INFLUENCE OF NUTRITION ON THE MUSCLE TRANSCRIPTOME AND RUMINAL MICROBIOME IN CATTLE

BY

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DISSERTATION

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Abstract

Beef cattle nutrition research has historically focused on formulating diets to address nutrient requirements of cattle for given level of animal performance. While predictive models account for many factors that may affect nutrient requirements, additional physiological effects can alter the animal's ability to utilize dietary nutrients. Several of these factors include the ruminal microbiome composition and epithelial tissue function, and nutrient provision in utero. The objective of this dissertation was to evaluate the effects of nutrition on these physiological effects to determine their potential to influence nutrient utilization.

Supplemental sources of fat, such as condensed distillers solubles (**CDS**), are often added to the diets of growing cattle to increase the energy density of the diet, but these products may negatively impact rumen bacteria at high inclusion levels. Five ruminally-fistulated steers were used in a 5 × 5 Latin square design to determine the effects of increasing dietary fat and sulfur from (**CDS**) on the ruminal microbiome. Alpha-diversity and species richness decreased (linear; $P < 0.05$) in the liquid fraction for steers fed greater CDS. At the phyla level, relative abundance of Bacteroidetes decreased in steers fed increasing dietary inclusion of CDS as Firmicutes increased to 82% of sequences for the 27% CDS treatment. The most abundant family of sulfate-reducing bacteria, Desulfovibrionaceae, increased ($P < 0.03$) in the solid and liquid fraction in steers fed additional dietary CDS and sulfur. There were no effects ($P > 0.10$) of feeding increasing dietary fat from CDS on fibroylytic phyla Fibrobacteres in either fraction.

Rapid consumption of a highly digestible diet causes rapid fermentation and may lead to the onset of subacute ruminal acidosis (SARA), a condition that negatively impacts the dairy industry by decreasing dry matter intake, milk production, and profitability. Six ruminally-fistulated, lactating Holstein cows were used in a replicated incomplete Latin square design to

determine the effects of SARA induction on the ruminal microbiome and epithelium using a SARA induction model. Ruminal contents and epithelial biopsies were collected on d 1 and 6 of each period prior to feeding. Principal coordinate analysis of beta-diversity indicated samples within the liquid fraction separated by day and coincided with an increased relative abundance of genera *Prevotella*, *Ruminococcus*, *Streptococcus*, and *Lactobacillus* on d 6 ($P < 0.06$). Phylum Bacteroidetes increased on d 6 ($P < 0.01$) for SARA cows driven by greater genera *Prevotella* and YRC22 ($P < 0.01$). *Streptococcus bovis* and *Succinivibrio dextrinosolvens* populations tended to increase on d 6 but were not affected by the severity of acidotic bout. In ruminal epithelium, *CLDN1* and *CLDN4* expression increased on d 6 ($P < 0.03$) 24 h after SARA induction, but overall effects on ruminal epithelium were modest.

Maternal nutrition provided during mid-gestation may influence skeletal muscle development and long-term metabolism. Three planes of nutrition were provided to cows to address 70% (**70%REQ**), 100% (**REQ**), and 130% NRC energy and protein requirements (**130%REQ**) during mid-gestation. All calf progeny were managed as a single contemporary group and longissimus muscles biopsies were taken on 99, 197, and 392 d of age. The skeletal muscle transcriptome analysis at d 392 indicated over 2,000 co-expressed genes were downregulated in progeny born to 130%REQ-fed compared with REQ-fed dams. These genes were annotated to many lipid-associated pathways including steroid and steroid hormone biosynthesis, sulfur metabolism, retinol metabolism, ketone synthesis and degradation, fat digestion and absorption, and PPAR signaling pathways. Another set of genes (342) was activated in progeny born to 130%REQ-fed compared with 70%REQ-fed dams and correlated negatively with marbling score. These genes were annotated to pathways centered on glycolysis/gluconeogenesis, energy metabolism, and calcium signaling to support increased

glycolytic muscle fibers (type 2x) in progeny born to 130%REQ-fed dams. Skeletal muscle miRNA were tightly regulated over time suggesting various roles in postnatal hypertrophy. Maternal plane of nutrition effects ($P < 0.1$) were observed for miR-376d and miR-381. Results indicate that maternal plane of nutrition has a long-term impact on the skeletal muscle transcriptome and may be linked to effects on meat quality.

TABLE OF CONTENTS

CHAPTER 1

LITERATURE REVIEW

Overview

Nutrients are essential for life for all animals, and the digestive tract is tasked with capturing these nutrients. In the animal kingdom, the specialized digestive tract of ruminants uniquely facilitates their exploitation of the most widely available organic compound on the planet, cellulose. A symbiotic relationship with bacteria in the rumen enables ruminants like cattle to digest a wide array of feedstuffs. Compared with other livestock species, meeting the nutrient requirements of rumen microbiota is a priority for ruminant nutritionist. Nutrition is the largest variable cost in modern cattle production and nutrition related decisions are principally motivated by animal performance outcomes in growth, milk production, reproductive success, etc. Despite the fact ruminant nutrition research has been conducted in some manner for hundreds of years, constant changes in feedstuff availability, industrial coproducts, commodity market dynamics, and cattle genetics necessitate continuous research in this area.

