the vast nutrients the rumen microbiota provide the host, understanding the rumen microbiome is imperative for improving beef cattle production.

Divergences in the abundance and diversity rumen microbial communities are associated with a number of host phenotypes, including feed efficiency (Myer, Smith et al. 2015), diseased states (McCann, Luan et al. 2016), and methane emissions (Wallace, Rooke et al. 2015). Of particular interest is the contribution of differences in the rumen microbiome to divergences in feed efficiency phenotypes. Feed is the largest input cost in beef cattle enterprises, representing 50 to 70% of overall cost of production (Montano-Bermudez, Nielsen et al. 1990). Efficiently converting feed to animal protein could reduce input costs, improve production,

and decrease negative byproducts of inefficient metabolism, such as methane emissions (Fox, Tedeschi et al. 2001, Herd, Archer et al. 2003, Kerley, Schnabel et al. 2012). Differences in the rumen microbiome among animals with divergent feed efficiency phenotypes have been identified (McCann, Wiley et al. 2014, Myer, Smith et al. 2015). However, the cause and impact of these associative differences are not yet known. Moving beyond correlation to causation to understand the complexities of the relationship between the rumen microbiome and the host metabolism will ultimately lead to better tools to improve beef cattle production.

Feed efficiency can be defined and calculated multiple ways, but one of the optimal and most common methods for expressing feed efficiency currently is as residual feed intake (RFI), which is described further below. Feed efficiency phenotypes are a culmination of many factors, including physiological, biochemical, and microbial factors. However, few studies have integrated these approaches to provide a global perspective of feed efficiency and the factors contributing to it. This manuscript serves to review currently known factors affecting divergences in feed efficiency in ruminants and recent advances in this field.

Residual Feed Intake as a Measure of Efficiency

Residual feed intake (RFI) is a measurement of expected feed intake compared to actual feed intake. It is defined as the difference between actual feed intake and expected feed intake, in which low-RFI is the result of less feed consumption than is expected for growth or maintenance requirements without loss of production, and high-RFI is the result of greater feed consumption that is expected to meet growth or maintenance requirements (Koch, Swiger et al. 1963). Residual feed intake is utilized as a measurement of feed efficiency because it is not dependent on body weight and average daily gain (ADG), which affects their expected maintenance requirements (Koch, Swiger et al. 1963). Utilization of RFI as a measurement of feed efficiency is beneficial because animals of different production stages can be compared (Table 1). Additionally, RFI has been demonstrated to be moderately heritable, and several single nucleotide polymorphisms (SNP) have been found in relation to RFI in cattle (Herd and Bishop 2000, Barendse, Reverter et al. 2007, Sherman, Nkrumah et al. 2008). Because of these relationships, RFI is routinely used in modern agriculture as a measurement of feed efficiency.

Physiological Differences Associated with RFI

The most well understood impacts of RFI as calculations of feed efficiency are likely those associated with host physiology. Divergences in RFI in cattle are associated with differences in a variety of physiological parameters, such as protein utilization (Richardson and Herd 2004), and organic acid production (Hernandez-Sanabria, Goonewardene et al. 2010). These physiological

Table 1.1. Common methods of measuring feed efficiency in ruminants.

Name	Abbreviation	Description	Source
Feed conversion ratio	FCR	Ratio of DMI[2] to live weight gain	(Woodward, Clark et al. 1942)
Residual feed intake	RFI	Difference between actual and predicted DMI[2]	(Koch, Swiger et al. 1963)
Residual gain	RG	Difference between actual and predicted gain	(Berry and Crowley 2012)
Gross efficiency	GE	Ratio between gain and feed intake	(Salmon, Bailey et al. 1990)
Lean feed conversion ratio	LCFR	Ratio of DMI[2] to lean muscle gain	(Mrode, Smith et al. 1990)
Quadrant Intake to Gain	n/a	Bivariate distribution of low and high ADG[3] and low and high ADFI[4]	(Myer, Smith et al. 2015)[1]

[1]Earliest identified utilization of method
[2]DMI = Dry Matter Intake
[3]ADG = Average Daily Gain
[4]ADFI = Average Daily Feed Intake

differences may, in part, be explained by genotypic variance in the host ruminant, as RFI is a moderately heritable trait (Arthur, Archer et al. 2001). Additionally, ruminants with divergences in RFI may vary in their nutrient absorption and utilization, which would ultimately alter production.

Protein. Protein is required in ruminants to build muscle and perform many biological functions, and most vertebrates in general. Because of its importance, the impact of variation in protein utilization has been analyzed in the context of feed efficiency. Both blood urea and creatinine have been used to assess protein and muscle status in cattle. Blood urea nitrogen (Barendse, Reverter et al. 2007) is used as a measurement of protein degradation in steers (Cameron and Bracken 1992), creatinine is used as a measurement of muscle mass (Rennie and Millward 1983, Virgili, Maiani et al. 1994), and both blood creatinine and BUN have been examined in relation to RFI. In a study conducted by Richardson et al (2004), RFI tended to negatively correlate with BUN and was negatively correlated with creatinine for the course of the entire study, indicating that more efficient animals had greater concentrations of BUN and creatinine (Richardson and Herd 2004). In agreement with the study conducted by Richardson et. al. (2001), BUN has been shown to decrease as feed intake increases (Vercoe 1967, Berschauer, Close et al. 1983); thus, because animals with high-RFI have greater DMI, it would be expected that BUN would be negatively correlated with RFI. Although Richardson and colleagues found a, albeit weak, positive correlation between BUN and RFI, a study by Kelly et al (2009) found no association between RFI and BUN in growing beef heifers (Kelly, McGee et al. 2010), which suggests that BUN may not be an effective biomarker for RFI in cattle; however, the differences observed could be the result of sex differences as Richardson et al (2001) used steers rather than heifers.

Differences in primary nutrients, including protein, fats, and carbohydrates, provide some insight as to the functional variation in animals differing in feed efficiency. The impact of variation in protein utilization has been analyzed in the context of feed efficiency. Both blood urea and creatinine have been used to assess protein and muscle status in cattle. Blood urea nitrogen (Barendse, Reverter et al. 2007) is used as a measurement of protein degradation in steers (Cameron and Bracken 1992), creatinine is used as a measurement of muscle mass (Rennie and Millward 1983, Virgili, Maiani et al. 1994), and both blood creatinine and BUN have been examined in relation to RFI. In a study conducted by Richardson et al (2004), RFI tended to negatively correlate with BUN and was negatively correlated with creatinine for the course of the entire study, indicating that more efficient animals had greater concentrations of BUN and creatinine (Richardson and Herd 2004). In agreement with the study conducted by Richardson et. al. (2001), BUN has been shown to decrease as feed intake increases (Vercoe 1967, Berschauer, Close et al. 1983); thus, because animals with high-RFI have greater DMI, it would be expected that BUN would be negatively correlated with RFI. Although Richardson

and colleagues found a, albeit weak, positive correlation between BUN and RFI, a study by Kelly et al (2009) found no association between RFI and BUN in growing beef heifers (Kelly, McGee et al. 2010), which suggests that BUN may not be an effective biomarker for RFI in cattle; however, the differences observed could be the result of sex differences as Richardson et al (2001) used steers rather than heifers.

Previous studies, including Richardson et al (2001), have found negative correlations between creatinine and RFI. Low-RFI (more efficient) animals had greater concentrations of blood creatinine concentrations in several studies including cattle of different sexes at different production stages (Richardson and Herd 2004, Santana, Rossi Junior et al. 2013, Karisa, Thomson et al. 2014). Creatinine has been previously demonstrated to increase with increased lean muscle mass and decreased with adipose tissue deposition (Cameron and Bracken 1992, Clarke, Binnie et al. 1996). Richardson et al (2001) also found that beef cattle with greater feed efficiency also had increased lean muscle mass and decreased fat deposition compared to less efficient cattle, which is in agreement with previous studies (Arthur, Archer et al. 2001, Richardson, Herd et al. 2001). The agreement between multiple studies regarding the negative correlation between RFI and creatinine suggests it may be a viable tool in assessing protein status and utilization in beef cattle, and illustrates the physiological differences observed in ruminants with divergence RFI.

A study conducted by Chen et al (2011) analyzed global gene expression differences in bulls with divergent RFI. Chen and colleagues (2011) found that bulls with low-RFI had increased gene expression of genes involved in growth and differentiation, as well as elevated protein synthesis. Additional studies, including those conducted by Sherman et al (2008) and Barendse et al (2007) found single nucleotide polymorphisms (SNP) and quantitative trait loci (QTL) that were related to growth traits and protein utilization that differed in cattle of divergent RFI (Barendse, Reverter et al. 2007, Sherman, Nkrumah et al. 2008). These studies provide support of a genetic relationship between RFI and protein utilization in cattle; however, the extent of these relationships are yet unknown, and cannot account for all variation in cattle protein utilization.

Fat. Fats are a unique nutrient with regards to ruminants because fats are not metabolized similarly in ruminants as they are in monogastric animals. Fats that enter the rumen are typically biohydrogenated or broken down by the rumen microbiota (Jenkins 1993). In most research, analysis of fat in relation to RFI in ruminants is that of an end product, rather than as an energy source. Greater feed intake, particularly in excess of energy requirements, often results in fat deposition (Robinson and Oddy 2004). Previous studies have found a positive correlation between RFI and fat deposition, particularly subcutaneous fat deposition, in cattle and other ruminants (Herd and Bishop 2000), and negative correlations have been

identified between RFI and lean muscle mass (Cameron and Bracken 1992, Clarke, Binnie et al. 1996). Because high-RFI have greater DMI beyond that expected for maintenance or growth, this may account for the greater body fat observed in those animals. Additional studies have also illustrated a positive correlation between DMI and empty fat deposition (Richardson, Herd et al. 1998, Basarab, Price et al. 2003). Basarab and colleagues examined carcass quality of steers differing in RFI and found that steers with lower RFI had slower rates of empty body fat accretion and less intermuscular fat (Basarab, Price et al. 2003). Richardson et. al. found that steers selected for low-RFI had less whole body fat and greater protein than their high-RFI counterparts (Richardson and Herd 2004). These traits are also genetically correlated, providing additional methods for selection as well as possible explanations for divergences in RFI.

While fat is often associated with adiposity and regarded as a tissue, it also serves as a macronutrient. As a nutrient, dietary fats are predominantly hydrogenated in the rumen and absorbed in the lower gastrointestinal tract (Doreau and Ferlay 1994). The majority of fats in the rumen are free fatty acids (Harfoot 1981). Microbial species in the rumen are unique in that they are able to synthesize odd chain fatty acids, which mammals are unable to produce, but are essential in mammalian systems (Patton, McCarthy et al. 1970, Ferlay, Chabrot et al. 1993). Fatty acids are primarily absorbed in the small intestine. Long chain fatty acids (LCFA), particularly polyunsaturated fatty acids (PUFA), have a toxic effect on many of the rumen microbiota (Maia, Chaudhary et al. 2007), thus increasing their abundance in the rumen is not ideal. Because most fats that enter the rumen are hydrogenated by the rumen microbiota, most fat accumulation is either due to microbial production or the result of host lipogenesis.

Status of energy balance can be assessed using blood non-esterified fatty acid (NEFA) concentrations. Non-esterified fatty acids are mobilized from adipose tissue during times of stress or negative energy balance and can subsequently be measured in the blood (Wathes, Cheng et al. 2007). Although NEFA are frequently used in cattle enterprises as a measurement of energy balance, NEFA is typically more useful in dairy systems because beef cattle, including heifers and cows, do not undergo the same physiological stress as dairy cows during pregnancy and lactation because beef cows do not produce milk to the same extent as dairy cattle (Bellmann, Wegner et al. 2004, Pfuhl, Bellmann et al. 2007). A study conducted by Wood et al (2014) examined the relationship between NEFA and RFI in beef cows on different diets (Wood, Montanholi et al. 2014). Residual feed intake was not correlated with NEFA concentrations for cows when RFI was calculated over the whole study, but a weak negative correlation between RFI and NEFA was found when treatment was added as a covariate (Wood, Montanholi et al. 2014). However, the results of correlation between RFI in beef cows or heifers and NEFA has been mixed in other studies (Kelly, McGee et al. 2010, Lawrence, Kenny et al. 2011, Lawrence, Kenny et al. 2013). Thus, while blood NEFA may be a useful tool

in the dairy industry for assessing energy balance, using blood NEFA as a biomarker for energy balance or RFI in beef cattle is likely less effective than other possible biomarkers.

Carbohydrates. In ruminants, carbohydrates are almost entirely fermented in the rumen by anaerobic microbiota. Carbohydrates, predominantly in the form of plant fibers or concentrates, depending on diet, are fermented to volatile fatty acids (VFA), such as acetate, propionate, and butyrate, among others. These VFA provide approximately 70% of the energy by serving as glucogenic precursors for the host (Bergman 1973, Seymour, Campbell et al. 2005). Acetate primarily serves as a precursor for fats, whereas butyrate and propionate primarily serve as glucose precursors (Bergman 1990). Propionate serves as the primary glucogenic precursor for ruminants, and often is used as a metric of microbial activity in the rumen (Bergman, Roe et al. 1966, Leng, Steel et al. 1967). Production of VFA are highly dependent on diet, which dictates substrate availability for the rumen microbiota. Due to the relationship between the rumen microbiota and VFA production, the relationship between the rumen microbiota, VFA production, and RFI has been extensively explored by previous research.

The utilization and fate of VFA in ruminants has been well-studied. As previously mentioned, propionate is the major glucogenic precursor in ruminants, followed by butyrate as a glucogenic precursor (Danfær, Tetens et al. 1995). Gluconeogenesis is the main source of glucose for ruminants and occurs predominantly in the liver of cattle (Young 1977). Acetate primarily serves as a fatty acid precursor and is incorporated initially as acetyl-CoA in adipose tissue (Bergman 1990). Acetyl-CoA is also necessary for the citric acid cycle, as well as other biochemical reactions that occur in protein and lipid metabolism (Davis, Solbiati et al. 2000, Zhang, Knight et al. 2010, Shi and Tu 2015). Butyrate can be used as a precursor for other VFA, including acetate and propionate, or is commonly metabolized into 3-hydroxybutyrate (Bergman, Reid et al. 1965, Sutton, Dhanoa et al. 2003). Butyrate can be used for ketogenesis or lipogenesis (Bergman and Kon 1964, Katz and Bergman 1969). Ketogenesis in cattle typically occurs during times of great stress, such as starvation or peak lactation (Kronfeld 1971, Heitmann, Dawes et al. 1987). Utilization of butyrate occurs as a result of lack of sufficient glucose production from propionate or adipose tissue mobilization (Heitmann, Dawes et al. 1987). Although propionate, acetate, and butyrate serve various functions in host metabolism, each is important for successful production in beef systems.
Hernandez-Sanabria et al (2010) analyzed differences in ruminal VFA concentrations in relation to diet and RFI. In the study, total VFA concentration on a high energy diet was greatest in high-RFI steers, while isobutyrate was lower. In low-RFI animals, concentrations of propionate, butyrate and isovalerate did not differ as a result of high-energy compared to low-energy diet, and concentrations of propionate, valerate, and total VFA were negatively correlated with RFI (Hernandez-Sanabria, Goonewardene et al. 2010). However, other studies have

found no difference in propionate concentrations among animals differing in RFI, including a previous study conducted by Hernandez-Sanabria et al (Hernandez-Sanabria, Goonewardene et al. 2010)(Guan, Nkrumah et al. 2008, Hernandez-Sanabria, Goonewardene et al. 2010, Lam, Munro et al. 2017). Propionate is the major glucose precursor for ruminants, and consequently improving its production is desired. Unfortunately from a research perspective, when accounting for age, sex, and diet of the ruminant subjects, considering differences in propionate, or other VFA, production is difficult. While diet and inter-animal variation appear to have the greatest effect on differences in VFA production (Leng and Brett 1966, Hristov, Ivan et al. 2001, Sutton, Dhanoa et al. 2003), elucidating the mechanisms that dictate differences in VFA production in ruminants could contribute to improvements in feed efficiency.

A common measurement of host-level carbohydrate status is blood-glucose concentrations. Glucose in ruminants is tightly regulated, primarily because glucogenesis is the predominant source of glucose in ruminants. A study conducted by Kolath et al in 2006 examined the differences in plasma glucose and insulin concentrations in Angus steers classified by RFI (Kolath 2006). Plasma glucose levels were greater in high-RFI steers than low-RFI steers; however, plasma insulin concentrations and glucose:insulin ratio did not differ between low-RFI and high-RFI. The increased glucose in high-RFI steers may simply have been a function of greater feed intake, as is characteristic of high-RFI steers (Kolath 2006). The lack of observed differences in plasma insulin concentrations and the glucose:insulin ratio between low- and high-RFI steers has also been observed in other studies (Xi, Zhao et al. 2016), and other studies have not observed differences in blood glucose concentrations between cattle that varied in feed efficiency phenotype (Richardson and Herd 2004, Kelly, McGee et al. 2010). Variation in results of glucose and insulin and their relationship with RFI may have been the result of differences in sampling time and method, bodily fluid from which it was measured (e.g. serum vs. plasma), as well as age and sex of the animals used. Although some studies have suggested that serum or plasma glucose concentrations may be used as an indicator of RFI in cattle, results are mixed, and blood glucose concentrations are likely the result of other functional differences between low- and high-RFI cattle.

Gastrointestinal Microbial Differences Associated with RFI

Given the importance of the rumen and lower gastrointestinal tract microbiome to host nutrient utilization, the implications of this microbiome have been explored. Particularly, the advent of next-generation sequencing technologies have allowed deeper interrogation of the relationship between RFI, as well as other measurements of feed efficiency, and the gut microbiota.

Variation in the populations of bacteria, including variation in abundance, diversity, and individual taxa, can provide insight into the contributions of those bacteria to differences observed in RFI in cattle. Guan et. al. (2008) analyzed the dissimilarities in bacterial community profile in divergent-RFI steers on a finishing diet based on PCR-DGGE banding patterns (Guan, Nkrumah et al. 2008). The rumen bacterial signatures clustered by low- and high-RFI using polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE). It was found that the rumen bacterial communities in steers with low-RFI (greater efficiency) were more closely related to each other (91%) contrasted to steers with high-RFI (73%). Another study conducted by Hernandez-Sanabria et al (2011) found similar results using analogous methods of bacterial community analysis in animals differing in RFI. This study found too that steers fed a finishing diet had rumen bacterial communities that clustered by RFI group (Zhou, Hernandez-Sanabria et al. 2009). Similar differences have been observed in bacterial taxa and communities in other studies using various techniques for microbial interrogation and feed efficiency definition (Myer, Smith et al. 2015).

At the phylum level, Bacteroidetes and Firmicutes are the predominant bacteria identified in the rumen, often accounting for greater than 70% of the total relative bacterial abundance in the rumen. (McCann, Wiley et al. 2014, Myer, Smith et al. 2015). Members of Bacteroidetes tend to dominate the bacterial community composition when the host ruminant is fed a diet consisting of greater concentrate proportions (Myer, Smith et al. 2015) , whereas Firmicutes are often more abundant when the ruminant diet consists primarily of forage (McCann, Wiley et al. 2014). The differences in abundance under these two conditions provides insight as to the functional relationship between these two phyla. The relationship between Bacteroidetes and Firmicutes is often quantified as a Firmicutes to Bacteroidetes ratio, or vice-versa. The Firmicutes:Bacteroidetes ratio has been used to identify differences in energy utilization in humans (Ley, Turnbaugh et al. 2006), mice (Turnbaugh, Ley et al. 2006), and ruminants (Jami, White et al. 2014). However, the value of the Firmicutes:Bacteroidetes ratio is yet unknown, thus it is of interest but not of primary concern in feed efficiency studies.

Recent research has suggested that dramatic shifts in abundance of bacterial populations among animals differing in feed efficiency may not be the underlying cause of variation in feed efficiency but rather the result of lower abundant, keystone species that are functionally superior or fill a specific niche. A study conducted by Shabat et al (2016) found that more efficient cows had greater abundances of *Megasphaera elsdenii* in the rumen. *M. elsdenii* is a lactate-consuming bacterial species that is often found in association with high-grain diets due to the production of lactate by other bacteria such as *Streptococcus bovis*. The major byproducts of *M. elsdenii* include butyrate and propionate of which greater concentrations or abundances, as mentioned previously, have been associated with increased feed efficiency in ruminants (Guan, Nkrumah et al. 2008,

Hernandez-Sanabria, Goonewardene et al. 2012). In the same study by Shabat et al (2016), it was observed that less efficient animals did not have any taxa that dominated in phylogenetic annotations of genes, suggesting that possibly greater diversity or lack of dominant functionality results in decreased feed efficiency (Shabat, Sasson et al. 2016).

Another genus of interest in relation to RFI in cattle is *Prevotella*. *Prevotella* is one of the most diverse genera in the rumen, and is often the most abundant genus in the rumen (Stevenson and Weimer 2007, Jami, White et al. 2014, McCann, Wiley et al. 2014, Myer, Smith et al. 2015). Species within *Prevotella* perform a diverse range of functions, including fibrolytic, amylolytic, and proteolytic functions, and exhibit great variation at the genetic level (Wallace and BRAMMALL 1985, Wallace, McKain et al. 1993, Avguštin, Wallace et al. 1997). Greater *Prevotella* abundances in the rumen have been associated with lower feed efficiency in cattle (Carberry, Kenny et al. 2012, Myer, Smith et al. 2015). The increased abundances and functional diversity of *Prevotella* may lead to decreased efficiency in the cattle rumen. In other microbiome systems, increased diversity can be associated with negative phenotypes. In human vaginal microbiomes, increased diversity within species is associated with increased instances of preterm birth (DiGiulio, Callahan et al. 2015), and this is also observed in cattle (Laguardia-Nascimento, Branco et al. 2015). However, little is still known about the contributions of *Prevotella* or other genera towards divergences in RFI in cattle, and the studies presented provide only correlation, not causation, of taxa-level associations with RFI. In addition, other taxa have been associated with differences in feed efficiency, and interrogating the relationship between specific taxa, including those at lower abundances, and cattle that vary in feed efficiency phenotypes could provide insight as to the contributing factors causing those differing phenotypes.

Beyond individual species or genera of microbes, groups of microbes can impact feed efficiency and nutrient utilization in cattle. Archaea in the rumen are the primary generators of methane. Methanogenesis in ruminants is a highly debated topic, predominantly due to the negative impact of methane as a greenhouse gas on the environment. Methanogenesis from livestock contributes an estimated 28% to anthropomorphic greenhouse gas emissions (Beauchemin, Kreuzer et al. 2008). In addition, it is estimated that methanogensis in cattle results in a 2-12% reduction in feed efficiency (Johnson and Johnson 1995, Pen, Sar et al. 2006). Due to its conceived contribution to reductions in feed efficiency in ruminants, methane mitigation strategies have been assessed, including as they relate to the rumen microbiome. Several studies have found relationships between methane production, the rumen microbiome, and feed efficiency. A study conducted by Hernandez-Sanabria et al (2009) examined the effect of low- or high-energy diets on methanogen abundance in steers (Zhou, Hernandez-Sanabria et al. 2009). This study found that total methanogen populations did not differ between diets, nor between low- and high-RFI steers, although differences were observed at the

genus level between both diet and RFI ranking (Zhou and Hernandez-Sanabria 2010). A later study conducted by Wallace et al (2015) measured methane production and methanogen populations in steers fed two different diets, and likewise found similar results with regards to differences observed in methanogen populations related with diet (Wallace, Rooke et al. 2015). However, Wallace and colleagues found that archaeal abundances were greater in steers with greater methane emissions (Wallace, Rooke et al. 2015), which is in contrast to the study conducted by Zhou et al (Zhou, Hernandez-Sanabria et al. 2009). These mixed results provide evidence that additional research is needed regarding the effects of methanogens on methane production and the relationship between methane production and feed efficiency in cattle.

Metabolomic Differences Associated with RFI

The advent of new technologies provides avenues for deeper exploration of the relationship between the microbiome and physiology of host animals. Metabolomics provides a snapshot of the physiological and metabolic status of the subject at a point in time (Fontanesi 2016). Metabolites are molecules that can be substrates, intermediates, or products of the on-going metabolism of the body or environment. In untargeted metabolomics, well over 100 known metabolites can often be identified as well as hundreds of unknown or unidentified metabolites (Saleem, Bouatra et al. 2013). Metabolomics in the context of host-microbial analyses can provide more information than other targeted approaches, such as immunoassays, because several fold more molecules can be measured (Fontanesi 2016). Metabolomics can be measured using different techniques, including mass spectrometry (MS), gas chromatography (GC), high performance liquid chromatography (HPLC), and/or nuclear magnetic resonance (NMR) (Saleem, Bouatra et al. 2013).

Only recently has metabolomics been applied to study ruminant physiology. Primarily, untargeted metabolomics has been used to extract metabolites from ruminant blood, though to a lesser extent has been applied to other bodily fluids from ruminants, such as rumen fluid, urine, and milk (Saleem, Bouatra et al. 2013, Sun, Wang et al. 2015). Untargeted metabolomics provides researchers with the opportunity to identify hundreds of metabolites in a single sample with one extraction technique, making a more effective and efficient method of interrogating host physiology, as well as allowing for deeper exploration of host physiology (Saleem, Bouatra et al. 2013). In cattle, untargeted metabolomic techniques have predominantly been used to analyze the effects of feeds or diseased states on the metabolome of the ruminant to gain insight into the effects that these treatments or states may have on the overall physiology and health of the animal (Ametaj, Zebeli et al. 2010, Saleem, Ametaj et al. 2012). However, few studies have utilized these techniques to investigate beef cattle nutrition and host-microbiome relationships.

Serum and rumen fluid metabolomics using several different techniques has been applied to examine the relationship between ruminal acidosis and the rumen metabolome of dairy cattle. Saleem and colleagues (2012) used NMR, GC-MS, and direct flow injection tandem mass spectrometry (DFI-MS/MS) to analyze the effect of varying levels of grain on the rumen fluid metabolome in dairy cattle (Saleem, Ametaj et al. 2012). In the study, the diet containing the greatest percentage of grain at 45% exhibited the greatest difference in the rumen metabolome as depicted by a principal component analysis, and had increased biogenic amines and changes in the concentrations of ethanol and ethanolamine (Saleem, Ametaj et al. 2012). The increase in biogenic amines at the higher grain content is likely the result of decarboxylation of amino acids by rumen microbes, which can occur at lower pH (Hill and Mangan 1964, Rice and Koehler 1976). This example provide evidence that it may be possible to identify metabolites that occur as a result of changes in microbial activity within the rumen, including as those changes relate to host physiology. Although the previously discussed research in dairy cattle is interesting, much of this has yet to be demonstrated in beef cattle. Because beef cattle differ physiologically from dairy cattle, the information gathered from dairy systems cannot necessarily be translated directly to beef systems, requiring additional research in beef cattle systems (Hart, Bines et al. 1975, Rowlands 1980, Pfuhl, Bellmann et al. 2007).

Recently, metabolomic techniques have been applied to a beef cattle system to characterize the rumen metabolome of animals exhibiting different feed efficiency phenotypes. A study conducted by Artegoitia et al (2017) examined the effect of feed efficiency status on the rumen and plasma metabolome (Artegoitia, Foote et al. 2017). To date, this is the only study using LC-MS to perform untargeted metabolomics on rumen fluid to assess differences in feed efficiency in beef cattle. After multiple test correction and use of false discovery rate (FDR) p-values, 33 metabolites differed between more and less efficient steers from the rumen fluid. Of these 33 metabolites, the authors chose several fatty acids to target in plasma as possible biomarkers for feed efficiency. The authors found that pentadecanoic acid was the only fatty acid that differed between low- and high-efficiency steers; however, when several fatty acids, including pentadecanoic acid, palmitic, linoleic, and α-linolenic acid, were used in combination, the authors were able to more effectively delineate low from high efficiency steers. The relationship between the rumen and blood metabolomes has been suggested by previous research in dairy cattle; yet, the association between the rumen and blood metabolomes are still poorly understood (Saleem, Bouatra et al. 2013). Because the rumen is more difficult to access, particularly in a production setting, identifying connections between the rumen and blood environments may allow researchers and producers to use blood samples, which are much more easily accessible on-farm, to assess rumen function and efficiency. The study conducted by Artegoitia and colleagues provides key evidence that it may be possible to use metabolomic biomarkers to identify, and possibly select for, more efficient animals. However, much more work

needs to be conducted in order to determine which metabolites may make appropriate biomarkers.

Conclusions and Future Directions

Given the importance of beef as a commodity in the United States and the potential to serve as an animal protein for an expected global population of greater than 9 million people by the year 2050 (FAO 2016), it is imperative to find and develop novel methods to improve production without increasing feed inputs, the greenhouse gas footprint, and land-use. Improvements in feed efficiency may lead to decreased methane emissions, lower input costs, as well as higher quality carcasses, among other benefits. Recent emphasis has been placed on microbial and molecular differences that occur in cattle that differ in feed efficiency phenotypes; however, the majority of these studies have failed to move beyond simple correlation to causation of host feed efficiency phenotypes. Cross-disciplinary research will provide novel insights into the relationships that mediate feed efficiency phenotypes in cattle, including microbiomics, metabolomics, and integration of physiological measurements.

CHAPTER II
TEMPORAL STABILITY OF THE RUMINAL BACTERIAL COMMUNITIES IN STEERS

A version of this chapter will be originally published by Brooke A Clemmons, Emily A Melchior, Mallory M Embree, Liesel Schenider, Cameron Martino, and Phillip R Myer:

"Temporal Stability of the Rumen Bacterial Community in Black Angus Steers." *Microbial Ecology* (in review).

This manuscript is undergoing peer review in the journal Microbial Ecology. BA Clemmons participated in experimental design, sample and data collection, sample and data analysis, and was primarily responsible for manuscript preparation. EA Melchior assisted in sample collection and manuscript preparation. L Schneider assisted with data analysis and manuscript preparation. MM Embree, C Martino, and PR Myer contributed to experimental design, data collection and analysis, and manuscript preparation.

Abstract

The rumen microbiome is critical in ruminant nutrition and contributes to nutrient utilization and feed efficiency in cattle. Nutritional studies in ruminants historically relied on limited wash-out periods between treatments and diet acclimation but few studies have accounted for the importance of the rumen microbiome, its stability over time, and its contribution to study results. Because of these critical gaps, the objective of this study was to interrogate the effects of diet changes on rumen bacterial stability and diversity over time. Fifty Black Angus steers of 7 months of age, weighing 264 ± 2.7 kg were acclimated to the GrowSafe© feeding system for 10d prior to intake measurement, and fed a step-up receiving diet 14d before receiving a growing ration. Steers were maintained on the diet for 70d. Weekly, rumen content was sampled via gastric tubing. Genomic DNA was extracted from rumen content and 16S rRNA amplicon sequencing was performed. Closed compositions were produced using a centered log-ratio transformation. Random Forests supervised machine learning and feature selection was performed on the raw sequencing counts. Normality was assessed with the UNIVARIATE procedure in SAS 9.4. Diversity metrics did not follow a normal distribution and were analyzed using the Kruskal-Wallis H test. Shannon's Diversity Index was greatest at the beginning of the trial (4.66±0.32), but decreased overall by the end of the trial (3.61±0.14; P=0.003). Observed OTU also was greater during the first week (160.14±12.83) compared to the final week (57.9±2.5; P<0.001). The β-diversity was greatest at the beginning of the trial but decreased by the end of the trial (R2=1; P=0.001). Three orders contributed to the shift to the final bacteriome, including Pasteurellales, Aeromonadales, and Bacteriodales, which occurred by week 4 (P≤0.1). Several physiological factors were predictive of the rumen bacteriome. Rumen pH was correlated with α-diversity (P=0.005) at week 5 and predictive of rumen bacteriome signature at week 10 (R^2=0.48; P=0.04). In this study, although the shift towards a final bacteriome began at approximately week 4, the rumen bacteriome did not stabilize until at least week 9 following the

transition to new feed. When conducting feeding and nutritional trials in ruminants with regard to the rumen microbiome, additional consideration must be addressed to account for sufficient time for the stabilization of the rumen microbiome.

Introduction

The composition, structure, and function of the rumen microbiome in cattle is critical to the host's health and nutrition, as these microbes are responsible for the breakdown of low-quality feedstuffs into energy substrates that can subsequently be utilized by the ruminant. Research continues to demonstrate that minor shifts in ruminal microbial taxa or abundances of specific microbes impacts livestock productivity. The rumen and gastrointestinal microbiomes in cattle have been linked to numerous aspects of host physiology and function, such as health (Khafipour, Li et al. 2009), nutrition (Patel, Patel et al. 2014), feed efficiency (Myer, Freetly et al. 2017), and management (Meale, Li et al. 2016). Such research has been critical in understanding the role of the rumen microbiome in cattle production.

Due to the complex network of the rumen microbial community and its interaction with the host and ruminal fermentation and metabolism, diet stands as an important factor in ruminal microbiome composition and structure (de Menezes, Lewis et al. 2011, Klevenhusen, Petri et al. 2017). For example, cattle fed an exclusively forage diet have a distinct microbial profile from that of a high-grain diet, which is also reflected within core operational taxonomic units (OTU)(Petri, Schwaiger et al. 2013). These outcomes are a direct result of the complexity of available substrates within the feed. Factors such as diet drive the day-to-day variation in microbiome stability, however, understanding the long-term variation in the ruminal microbiome is critical to maintaining ecological and functional equilibrium.

Thus far, the majority of research aimed at determining the influence of the microbiome on cattle production or the influence of cattle production on the ruminal microbiome has been conducted by examining short-term, end point sampling or single periods of sample collection. These single-point analyses have provided valuable insight to the influence of the rumen microbiome on livestock production but may confound the interpretation of study results and conclusions. Variation of the ruminal microbiota between animals is considerable (Ross, Moate et al. 2012, Myer, Smith et al. 2015), and point samples may be not be satisfactory to adequately define an existing state within a population. Importantly, studies examining bovine nutrition with regard to the ruminal microbiome routinely rely on differences in diet or diet transitions, where the length of the study is defined by traditional nutritional parameters and historical data, not taking into account microbial acclimation to the study ration. The ruminal microbial temporal stability following such perturbations has yet to be determined. Thus, diet acclimation prior

to experiments, diet re-acclimation among experimental periods, and other dietary changes in cattle gut microbiome studies may confound microbial characterization when temporal variation and stability are not taken into consideration. These patterns of variation may have significant implications when aiming to determine relationships between the gut microbiome and nutrition in cattle.

As microbial community stability following dietary changes may impact the length and results of in vivo research trials, as well as sampling frequency and interval, in this study, we examined the temporal diversity and stability of the ruminal bacteriome in cattle following diet transition. The objective of this study was to determine bacterial community diversity and composition following diet transition and the duration required to achieve restoration of microbial stability.

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.

Animal experimental design and sample collection. Fifty steers of approximately seven months of age were maintained at the Plateau Research and Education Unit in Crossville, TN. The steers weighed 264 ± 2.7 kg at the beginning of the trial. The steers grazed on cool-season grasses until being transferred to the GrowSafe© system for a 14d adaptation period. Animals were placed on a step-up diet during the 14d adaptation period and transitioned to a growing diet (11.57% crude protein and 76.93% total digestible nutrients on a dry matter basis) with 28 mg monensin/kg DM. A 70d feed efficiency trial was administered following the acclimation period. Weekly, body weight (BW) via chute scale, rumen fluid samples via gastric tubing, and blood samples via coccygeal venipuncture were collected (Krysl and Hess 1993). Approximately 100mL of rumen content were transferred to 50mL conical tubes, rumen content pH determined, and samples stored at -80°C. Feed intake was continually monitored via the GrowSafe© system throughout the 70d feed efficiency trial.

DNA extraction and amplification. Rumen samples were centrifuged at 4,000 rpm for 15 min, the supernatant was decanted and 0.5 mL was aliquoted for DNA extraction using the PowerViral® Environmental RNA/DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). The 16S rRNA gene was amplified using 27F (Lane 1991) and 534R (Muyzer, De Waal et al. 1993) primers modified for Illumina sequencing, following standard protocols Q5® High-Fidelity DNA Polymerase (New England Biolabs, Inc., Ipswich, MA, USA). Following amplification, PCR products were verified with a standard agarose gel electrophoresis and purified using AMPure XP bead (Beckman Coulter, Brea, CA, USA). The purified amplicon library was quantified and sequenced on the MiSeq

Platform (Illumina, San Diego, CA, USA) according to standard protocols (Flores, Caporaso et al. 2014). Raw fastq read were de-multiplexed on the MiSeq Platform (Illumina, San Diego, CA, USA)

Phylogenetic analysis. All raw sequencing data was trimmed of adapter sequences and phred33 quality filtered at a cutoff of 20 using Trim Galore (Krueger 2015). All remaining sequences were then filtered for PhiX, low-complexity reads and cross-talk (Edgar 2016). 16S taxonomic sequence clustering and classification was performed with the USEARCH's UNOISE and SINTAX(v10.0.240) (Edgar and Flyvbjerg 2015, Edgar 2016) with the RDP 16S rRNA database (Cole, Wang et al. 2013). Each sample was filtered for sequencing depth at a minimum of 2,000 reads per sample (Jovel, Patterson et al. 2016).

Statistical analysis. Downstream analysis was performed in python. PCoA was performed on Bray-Curtis distances and statistical significance was assessed through Analysis of Similarities (ANOSIM) (CLARKE 1993). Alpha diversity was measured both in dominance (Hammer, Harper et al. 2001) and singletons. OptSpace, a spectral based manifold optimized matrix completion (Keshavan, Montanari et al. 2010) was performed with a rank of 2 and converged in 30 iterations. Compositional normalization was performed through the centered log-ratio transform. Completed OTU tables were then sorted in rows (OTUs) through spectral clustering (Pentney and Meila 2005). Regression based analysis was performed through Ordinary Least Squares (OLS) regression (Shi, Zhang et al. 2016). Feature selection and supervised machine learning was performed on completed data through Random Forests (Breiman 2001). Matrix completion was performed through DEICODE (Martino and Morton 2017), compositional statistics through gneiss (Morton, Sanders et al. 2017), PCoA (bray-curtis) dimensionality reduction through scikit bio (http://scikit-bio.org/), data wrangling through pandas (McKinney 2011), visualization through seaborn (Waskom, Botvinnik et al. 2016) and matplotlib (Hunter 2007), OLS regression was performed through statsmodels (Seabold and Perktold 2010), and machine learning and feature selection through scikit learn (Pedregosa, Varoquaux et al. 2011).

Other measurements of α-diversity, including equitability, Simpson's Evenness E, Shannon's Diversity Index, and Observed OTU, were assessed for normality using SAS 9.4 using the PROC UNIVARIATE command (SAS Institute, Cary, NC). All variables were found to follow a non-normal distribution, and were analyzed using Wilcoxon Rank Sum and Kruskal Wallis test.

Results

A total number of 500 samples underwent microbial DNA extraction. Bacterial community composition was determined by amplifying and sequencing the V1-V3 hypervariable region of the 16S rRNA gene. A total of 21734148 sequences

remained following quality control and chimera removal. An average of 48048 ± 41628 sequences were present in each sample. Average number of sequences per week are presented in Table 2.1.

After binning at 97% similarity, a total number of 21401 OTU were detected. Average number of OTU per week are presented in Table 2.1. Alpha-diversity was measured using, equitability, Simpson's Evenness, chao1, Shannon's Diversity Index, and observed OTU (Table 2.1). Good's coverage was also measured to ensure satisfactory coverage of OTU for each week (Table 2.1). Shannon's Diversity Index was greatest at the beginning of the trial (4.66±0.32), but decreased overall by the end of the trial (3.61±0.14; P<0.0001; Table 2.1). Observed OTU also was greater during the first week (160.14±12.83) compared to the final week (57.9±2.5; P<0.0001, Table 2.1). Equitability was greatest during week 1 of the trial (0.66±0.03), fluctuated throughout the trial, but was lower at week 10 (0.61±0.02; P<0.0001). Whereas several metrics related to richness increased from week 1 to week 10, overall evenness fluctuated greatly (P<0.0001) but was numerically similar by the end of the trial (0.12±0.02) to the first week of the trial (0.13±0.01; Table 2.1). Greater phylogenetic diversity was observed at week 5 compared to week 1, but decreased by week 10 as evidence by spatial coclustering (Figure 2.1). The rumen bacteriome began to shift at week and reached stabilization by week 10 (Figure 2.2). Three orders were identified as changing significantly during the shift to the final bacteriome, including Pasteurellales, Aeromonadales, and Bacteriodales. The shift to the final bacterial community composition began to occur at week 4 (Figure 2.3). Several physiological factors were predictive of the rumen bacteriome. Rumen pH was correlated with α-diversity (P=0.005; Figure 2.4A) at week 5 and predictive of rumen bacteriome signature at week 10 (R^2=0.48; P=0.04; Figure 2.4B).

Discussion

Beef cattle are a critical source of protein on a global scale, and of particular interest from both basic and applied research perspectives due to the nature of ruminal. The rumen microbiome produces an estimated 70% of the energy precursors required by the host beef cattle, predominantly due to the microbial conversion of low-quality feedstuffs to glucogenic precursors (Young 1977). These glucogenic precursors, such as organic fatty acids including acetate and propionate, are subsequently absorbed by the animal. Nutritional studies involving beef cattle have historically relied on short adaptation and "wash-out" periods to diets or treatments. However, the research provided in this and other studies have demonstrated that short-duration dietary acclimation may not be sufficient time in order for the rumen microbiome to stabilize, which could influence the outcome of the trial, particularly as it relates the rumen microbiome and nutritional conclusions.

Table 2.1. Sequencing and diversity metrics of the rumen bacteriome for each week

Metric Week	Equitability[a]	Simpson's E[a]	Observed OTU[a]	Good's[a]	Chao1[a]	Shannon's Diversity Index[a]
1	0.66±0.03	0.12±0.02	151.08±9.27	0.92±0.04	153.14±9.26	4.71±0.22
2	0.58±0.02	0.14±0.03	101.31±6.42	0.94±0.04	102.11±6.41	3.62±0.16
3	0.51±0.03	0.07±0.02	125.12±8.92	0.94±0.04	126.85±8.9	3.39±0.17
4	0.61±0.02	0.09±0.01	89.25±2.32	0.99±0.00	89.34±2.29	3.97±0.11
5	0.52±0.02	0.03±0.00	301.17±7.97	0.98±0.02	315.63±8.06	4.27±0.14
6	0.59±0.02	0.14±0.01	45.60±1.64	0.99±0.00	45.63±1.64	3.22±0.11
7	0.60±0.01	0.11±0.01	74.29±2.75	0.99±0.00	74.38±2.75	3.74±0.10
8	0.61±0.01	0.10±0.01	94.92±1.87	0.99±0.00	94.48±1.86	4.02±0.10
9	0.59±0.03	0.10±0.01	84.06±2.97	0.96±0.03	84.23±2.97	3.83±0.16
10	0.61±0.02	0.13±0.01	60.48±1.74	0.99±0.00	60.66±1.66	3.58±0.11
P value	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

[a]Mean±SEM

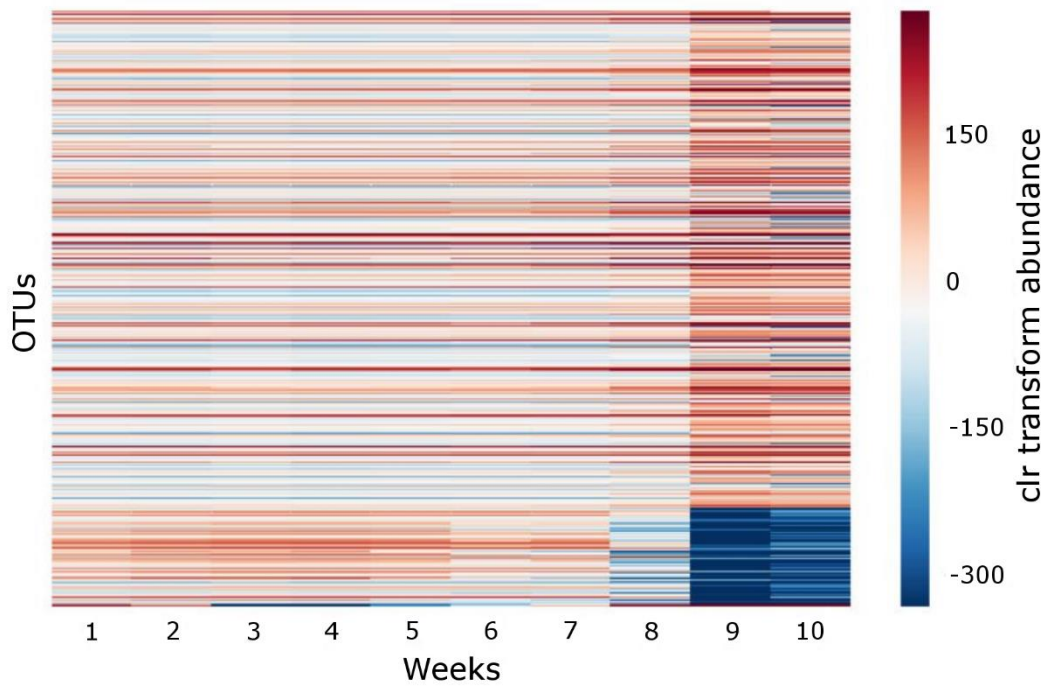

Figure 2.1. Spectral coclustering of composite average of OTU over time, indicating major microbial successions throughout the study in the rumen bacteriome.

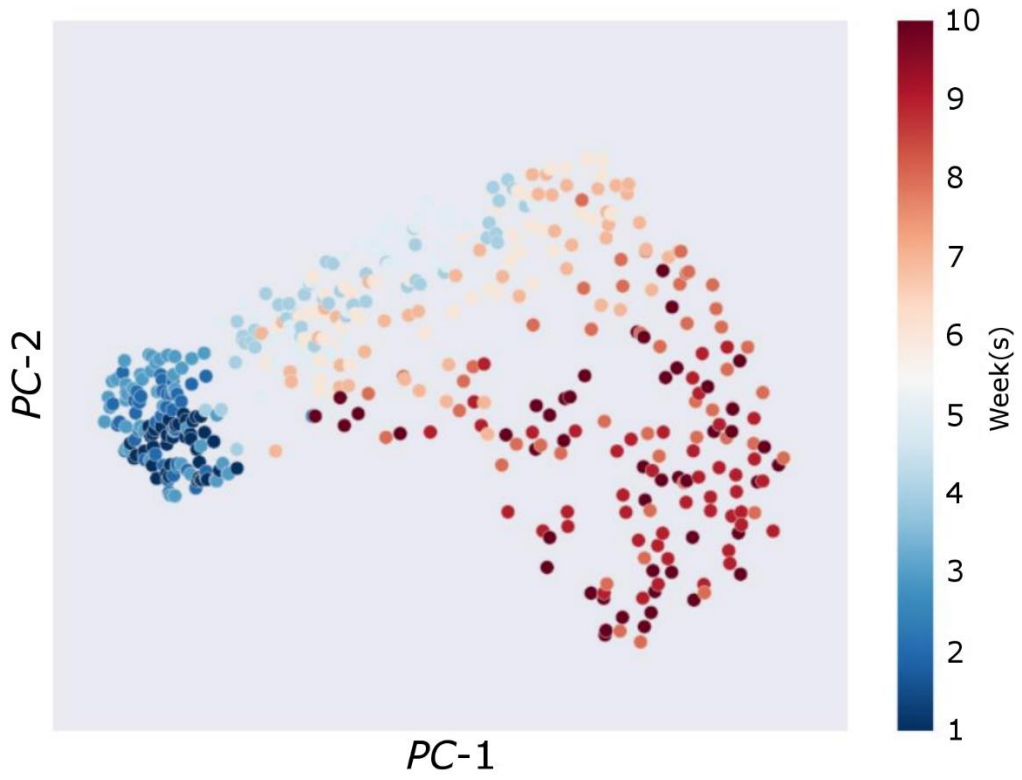

Figure 2.2. Principal coordinate analysis (PCoA) based on Bray-Curtis distances among the ruminal bacterial communities throughout the study. Weekly *n*=50.

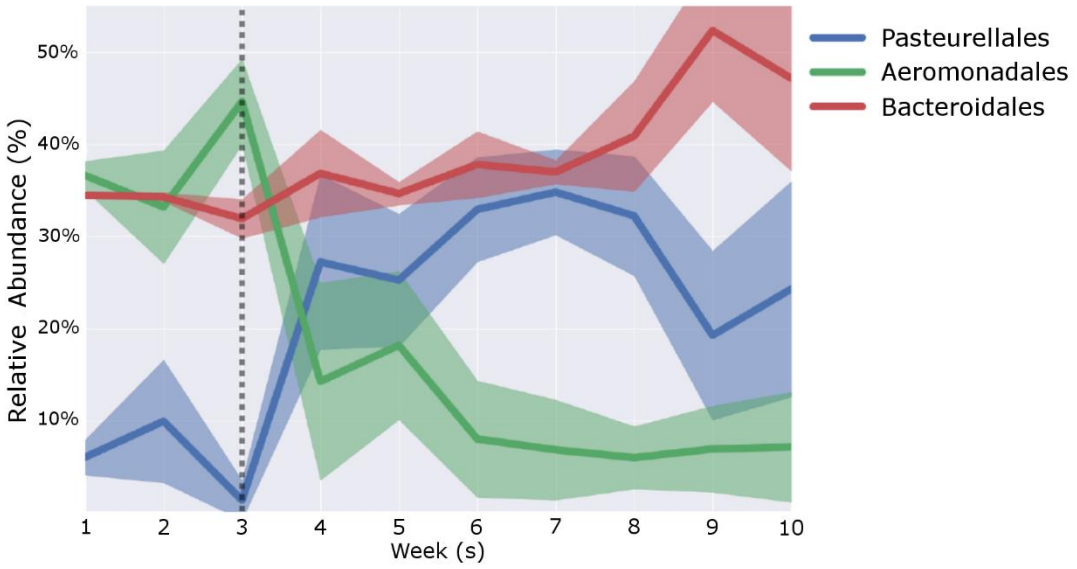

Figure 2.3. Relative abundance of three bacterial orders driving the shift in bacterial community composition throughout the 10-week trial. Shaded regions indicate SEM.

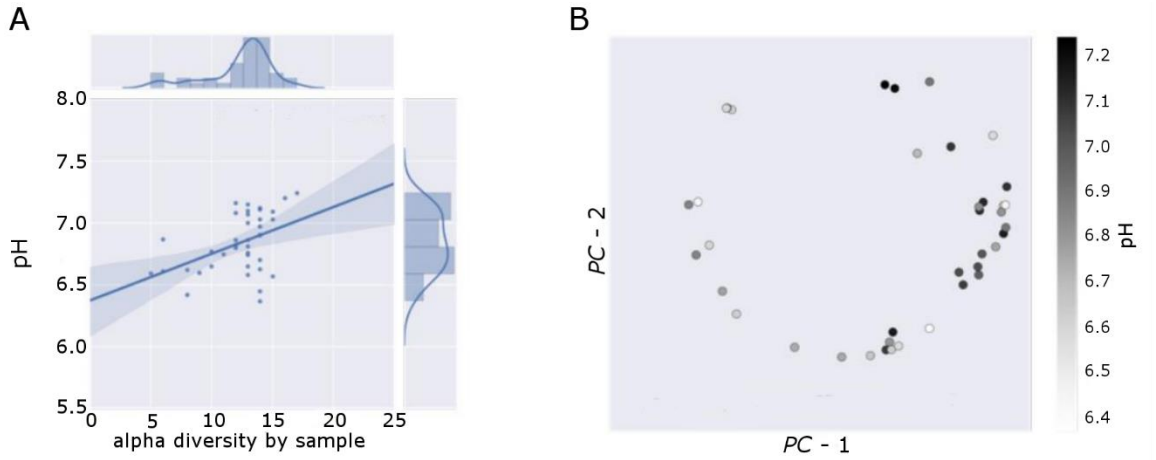

Figure 2.4. Rumen pH is an important factor in establishing bacterial community structure in week 5. A) Rumen α-diversity correlated with pH (Pearson R=0.44; P=0.0051) B) Rumen pH is predictive of bacterial community structure at week 5 (R^2=0.48; P=0.04).

In this study, the diversity of the rumen bacterial community was greatest at the start of the trial, following a field-standard two week adaptation period to the growing diet and was greatly variable throughout much of the trial. Rumen bacterial community diversity was lowest by the end of the trial, at ten weeks following the adaptation period. The rumen bacterial diversity was expectedly greatest at the start of the trial following the adaptation period, but reached stability by ten weeks. Other studies have established that diet is one of the greatest contributors to differences in the gastrointestinal microbiome (Petri, Schwaiger et al. 2013, David, Maurice et al. 2014, Henderson, Cox et al. 2015). The transition from a predominantly forage-based diet to a diet incorporating concentrates causes a shift in bacterial taxa due to changes in substrate type (Tajima, Aminov et al. 2001, Fernando, Purvis et al. 2010). These differences in nutrient availability to the microbes may have resulted in a state of microbial community dysbiosis as bacterial populations competed for nutritional sources, increased functional redundancy occurred, and the physical environment of the rumen, such as pH, changed (Whittaker 1972).

Besides individual animal variation, diet is one of the greatest contributing factors to rumen microbial diversity (Henderson, Cox et al. 2015). Dietary transitions, adaptation periods, and wash-out periods are often incorporated into nutritional studies, including those involving the rumen microbiome (Brown, Ponce et al. 2006). These adaptation and wash-out periods have historically spanned anywhere from several days to four weeks, which previous studies have suggested is adequate time for acclimation, as reviewed by Brown et. al. (2006) (Brown, Ponce et al. 2006) and supported by recent nutritional microbial studies (Anderson, Schneider et al. 2016) However, in this study, it took approximately ten weeks for the rumen bacteriome to stabilize, a time frame much longer than traditionally utilized for adaptation or wash-out periods. Although cattle may physically acclimate to feed within a two week period, the results of this study suggest the rumen microbiome requires additional time to stabilize. At week 4 of this study, three orders appeared to drive the shift to a stable bacterial community composition, including Aeromonadales, Pasteurellales, and Bacteroidales. Two of these orders, Aeromonadales and Pasteurellales, belong to the phylum Proteobacteria, and Bacteroidales belongs to the phylum Bacteroidetes. Proteobacteria and Bacteroidetes are typically two of the three most prevalent bacterial phyla found in the rumen but bacteria belonging to the phylum Firmicutes are frequently found to be the most abundant in the rumen, followed by Bacteroidetes and Proteobacteria in cattle on a primarily forage-based diet (Fernando, Purvis et al. 2010, Pitta, Pinchak et al. 2010, McCann, Wiley et al. 2014). However, as cattle transition to incorporate more readily-fermentable feedstuffs, Bacteroidetes becomes the dominant phylum (Fernando, Purvis et al. 2010, Myer, Smith et al. 2015). This information may suggest that, although members of Proteobacteria are typically less abundant in the rumen overall, those microbes may play a larger role in transition of the rumen microbiome from a forage

to concentrate diet (Fernando, Purvis et al. 2010, Chen, Penner et al. 2011). Proteobacteria are a large and greatly diverse phylum, further suggesting that low abundances of a variety of bacterial species may contribute significantly to divergences in host phenotypes in ruminants (Mukhopadhya, Hansen et al. 2012)

The changes in abundances of these orders also provides additional insight given their function in the rumen. At week 4, relative abundance of Aeromonadales sharply decreased, whereas Bacteroidales and Pasteurellales both increased at week 4. Some rumen microbes belonging to Aeromonadales, including *Ruminobacter* and *Succinovibrio,* are fibrolytic in nature and found in conjunction with high-fiber diets (Bryant and Small 1956, Bryant 1973), which may account for the decrease in abundance of those microbes as the rumen microbiome adapted to the more readily digestible diet. Genera found in Bacteroidales and Pasteurellales, including *Prevotella* and *Actinobacillus*, respectively, are important for digestion of protein and carbohydrates (Avguštin, Wallace et al. 1997, Wallace, McKain et al. 1997, Guettler, Rumler et al. 1999). Rapid production of byproducts of metabolism, such as organic acids, by Bacteroidales and Pasteurellales may be responsible for the shift in pH seen in week 5, which was also indicative of rumen microbiome signature (Avguštin, Wallace et al. 1997, Guettler, Rumler et al. 1999, Fernando, Purvis et al. 2010). The diet fed in this study included feedstuffs that are more readily fermentable, which result can cause decreases in rumen pH due to increased production of organic acids such as lactate (Goad, Goad et al. 1998). This can shift the bacterial community composition towards those bacteria that are more tolerant of low pH, including those in Bacteroidales and Pasteurellales (Fernando, Purvis et al. 2010, McCann, Luan et al. 2016).

Conclusion

Although previous studies have suggested that a two week adaptation period may be adequate for nutritional studies in ruminants, in this study, the rumen microbiome began to shift towards a stable community four weeks following the transition to the new diet and continued to stabilize in the subsequent five weeks. In nutritional studies involving cattle, a two week adaptation and/or wash-out periods may not permit sufficient time for the rumen microbiome to stabilize. Instability of the rumen microbiome will likely influence nutritional and physiological production, and appropriately, study results. Thus, consideration should be given in the experimental design to account for ruminal stability.

CHAPTER III
SERUM METABOLITES ASSOCIATION WITH FEED EFFICIENCY IN BLACK ANGUS STEERS

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Abstract

Improving feed utilization in cattle is required to reduce input costs, increase production, and ultimately improve sustainability of the beef cattle industry. Characterizing metabolic differences between efficient and non-efficient animals will allow stakeholders to identify more efficient cattle during backgrounding.This study used an untargeted metabolomics approach to determine differences in serum metabolites between animals of low and high residual feed intake.Residual feed intake was determined for 50 purebred Angus steers and 29 steers were selected for the study steers based on low vs high feed efficiency. Blood samples were collected from steers and analyzed using untargeted metabolomics via mass spectrometry. Metabolite data was analyzed using Metaboanalyst, visualized using orthogonal partial least squares discriminant analysis, and p-values derived from permutation testing. Non-esterified fatty acids, urea nitrogen, and glucose were measured using commercially available calorimetric assay kits. Differences in metabolites measured were grouped by residual feed intake was measured using one-way analysis of variance in SAS 9.4.Four metabolites were found to be associated with differences in feed efficiency. No differences were found in other serum metabolites, including serum urea nitrogen, non-esterified fatty acids, and glucose.Four metabolites that differed between low and high residual feed intake have important functions related to nutrient utilization, among other functions, in cattle. This information will allow identification of more efficient steers during backgrounding.

Introduction

Improving the feed efficiency of cattle is vital to increase the amount of beef produced per unit of feed, thus increasing production and decreasing input costs for producers in the United States. Feed is the largest cost to producers in the beef industry, representing approximately 60% of overall cost of production (Norton 2005; Holmgren and Feuz 2015). As resources become more limited and feed costs increase, the overall cost of production is likely to grow in subsequent decades (Lawrence et al. 2008). Therefore, the selection and optimization of economically important phenotypes, such as feed efficiency, must be evaluated.

Feed efficiency is moderately heritable (Berry and Crowley 2013), and genetic selection has been used to improve the efficiency of beef herds (Arthur et al. 2001). However, non-genetic factors such as the gut microbiome (Myer 2017) and the environment (Mader and Davis 2004) also contribute to efficiency. Additionally, feed intake and body weight gain, key components of efficiency, are themselves polygenic traits with complex patterns of inheritance (Elzo et al. 2012). Methods beyond genetic selection are therefore needed to optimize efficiency in beef cattle. Biochemically, efficiency results from composite changes in metabolism of macronutrients that increase the yield of energy from feed. Markers of this variation in metabolism that results in difference in efficiency could therefore be used as phenotypic traits for selection. However, relatively little is known at the molecular level about the basis for efficiency.

Metabolomics is a tool that produces a snapshot of cellular metabolism by comprehensively profiling metabolite abundance in biological samples. Accordingly, metabolomic profiling provides insight into nutrient utilization in humans and animals. It provides finer resolution of factors affecting phenotypic variation in growth and physiological parameters (Fontanesi 2016). Because metabolites are the result of combined endogenous and exogenous production, metabolomic studies reveal information beyond that provided solely from genetics or genomics, revealing relationships between animal genetics and physiological phenotypes (Fontanesi 2016). With respect to beef cattle, metabolomics is a dual-purpose tool that can both increase the fundamental understanding of efficiency and identify metabolites that are potential biomarkers for selection.

While previous studies have focused on phenotypic or genetic selection for feed efficient animals, understanding molecular-level changes that distinguishes animals that utilize feed more efficiently could provide valuable insight for the selection of efficient animals. In this study, we hypothesized that steers that differ in feed efficiency would have different levels of metabolites associated with nutrient utilization. The objective was to determine serum metabolites that differed between low and high feed efficient animals, utilizing untargeted metabolomics.

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.

Animal experimental design and sample collection. Fifty purebred Angus steers were used in this study. Steers from the University of Tennessee Institute of Agriculture Plateau Research and Education Center (Lamprecht and Williams) in Crossville, TN 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. 1963). The average value of RFI (actual dry matter intake (DMI) vs. expected DMI) and standard deviation (Elzo et al.) were calculated for individual animals and were divided into two groups of low (n = 14), and high (n = 15) RFI. High RFI was defined as RFI ≥ 0.5 SD above the mean; and Low RFI was RFI ≤ −0.5 SD below the mean.

Weekly, approximately 9 mL of blood was collected from the coccygeal vein into serum separator tubes (Corvac, Kendall Health Care, St. Louis, MO) and centrifuged at 4°C for 20 minutes at 2,000xg. Serum was transferred via aspiration to plastic tubes and frozen at -80°C for further analysis.

LC-MS analysis. Serum samples (50 µl) from each steer were extracted for metabolomic analysis using 0.1% formic acid in acetonitrile:water:methanol (2:2:1), as described previously (Kamphorst et al. 2011). Metabolites were separated using a Synergy Hydro-RP column (100 x 2 mm, 2.5 µm particle size). Mobile phases consisted of A: 97:3 H2O:MeOH with 11 mM tributylamine and 15 mM acetic acid and B: MeOH. The 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. Flow rate was set to a constant 0.200 mL/min and the column temperature was kept at 25 °C. The autosampler tray was kept at 4 °C and 10 µL of sample was injected into the Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific, Waltham, MA). Electrospray ionization was used to introduce the samples into an Exactive Plus Orbitrap MS (Thermo Fisher Scientific, Waltham, MA), using an established method (Kamphorst et al. 2011; Lu et al. 2010).

Data analysis. Raw files obtained from Xcalibur MS software (Thermo Electron Corp., Waltham, MA) were converted into the mzML format using ProteoWizard (Chambers, Maclean et al. 2012). The converted files were imported into MAVEN (Metabolomic Analysis and Visualization Engine for LC-MS Data), a software package (Clasquin, Melamud et al. 2012). Peaks for the known metabolites were picked in MAVEN, which automatically performs non-linear retention time correction and calculates peak areas across samples, using a preliminary mass error of ±20 ppm and retention time window of five min. The UTK Biological and Small Molecule Mass Spectrometry Core (BSMMSC) has replicated and expanded the method of Rabinowitz and coworkers (Lu, Clasquin et al. 2010) and final metabolite annotations were made using a library of 263 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 two min of the expected retention time, and the metabolite mass had to be within ±5 ppm of the expected value. Metabolite identities were confirmed using the MAVEN software package (Clasquin, Melamud et al. 2012), and peak areas for each compound were integrated using the Quan Browser function of the Xcalibur MS Software (Thermo Electron Corp., Waltham, MA). Metabolomics data were pre-processed and analyzed using Metaboanalyst (Xia, Sinelnikov et al. 2015). Variables with missing values for > 20% of samples were removed from the dataset prior to statistical analyses. Missing data values were imputed using K-Nearest Neighbors (Stacklies, Redestig et al. 2007). Peak areas were normalized by median values, transformed logarithmically, and scaled using Pareto scaling prior to statistical testing. Metabolites differing significantly ($P< 0.05$) between low- and high-RFI steers were identified using t-test. Multiple testing was addressed by setting a false discovery rate of 5%, using the method of Benjamini-Hochberg (Benjamini 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-PLS-DA model. Validity of model estimates was evaluated using permutation testing (van Velzen, 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.

Serum samples were analyzed using a 96-well EPOCH 2 microplate reader (BioTek Instruments, Winooski, VT) with commercial kits for non-esterified fatty acids (NEFA; Wako Chemicals, Richmond, VA; sensitivity of 0.01 mmol/L), glucose (Thermo Electron Corp., Waltham, MA; sensitivity of 0.3 mg/dL), and serum urea nitrogen (SUN; Thermo Electron Corp., Waltham, MA; sensitivity of 2.0 mg/dL). The intra- and interassay CV were, respectively, 4.08% and 3.73% for serum NEFA, 0.53% and 4.31 % for serum glucose, 0.30% and 0.35% for SUN.

Measurements were tested for normality using the PROC UNIVARIATE command in SAS 9.4 (SAS Institute, Cary, NC) and did not follow normal distribution. Non-parametric one way ANOVA was calculated using Wilcoxon rank-sum test in SAS 9.4. Statistical significance was set at $P \leq 0.05$.

Results

A total of 109 known metabolites were identified in serum samples from low- and high-RFI steers. Multivariate analysis (O-PLS-DA) was used to visualize the extent to which serum metabolomes discriminated steers with low vs. high RFI. As shown in Figure 3.1, steers tended to separate according to RFI, based on lack of overlap of Hotelling's 95% confidence intervals. Statistically significant differences in metabolite abundance between the two groups of animals were identified by t-test. After controlling for multiple testing, four metabolites (homocysteine, pantothenate, carnitine and glutamate) differed significantly between low- and high-RFI steers (FDR < 0.05; Fig. 3.2). Each of the four metabolites was present at lower levels in serum of high- vs. low-RFI animals (Table 3.1).

Circulating serum glucose, NEFA, and SUN concentrations did not differ significantly between low- and high-RFI steers ($P \geq 0.05$; Table 3.2).

Discussion

Understanding the mechanisms driving the variation in feed efficiency in animals of similar genetic backgrounds could lead to substantial innovations in agriculture. Complex phenotypes such as feed efficiency are attributed to many confounding factors that are not completely understood. We hypothesized that steers differing in feed efficiency during a backgrounding phase would produce different levels of metabolites associated with nutrient utilization. Using LC-MS to perform untargeted metabolomics, it was found that metabolites differed between low- and high-RFI steers were pantothenate, carnitine, homocysteine, glutamine (Table 1), while no differences were observed in circulating serum NEFA, SUN, or glucose concentrations between the two groups ($P > 0.05$). Delineating discrepancies in metabolite production in steers of dissimilar feed efficiencies may provide critical information critical for understanding factors associated with diverse feed efficiency phenotypes.

Pantothenate (Seal et al.), or vitamin B5, was greater in low-RFI steers. Ruminants get PA in several ways; via absorption directly from feed, endogenous synthesis, or microbial production (Wegner et al. 1940). PA is required by all living organisms as it is necessary for the production of coenzyme A (Ball 2006; Bender 2003; Pietrzik et al. 2008; Begley et al. 2001; McGinn et al. 2004). Coenzyme A is key in intermediary metabolism, necessary for many amino acid, carbohydrate, and fat metabolism reactions (Leonardi et al. 2005). This coenzyme typically acts as a

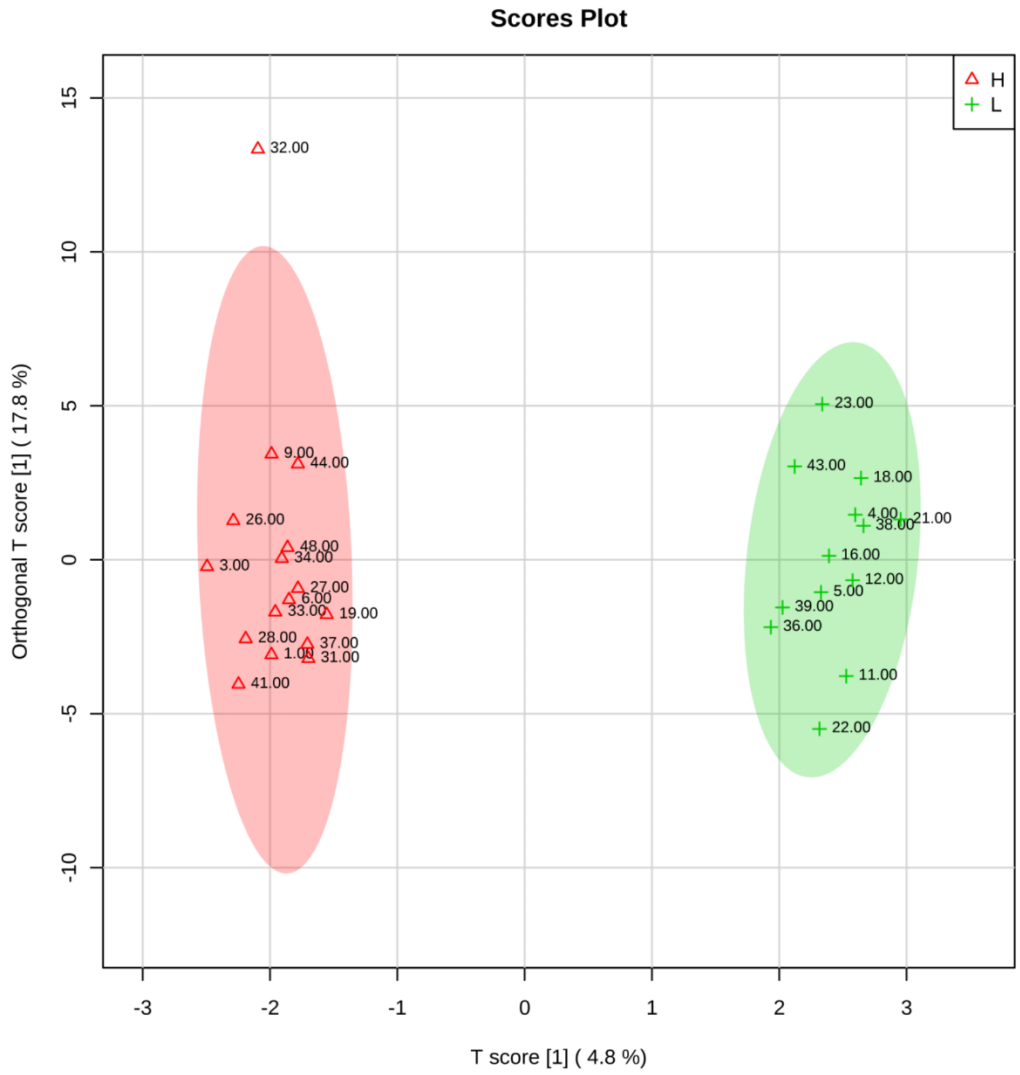

Figure 3.1. 2D score plot from orthogonal PLS-DA of serum metabolomes from low (green plus symbol) and high (red triangle) RFI steers. Model fitting based on R^2Y (0.98) and Q^2 (0.41) was validated using permutation testing (P = 0.004 and 0.058, respectively) using 1000 permutations. Ellipses represent 95% confidence intervals.

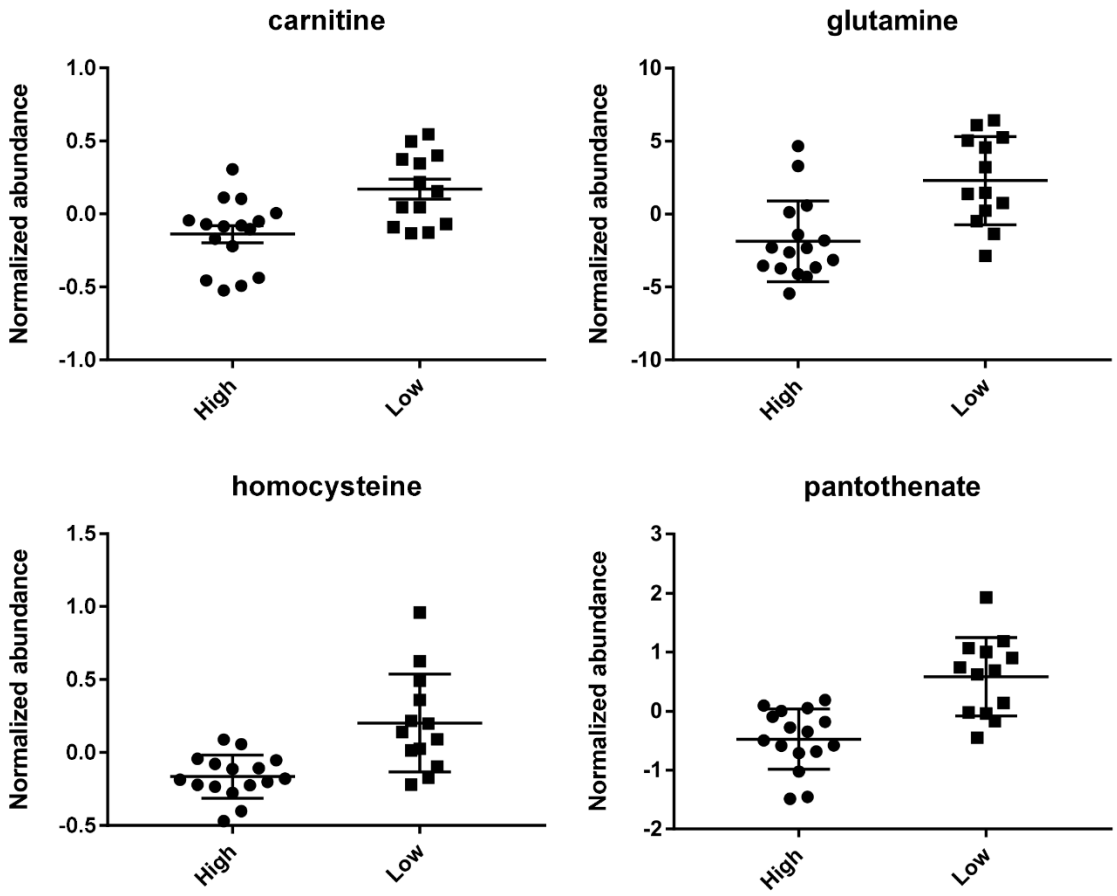

Figure 3.2. Dot plot representation of carnitine, glutamine, homocysteine and pantothenate levels in low- and high-RFI steers. Data values shown were normalized using median normalization and pareto scaling. Horizontal lines represent mean ± standard deviation of normalized data.

Table 3.1. Blood serum metabolites associated with high- and low-RFI steers at week 10.

Metabolite	**FDR *P* value[1]**	**Fold Change (High/Low RFI)[2]**
Pantothenate	0.009586	0.7635
Homocysteine	0.01677	0.7610
Glutamine	0.01707	0.7496
Carnitine	0.08253	0.7220

[1] FDR controlled by the method of Benjamini-Hochberg
[2] Based on normalized data

Table 3.2. Concentrations of serum NEFA, glucose, and SUN

Metabolite	Low-RFI Concentrations	High-RFI Concentrations	P-value
NEFA[a]	262.6±15.24	263.9±9.28	0.1970
Glucose[b]	47.48±7.47	39.43±3.47	0.2845
SUN[b]	2.14±0.19	2.15±0.20	0.4614

[a] measured as mmol/L
[b] measured as mg/dL

metabolism, respectively (Leonardi et al. 2005). Acetyl-CoA introduces an acetate group into the tricarboxylic acid (Morton, Sanders et al.) cycle, while acyl-CoA assists with transport of fatty acids into and out of mitochondria (Begley et al. 2001; Leonardi et al. 2005). Coenzyme A can also be esterified to propionate, the most common glucogenic precursor in healthy and normally functioning cattle (Yost et al. 1977). Propionyl-CoA is converted to succinyl-CoA through a series of enzymatic reactions, which can then enter the TCA cycle (Young 1977). Increased coenzyme A acts as an inhibitory agent in the formation of coenzyme A from PA, but carnitine can reverse this inhibition (Miller et al. 2001). Because carnitine was also greater in low-RFI steers, it may help to counteract the inhibitory processes of the increased concentrations of PA or coenzyme A (Miller et al. 2001). Ruminants are unique in that gluconeogenesis is the primary source of glucose in the ruminant (Bergman et al. 1974; Young 1977; Freetly and Klindt 1996), compared to the non-ruminant, which relies on direct glucose absorption from the diet (Young 1977; Nafikov and Beitz 2007). In amphibians and monogastric species, supplementation of PA results in increased muscle performance, though results are inconsistent (Shock and Sebrell 1944; Smith et al. 1987; Nice et al. 1984; Litoff et al. 1985; Miller et al. 2001). Although physiology of monogastric and ruminant species differ greatly, increased concentrations of PA may increase muscle performance and growth of animals (Karasov and Douglas 2013).

Carnitine concentrations were elevated in low-RFI steers. Carnitine provides several important functions related to metabolism in the body, including import of long chain fatty acids (LCFA) into the mitochondria for β-oxidation via interaction with the coenzyme A attached to the fatty acid (Bremer 1983). Carnitine is also involved in controlling the acetyl-CoA to coenzyme A ratio, transporting long and medium chain fatty acids into the mitochondria, and mediating energy-associated molecules (Arslan 2006). Carnitine is synthesized endogenously, but several precursors and cofactors are required, including lysine, methionine, vitamin C, niacin, vitamin B12, choline, and reduced iron (Bremer 1983). Increasing carnitine in dairy cow hepatic cells stimulated LCFA β-oxidation in vitro (Drackley et al. 1991), and this has been demonstrated in vivo in other species, including beef cattle (Cetin et al. 2003; R. Greenwood et al. 2010). Carnitine supplementation in vivo also increased plasma glucose concentrations (Cetin et al. 2003; R. H. Greenwood et al. 2001). Increased carnitine and subsequent metabolism may result in more efficient nutrient utilization (Karisa et al. 2014). In our study, though no significant differences were reported between and low- and high-RFI steers in serum glucose concentrations, low-RFI steers had numerically greater glucose concentrations than high-RFI steers. Greater carnitine levels may have led to increased concentration of glucose in the steers in this study, though additional studies should be conducted to confirm.

Low-RFI steers also had greater concentrations of homocysteine than high-RFI steers. Homocysteine is an intermediate metabolite in the interconversion pathway

of methionine and cysteine and concentrations differ based on physiological need (Lehninger 1977). Homocysteine supplementation increases methionine synthase, the enzyme responsible for conversion of homocysteine to methionine, in vitro (Ortiou et al. 2004). Abomasal infusion of homocysteine increased plasma concentrations of methionine in wethers (Amos et al. 1974). Methionine is required for protein synthesis and other metabolic processes in ruminants (Seymour 2016; Lehninger 1977) and can be an indicator of energy balance in cattle (Pedernera et al. 2010). Methionine is usually the first limiting amino acid, particularly in growing steers (Richardson and Hatfield 1978; Titgemeyer and Merchen 1990). Though no differences were observed between low- and high-RFI steers in methionine or cysteine using the methods described in this study, greater concentrations of homocysteine may indicate increased metabolic reactions involving methionine as a methyl donor (Ditscheid et al. 2005) or cysteine. It could also indicate more substrate availability for methionine-dependent reactions (Amos et al. 1974; Ortiou et al. 2004).

Greater concentrations of glutamine were measured in low-RFI steers. In livestock species, the goal of production is to promote muscle synthesis to increase available animal protein for consumption. In growing ruminants, glutamine may act as an anabolic mediator, increasing muscle growth (Lobley et al. 2001); at the very least, it has been linked to decreased catabolism of muscle (Reecy et al. 1996). Glutamine synthetase, the enzyme responsible for synthesizing glutamine from glutamate and ammonia, increases during time of backgrounding and weaning in steers, but decreases during the finishing stage (Matthews et al. 2016). This could be indicative of decreased need for glutamate for muscle growth, which is responsible for approximately half of circulating glutamine (Matthews et al. 2016). Glutamine is a very potent cause of protein swelling, which leads to protein synthesis (Bequette 2003). Glutamine is also a gluconeogenic amino acid, and it has been hypothesized that greater circulating levels of some gluconeogenic amino acids may moderate apparent energy status of beef cattle, and affect feed intake (Karisa et al. 2014).

A lack of differences in glucose, NEFA, SUN was unexpected given their use as markers of health and nutrient status in cattle (Adewuyi et al. 2005; Ørskov et al. 1999; Gleghorn et al. 2004). Attempts to correlate NEFA, SUN, and glucose with other metabolites, such as carnitine, have yielded inconsistent results (Adewuyi et al. 2005; R. H. Greenwood et al. 2001; Kelly et al. 2010). Glucose is highly regulated in ruminants and is constantly being produced via gluconeogenesis (Young 1977). Glucose concentrations can remain the same in growing steers regardless of diet (Seal et al. 2007). The tight regulation of glucose in ruminants may explain the lack of differences found between low- and high-RFI steers in this study. NEFA concentrations are used to infer information about the nutrient status of cattle (Bowden 1971). NEFA in blood is a result of mobilization of adipose tissue, particularly during times of fasting or high energy requirements, such as lactation

(Bowden 1971; Reid and Hinks 1962). However, because the steers used in this study were growing steers, they would have little need to mobilize adipose tissue and would have lower concentrations of NEFA in serum. SUN is used in ruminants and other production species as an indicator of protein intake (Preston et al. 1965), nitrogen utilization (Egan and Kellaway 1971; Kohn et al. 2005), and nitrogen intake (Nolan et al. 1970). It is currently regarded as one of the most effective measurements of protein status in ruminants (Herdt 2000). In dairy cows, SUN concentrations increase after feeding (Miettinen and Juvonen 1990). Blood sample collections occurred prior to morning feeding in this study, which may partially account for the decreased concentrations in SUN.

Based on the findings of this study, Black Angus beef steers differing in feed efficiency exhibit varying levels of serum metabolites associated with nutrient utilization and energy status. The majority of the metabolites that were found to be in greater blood circulation in low-RFI steers are associated with energy usage, including pantothenate, glutamine, carnitine, and homocysteine. In addition, these four metabolites are intimately related, as they either inhibit or facilitate nutrient metabolism reactions. Determining causes of divergence in the production of these metabolites may, in part, account for some of the variation in feed efficiency of growing Black Angus beef steers. Subsequent analyses utilizing different techniques, such as transcriptomics, could bridge the gap between correlation and causation. Additional analyses should also be conducted across breeds and species to determine metabolite biomarkers that may be unique to those animals. Further understanding of the mechanisms driving these trends will result in improved nutrient utilization, increased feed efficiency, and reduction of production costs.

CHAPTER IV
MICROBIAL AND BIOCHEMICAL DYNAMICS MEDIATING FEED EFFICIENCY PHENOTYPES IN BEEF CATTLE

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This article is undergoing peer review in the journal Frontiers in Microbiology. BA Clemmons contributed to experimental design, sample collection and processing, data analysis, and was responsible for primary preparation of the manuscript. JB Powers, SR Campagna, and BH Voy assisted with metabolomics sample processing and data collection, as well as assisted in preparation of manuscript. EA Melchior, PR Myer assisted with sample collection and analysis, as well as manuscript preparation. MM Embree, BH Voy, and PR Myer developed experimental design and manuscript preparation. C Martino contributed to sample and data processing and analysis, as well as manuscript preparation.

Abstract

As the global population is expected to exceed 9 billion people by 2050, finding novel methods of improving food production is imperative. The rumen microbiome is critical in ruminant nutrition and contributes to nutrient utilization and feed efficiency in cattle. Therefore, the objective of this study was to interrogate microbial and biochemical factors affecting divergences in feed efficiency in Black Angus steers. Fifty Black Angus steers of 7 months of age, weighing 264 ± 2.7 kg were acclimated to the GrowSafe© feeding system for 10d prior to intake measurement, and fed a step-up receiving diet 14d before receiving a growing ration (11.57% CP and 76.93% TDN DM) with 28 mg monensin/kg DM. Steers were maintained on the diet for 70d. Weekly BW was measured, serum collected, and rumen content was obtained via gastric tubing. Based on performance and FI measured from 0 to 70d, the average RFI was calculated and steers were divided into low- (n=14) and high-RFI (n=15) groups based on 0.5 SD below and above the mean RFI, respectively. Untargeted serum metabolomics was conducted utilizing the Dionex UltiMate 3000 UPLC system and electrospray ionization was used to introduce the samples into an Exactive Plus Orbitrap MS. Genomic DNA was extracted from rumen content and the amplified V1-V3 hypervariable region of the bacterial 16S rRNA gene was sequenced for analyses. Missing values were approximated through matrix completion and data was normalized using a centered log-ratio transformation. Random Forests supervised machine learning and feature selection was performed on the bacterial compositions. Residual feed intake was associated with several attributes of the rumen bacteriome. Low-RFI steers were associated with decreased bacterial α- (P=0.03) and β- diversity (R^2=1, P=0.001). Several serum metabolites were associated with RFI. Based on

fold change (high/low RFI), low-RFI steers had greater abundances of pantothenate (0.375; *P*=0.04) and reduced abundances of glucose-6-phosphate (2.13; *P* = 0.02) and glucose-1-phosphate (2.13; *P*=0.03). Machine learning on RFI was highly predictive of both serum metabolomic signature and rumen bacterial composition (accuracy ≥0.7). Fold change *Flavobacteriia* abundances were greater with increased pantothenate contrasted to reduced pantothenate (5.06; *P*=0.04). Greater abundances of pantothenate-producing bacteria, such as *Flavobacteriia*, may result in improved nutrient utilization in low-RFI steers. Pantothenate and/or *Flavobacteriia* may serve as potentially novel biomarkers to assess or predict feed efficiency in Black Angus steers on a backgrounding diet.

Introduction

The United States is the largest producer of beef, and the beef industry accounts for a retail equivalent of $105 billion (ERS 2015). Over the next decade, demand for US exportation of beef is expected to increase (USDA 2017
). Given the rapid reduction in natural resources and substantial growth of the human population expected in the coming decades, it is imperative to develop novel agricultural approaches in order to increase the global food supply with limited resources (Alexandratos and Bruinsma 2012). Ruminants, including beef cattle, rely on the fermentation of feedstuffs to provide energy for the animal. The rumen microbiome in cattle is fundamental for the successful conversion of plant matter to energy substrates for the animal via fermentation (Hungate 1966). This microbiome also supplies the host animal with other important nutrients such as vitamins and protein (Hungate 1966). Identifying and exploiting factors that affect the efficiency of this conversion in beef cattle will result in increased animal protein supply without increasing input resources.

Rumen microbes produce metabolites that are released into the rumen lumen and can be absorbed through the rumen epithelium or through the epithelium in the lower gastrointestinal tract (Hungate 1966). The rumen microbes are responsible for the production of approximately 70% of the energy supply to the ruminant, including production of organic acids such as acetate and propionate (Seymour, Campbell et al. 2005). Differences in the production of these metabolites as well as variation in rate and quantity of absorption can contribute to divergences in nutrient utilization and efficiency of the ruminants, and may lead to physiological or phenotypic changes (Huntington 1990, Okine and Mathison 1991). However, it can be difficult to distinguish the origin of many metabolites between those of endogenous origin and metabolites of microbial origin. Although associations between the rumen microbiome and physiological changes in the host have been identified (Hungate 1975, Fernando, Purvis et al. 2010), it has yet to be determined the mechanisms driving these changes and whether foundational, or keystone, species are responsible for the divergences in feed efficiency and other phenotypes.

In order to address these critical knowledge gaps, we used a combination of microbial genomics, metabolomics, and bioinformatics to further define variations in feed efficiency as determined by the divergences in residual feed intake (RFI). Determination of the complex associations and networks between the rumen microbiome, host metabolome, and differences in host phenotypecan be facilitated by novel utilization of bioinformatics and machine learning to discover physiological patterns and identifying microbial factors. By taking a multidisciplinary approach, researchers can move beyond correlation to identify causation accounting for differences in host phenotype.

The objective of this study was to determine the microbial and biomarkers mediating variation in feed efficiency in cattle. To accomplish this we analyzed the relationship among RFI, the rumen bacteriome, and serum metabolome in order to identify potential biomarkers for feed efficiency in beef steers.

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.

Animal experimental set up and sample collection. Fifty weaned steers of approximately 7 months of age were housed at the Plateau Research and Education Center in Crossville, TN. Animals weighed 264 ± 2.7 kg at the beginning of the study and transitioned to a backgrounding diet (11.57% crude protein and 76.93% total digestible nutrients with 28 mg monensin/kg on a dry matter basis) for 14 days prior to the start of the trial. Steers were adapted to the GrowSafe© system during that adaptation period. Body weight (BW) was measured at 7 day intervals and daily feed intake measured using the GrowSafe© system for the length of the 70 day feed efficiency trial. Feed efficiency was determined using RFI (Koch, Swiger et al. 1963). At the conclusion of the trial, steers were ranked based on RFI. Low- or high-RFI was determined as 0.5 SD below or above the mean RFI, respectively.

Weekly, approximately 9mL of blood was sampled via venipuncture from the coccygeal vein into serum separator tubes (Corvac, Kendall Health Care, St. Louis, MO). Blood samples were centrifuged at 2,000x*g* for 20 min at 4°C. Serum was decanted into 5mL plastic culture tubes and stored at -80°C for further analyses.

DNA extraction and amplification. Rumen samples were centrifuged at 4,000 rpm for 15 min, the supernatant was decanted and 0.5 mL was aliquoted for DNA extraction using the PowerViral® Environmental RNA/DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). The 16S rRNA gene was amplified using 27F (Lane 1991) and 534R (Muyzer, De Waal et al. 1993) modified for Illumina

sequencing following standard protocols Q5® High-Fidelity DNA Polymerase (New England Biolabs, Inc., Ipswich, MA, USA). Following amplification, PCR products were verified with a 2% agarose gel electrophoresis and purified using AMPure XP bead (Beckman Coulter, Brea, CA, USA). The purified amplicon library was quantified and sequenced on the MiSeq Platform (Illumina, San Diego, CA, USA) according to standard protocols (Flores, Caporaso et al. 2014). Raw fastq reads were de-multiplexed on the MiSeq Platform (Illumina, San Diego, CA, USA).

Phylogenetic analysis. All raw sequencing data was trimmed of adapter sequences and phred33quality filtered at a cutoff of 20 using Trim Galore(Krueger 2015). All remaining sequences were then filtered for PhiX, low-complexity reads and cross-talk(Edgar 2016). 16S taxonomic sequence clustering and classification was performed with USEARCH UNOISE and SINTAX(v10.0.240) (Edgar and Flyvbjerg 2015, Edgar 2016)with the RDP 16S rRNA database(Cole, Wang et al. 2013). Each sample was filtered for sequencing depth at a minimum of 2,000 reads per sample(Jovel, Patterson et al. 2016).

LC-MS analysis. Serum samples (50 µL) from each steer were extracted for metabolomic analysis using 0.1% formic acid in acetonitrile:water:methanol (2:2:1), as described previous (Kamphorst, Fan et al. 2011). Metabolites were separated using a Synergy Hydro-RP column (100 ×2 mm, 2.5 µm particle size). Mobile phases consisted of A: 97:3 H2O:MeOH with 11 mM tributylamine and 15 mM acetic acid and B: MeOH. The 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. Flow rate was set to a constant 0.200 mL/min and the column temperature remained at 25°C. The autosampler tray was maintained at 4°C and 10 µL of sample was injected into the Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific, Waltham, MA). Electrospray ionization was used to introduce the samples into an Exactive Plus Orbitrap MS (Thermo Fisher Scientific, Waltham, MA), using an established method (Lu, Clasquin et al. 2010, Kamphorst, Fan et al. 2011).

Raw files obtained from Xcalibur MS software (Thermo Electron Corp., Waltham, MA) were converted into the mzML format using ProteoWizard (Chambers, Maclean et al.). The converted files were imported into MAVEN (Metabolomic Analysis and Visualization Engine for LC-MS Data), a software package (Clasquin, Melamud et al.). Peaks for the known metabolites were picked in MAVEN, which automatically performs non-linear retention time correction and calculates peak areas across samples, using a preliminary mass error of ±20 ppm and retention time window of five min. The UTK Biological and Small Molecule Mass Spectrometry Core (BSMMSC) has replicated and expanded the method of Rabinowitz and coworkers (Lu, Clasquin et al.) and final metabolite annotations were made using a library of 263 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 two min of the expected retention time, and the metabolite mass had to be within ±5 ppm of the expected value. Metabolite identities were confirmed using the MAVEN software package (Clasquin, Melamud et al.), and peak areas for each compound were integrated using the Quan Browser function of the Xcalibur MS Software (Thermo Electron Corp., Waltham, MA).

Statistical analysis. Downstream analysis was performed in python. PCoA was performed on Bray-Curtis distances and statistical significance was assessed through Analysis of Similarities (ANOSIM)(CLARKE 1993). Alpha diversity was measured both in dominance (Hammer, Harper et al. 2001) and singletons. OptSpace, a spectral based manifold optimized matrix completion (Keshavan, Montanari et al. 2010) was performed with a rank of 2 and converged in 30 iterations. Compositional normalization was performed through the centered log-ratio transform. Completed OTU tables were then sorted in rows (OTUs) through spectral clustering (Pentney and Meila 2005). Regression based analysis was performed through Ordinary Least Squares (OLS) regression (Shi, Zhang et al. 2016). Feature selection and supervised machine learning was performed on completed data through Random Forests (Breiman 2001). Matrix completion was performed through DEICODE (Martino and Morton 2017), compositional statistics through gneiss (Morton, Sanders et al. 2017), PCoA (bray-curtis) dimensionality reduction through scikit bio (http://scikit-bio.org/), data wrangling through pandas (McKinney 2011), visualization through seaborn (Waskom, Botvinnik et al. 2016) and matplotlib (Hunter 2007), ols regression was performed through statsmodels (Seabold and Perktold 2010), and machine learning and feature selection through scikit learn (Pedregosa, Varoquaux et al. 2011).

Other measurements of α-diversity, including equitability, Simpson's Evenness E, Shannon's Diversity Index, and Observed OTU, were assessed for normality using SAS 9.4 (SAS Institute, Cary, NC). All variables were found to follow a non-normal distribution, and were analyzed using Wilcoxon Rank Sum and Kruskal Wallis test.

Results

Sequencing information. A total number of 50 samples underwent microbial DNA extraction. Bacterial community composition was determined by amplifying and sequencing the V1-V3 hypervariable region of the 16S rRNA gene. 21734148 sequences were present following quality control and chimera removal. An average of 48048 ± 41628 sequences were present in each sample .

Bacterial community diversity. After binning reads at 97% similarity, a total of 21401 OTU were detected. Alpha-diversity was measured by number of singletons, equitability, Simpson's Evenness, observed OTU, Good's coverage,

chao1, and Shannon's Diversity Index. With the exception of number of singletons, α-diversity metrics did not differ between low- and high-RFI steers at the end of the study (Table 4.1). The number of singletons was greater in high-RFI steers than low-RFI steers (p=0.03; Table 4.1). Other α-diversity metrics did not differ between high- and low-RFI steers, including equitability (p=0.24; Table 4.1), Simpson's Evenness (p=0.19; Table 4.1), Observed OTU (p=0.78; Table 4.1), Good's coverage (p=0.14; Table 4.1), chao1 (p=0.78; Table 4.1), and Shannon's Diversity Index (p=0.07; Table 4.1). Phylogenetic diversity of the rumen bacterial communities also occurred over time (R^2=1, p=0.001,Figure 4.1A), with two distinct communities arising (Figure 1B).

Serum metabolites associated with RFI. A total of 109 known metabolites were identified. Residual feed intake was predictive of serum metabolomic signature and rumen bacteriome signature at week 10. Glucose-1-phosphate (p=0.03; Figure 4.2) and glucose-6-phosphate (p=0.02; Figure 4.2) differed between low- and high-RFI steers. Several other serum metabolites were predictive of rumen bacterial community structure, including pantothenate, aconitate, succinate, 2-hydroxy-2-methylsuccinate, allantoin, homocysteic acid, citate/isocitrate, and cytosine (Figure 4.3). Serum pantothenate abundance was the greatest predictor of the rumen bacterial community composition, and was also found to be significantly different between low- and high-RFI steers (p=0.04; Figure 4.3; Figure 4.4A).

Microbial and biochemical predictors of RFI. In addition to serum pantothenate abundance as a predictor of rumen bacterial community composition, panothenate abundances were also associated with a class of rumen bacteria, Flavobacteriia. Flavobacteriia were predictive of pantothenate abundance in serum (prediction accuracy = X). Mean Flavobacteriia abundance differed between steers with low and high pantothenate abundances (p=0.04; Figure 4.4B).

Discussion

Total beef consumption in the United States is greater than 20 billion pounds annually, and is the most consumed red meat product in the United States (ERS 2015); however, declining land resources and increasing human populations place pressure on producers to improve production efficiency. Therefore, researchers and producers are charged with finding novel approaches and methods for reducing inputs while increasing animal protein supply to meet the needs of an expected global population exceeding 9 billion people by the year 2050 (Alexandratos and Bruinsma 2012). Given this need, targeting phenotypes that improve efficiencies, such a feed and reproductive efficiencies, or reduce negative environmental impacts, including methane production and excess nitrogen release, will ultimately improve beef and livestock agriculture on a global scale.

Table 4.1. Sequence and alpha-diversity statistics of the 16S rRNA gene sequences for bacterial populations in low- and high-RFI steers.

	Low-RFI[a]	**High-RFI[a]**	***P*-value[b]**
Equitability	0.596±0.040	0.650±0.015	0.239
Simpson's Evenness	0.142±0.032	0.141±0.011	0.190
Observed OTU	55.9±4.62	59.9±2.43	0.777
Good's Coverage	0.985±0.015	0.999±0.001	0.140
Chao1	56.5±4.27	59.9±2.43	0.777
Shannon's Diversity Index	3.38±0.245	3.83±0.108	0.074

[a]Mean±SEM
[b]Significance determined at α ≤0.05

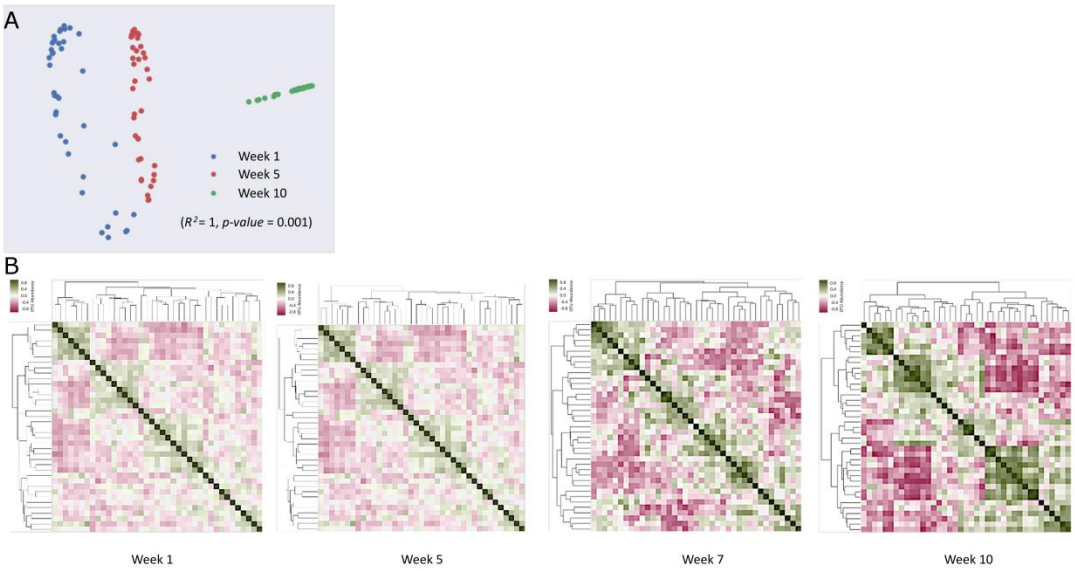

Figure 4.1. Phylogenetic diversity of rumen bacterial communities A) Phylogenetic diversity across all samples decreases as rumen microbiome adapts to the diet B) Two distinct bacterial communities diverge by week 10 of the study

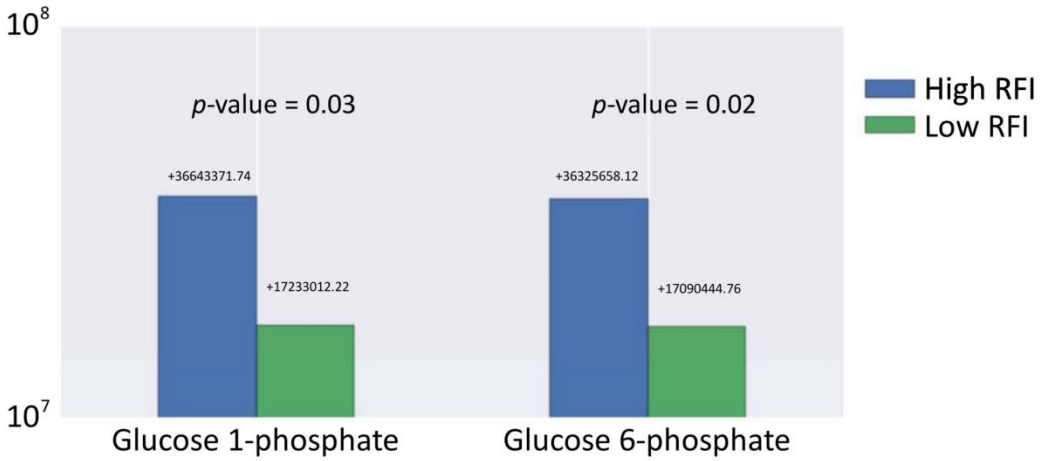

Figure 4.2. Mean predictive compounds between low- and high-RFI. Greater glucose-1-phosphate and glucose-6-phosphate abundances are indicators of high-RFI.

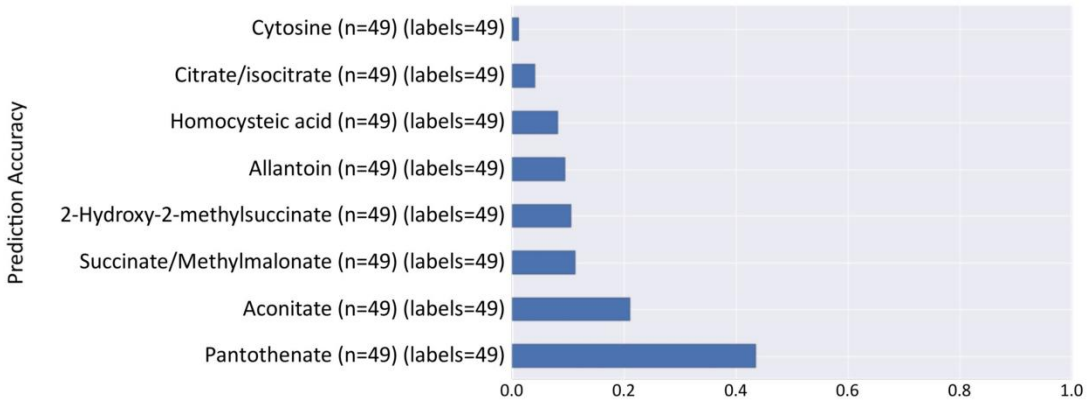

Figure 4.3. Serum metabolic signatures correlated to week 10 bacterial community composition.

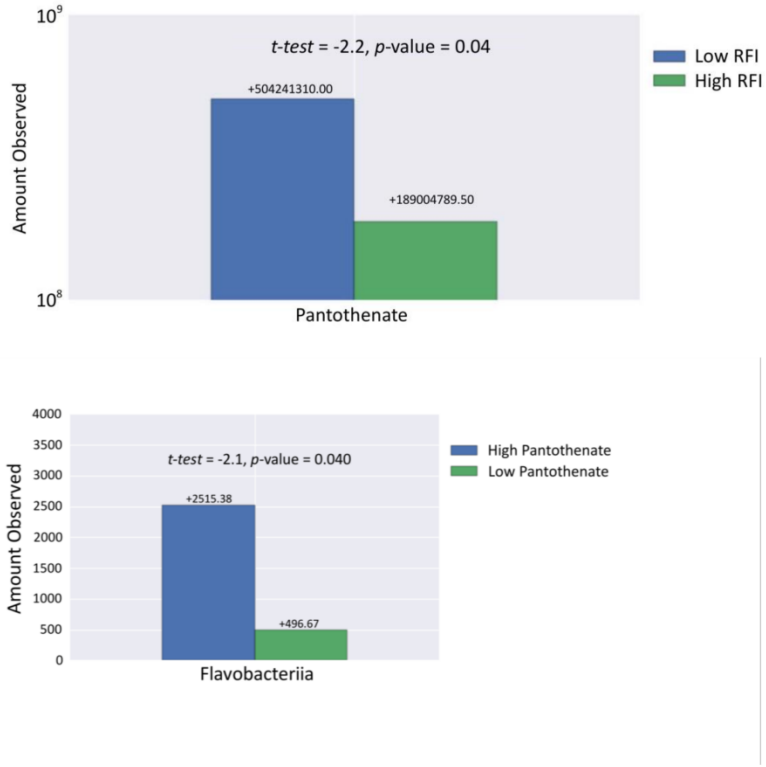

Figure 4.4. Week 10 rumen bacteriome correlated to serum metabolome A) Mean serum pantothenate abundance differs between low- and high-RFI steers B) Mean Flavobacteriia abundances associated with high pantothenate abundances.

In this study, although number of singletons in the rumen bacteria differed between low- and high-RFI steers, other measurements of α-diversity did not differ between the two groups. In other ecosystems, increased biodiversity is associated with greater success and resilience of the ecosystem (Cardinale, Palmer et al. 2002, Zak, Holmes et al. 2003); however, the data presented in this study suggest that α-diversity may not be a significant contributing factor to feed efficiency phenotypes in stable bacteriomes in growing beef steers. Previous studies have also observed the same relationships between rumen bacterial α-diversity and feed efficiency phenotypes in steers (McCann, Wiley et al. 2014, Myer, Smith et al. 2015). Other factors, such as divergences in individual taxa or functionality of the rumen microbiota, may play a greater role in dictation of host feed efficiency phenotypes.

Given the relationship between feed efficiency phenotypes and the rumen bacterial communities, it may be possible to identify specific rumen microbes and serum metabolites associated with RFI. Other studies, including those using various models of feed efficiency, have illustrated that α-diversity was similar between animals of divergent efficiency phenotypes (McCann, Wiley et al. 2014, Myer, Smith et al. 2015). The lack of differences in these studies suggest that divergences in feed efficiency phenotypes may be the result of dissimilarities at a finer resolution, such as individual taxa and metabolites, rather than global changes in the microbial communities and cumulative metabolites. Differences in serum and rumen metabolites may provide indications of feed efficiency, and could be developed into a method for on-farm detection of feed efficiency.

Glucose-1-phosphate and glucose-6-phosphate are both intermediate metabolites of the pentose phosphate pathway in which G6P undergoes several enzymatic reactions to generate NADPH, and is most common in pathways involved in fatty acid and steroid production (Cori, Cori et al. 1939). Glucose-6-phosphate dehydrogenase, the first enzyme of the pentose phosphate pathway, is the rate limiting enzyme of this pathway (Laliotis, Bizelis et al. 2007), which is estimated to supply 50 to 80% of the NADPH required to perform fatty acid synthesis in ruminants (Vernon 1981, Belk, Savell et al. 1993). Accumulation of G6P and G1P in less efficient animals may indicate less enzymatic activity or efficiency of the pentose phosphate pathway, which would decrease NADPH concentrations, resulting in less adipose accumulation. Both G1P and G6P in serum could potentially serve as biomarkers for feed efficiency, as increased concentrations are both are associated with decreased feed efficiency.

The rumen microbiome produces several vital nutrients for the host animals, including organic acids that serve as glucogenic precursors, as well as proteins and vitamins (Hungate 1966). A nutrient produced by the rumen microbiota is pantothenate. Pantothenate plays a significant role in the metabolism of fatty acids in ruminants and other species (Smith, Narrow et al. 1987, Palanker Musselman,

Fink et al. 2016). In this study, pantothenate was not only associated with greater feed efficiency, but was also predictive of rumen bacterial community composition. Pantothenate is a key component of coenzyme A (CoA), which is required to perform a variety of functions in intermediary metabolism of ruminants (Ragaller, Lebzien et al. 2011). Namely, CoA is responsible for transfer of fatty acid components into and out of the mitochondria (Ball 2006). Pantothenate is produced by several species of bacteria in the rumen, and can then be released into the rumen lumen to be absorbed by the host animal. One class of bacteria that can generate pantothenate in the rumen are Flavobacteriia. It was found in this study that greater Flavobacteriiaabundances were associated with greater pantothenate abundances, and Flavobacteriia abundances were predictive of pantothenate quantities, supporting that more efficient steers may have greater abundances of Flavobacteriia, which may lead to increased abundance of pantothenate.

As with G1P and G6P, pantothenate can be identified through serum; however, whereas G1P and G6P may serve as indicators of lower feed efficiency, pantothenate may indicate greater feed efficiency. The relationship between pantothenate and Flavobacteriia can not only provide insight as to mechanisms accounting for some variability in feed efficiency, but also serve as biochemical and microbial biomarkers in the serum and rumen, respectively. These biomarkers could allow producers to identify and select animals of greater feed efficiency. Metabolites and microbes predictive of efficiency phenotypes in cattle are not only imperative to partially explaining divergences in feed efficiency, but also to the selection of microbial communities related to efficient animals. These insights may also lead to the ability to select for an optimal rumen microbiome.

This study identified potential microbial and biochemical biomarkers that were used to determine extremes in feed efficiency in steers. Although, notable correlations between G1P and G6P and feed efficiency were identified, linking, and perhaps predicting, the functional capacity of the rumen and its microbiome, specifically Flavobacteriia, through serum pantothenate offers the potential to use serum biochemistry as an indicator in identification of feed efficient cattle. Additionally, although it has yet to be determined to what degree the rumen microbiome influences the host, or the host influences the rumen microbiome, the present study identified several key physiological elements that may impact or predict microbial community structure (e.g. RFI), or predictive of RFI (i.e. the serum metabolome and rumen bacteriome). As producers and researchers alike search for sources of variation in feed efficiency in cattle with the intent to optimize cattle productivity, methods to predict feed efficiency, such as use of microbial and biochemical markers could ultimately be used to improve the selection for feed efficient cattle.

CONCLUSION

Research Conclusions

The overall objective of the studies included in this thesis were to identify potential biochemical and microbial biomarkers, which were achieved. Several serum metabolites were identified that differed between steers of divergent RFI, including glucose-6-phosphate, glucose-1-phosphate, and pantothenate, among others. In addition to the serum metabolites, *Flavobacteriia* differed between low- and high-RFI steers in the rumen content. Pantothenate abundances were also correlated with *Flavobacteriia*, and *Flavobacteriia* was predictive of pantothenate abundance. Thus, we were able to identify several serum and rumen biochemical and microbial biomarkers that are associated with feed efficiency phenotypes in Black Angus steers.

In addition to addressing the main objective of this thesis project, another aim was to determine the temporal stability of the rumen bacteriome following transition to a growing diet in steers. This study revealed the ruminal bacteriome does the major transition to its stable bacteriome four weeks following transition to a diet, and does not stabilize until at least ten weeks following transition to a diet, which is a much longer timeframe than has been previously suggested by the literature. This could have implications in future study designs, particularly as they relate to diet acclimation and wash-out periods.

Future work should continue to interrogate the relationship between the ruminal and blood metabolomes to integrate how those may bridge the gap in knowledge between the ruminal microbiome and host physiology in order to explain differences in production-centric phenotypes, such as feed efficiency. This will ultimately lead to identification and selection of optimal livestock animals to expand food resources without diminishing environmental and economic resources.

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VITA

Brooke Ashley Clemmons (previously Howell) was born March 4, 1991 in Raleigh, NC. Brooke was active in athletics and honor societies until she graduated high school from Northeast High School in 2009. Following high school graduation, Brooke attended North Carolina State University. She obtainedher Bachelor of Science in Animal Science in May 2013. Following graduation, Brooke taught Chemistry and Physical Science at Corinth-Holders High School before becoming an Academic Support Associate and Graduate Services Coordinator in the Department of Biological Sciences at North Carolina State University. Brooke began her Master of Science in Animal Science at the University of Tennessee Institute of Agriculture. Brooke served under Dr. Phillip Myer during her graduate career, graduating with a Master of Science in May 2018.