Ruminant nutrition research has classically focused on increasing performance outputs by precisely addressing the nutritional requirements for gain or production goals. Based on the nutrients required, diets are typically formulated on a least-costs basis. Committees in countries across the globe have incorporated existing data to define the nutrient requirements for beef cattle. Diets are evaluated based on the composite nutrient values of individual feedstuffs. Although these models accounts for a wide variety of factors such as mature body weight, genetics, temperature, and intake, there are a host of additional factors that may affect nutrient

utilization. Interactions occurring within dietary feed ingredients that can affect their utilization are often called associative effects. Within the animal system, any physiological change affecting nutrient requirements will, by definition, also alter nutrient utilization at a constant rate of intake. While these effects abound, much more information is needed to understand how these effects are mediated so that they can subsequently be modeled. These factors can be termed physiological associative effects as they are innate to the animal itself. Examples of these effects include the epigenome, rumen microbiome, imprinted metabolism, epithelial permeability, and immune system activation. While decades of research has focused on the diet composition and animal performance measures, only within the last 15 years has the animal science community began to evaluate physiological associative effects in a nutritional context.

While the current condition of various physiological associative effects can shape nutrient utilization, nutrition may also be able to have a lasting impact on some of these effects. Therefore, prior nutrition may be one way to optimize subsequent nutrient utilization via physiological associative effects. For example, maternal nutrition is responsible for nutrient delivery to the fetus. The over- or under-provision of a particular nutrient during a critical developmental window may shape long-term metabolism of a given tissue and influence nutrient utilization. Epigenetics exemplify the potential opportunity to utilize physiological associative effects for long-term benefit. Alternatively, the effects may be more short-term such as the barrier function of ruminal epithelium. Acidotic ruminal pH values impair the barrier function in the rumen and may lead to systemic inflammation and decreased animal performance. Greater understanding of acidosis etiology and the role of the host-microbiome interaction may lead to further prevention and improved animal health. Many other opportunities exist to research

physiological associative effects for the benefit of efficient beef cattle production but a solid foundation of current literature is needed to direct future experiments.

Rumen Microbiome and Epithelium

Introduction

The fermentation abilities of microorganisms within the reticulorumen are the hallmark of the ruminant digestive tract. The continuous fermentation in the rumen is driven by a diverse and competitive microbiome consisting of bacteria, archaea, protozoa, and fungi (Mackie et al., 2000) and viruses (Gilbert and Klieve, 2015). Regardless of the dietary substrates, the primary end products of fermentation include volatile fatty acids (**VFA**), microbial crude protein (**MCP**), and ammonia, and are responsible for addressing a large portion of host energy and protein requirements (Russell et al., 1992). Additional end products, CO_2 and methane, are released via eructation and represent a loss of energy to the animal. Collectively, this fermentation system highlights the vast capabilities possessed by microorganisms and how they function in concert with the host.

Although historically dependent on anaerobic culture techniques pioneered by Robert Hungate (Hungate, 1966; Krause et al., 2013), the inception of nucleic acid-based, molecular technologies has redefined all fields of microbiology (McCann et al., 2014; Firkins and Yu, 2015). Microbial studies are no longer bound by the culturing aptitude of various microbial species. Recent estimates suggest that less than 10 % of rumen bacteria have been isolated in pure culture (Kim et al., 2011). Nevertheless, a rich history of rumen microbiology research has been foundational in our understanding of microbial function and metabolism (Krause et al., 2013).

From a nutrition perspective, the rumen sets the stage for everything to follow in terms of digestion and nutrient absorption. Therefore, the inhabitants of the rumen and their function are a primary consideration to help understand and augment classic nutritional concepts such as intake and digestion, ruminal fermentation, and rate of passage. Furthermore, as the use growth-promoting technologies in the beef industry appears to be more restricted in the future, the optimization of nutrition and ruminal fermentation will only become more vital to maintain production efficiency and profitability.

Lipid effects

Dietary lipids are included to increase the energy density of diets fed to high-producing ruminants, dairy cows in peak lactation, and beef steers on finishing diets. Unless in a protected form, dietary lipids undergo microbial lipolysis and biohydrogenation in the rumen (Jenkins, 1993). The final fatty acid profile of the digesta leaving the rumen is correlated to fatty acid profile in meat and milk as mammals lack hydrogenating enzymes. Beyond greater dietary energy, lipid inclusion may be increased due to coproduct inclusion (Berger and Singh, 2010), methane mitigation (Beauchemin et al., 2007), or to alter fatty acid profile of retail products (Alvarado-Gilis et al., 2015). Importantly, some microbes are sensitive to high levels of unsaturated fatty acids and the growth of cellulolytic bacteria in vitro was decreased by the presence of polyunsaturated fatty acids (Maia et al., 2007). Including fish oil, a highly unsaturated source of fatty acids, in dairy cattle diets decreased *Butyrivibrio fibrisolvens* and *Psuedobutyrivibrio*, but *Propionibacterium acnes* increased significantly at high levels of supplementation (Shingfield et al., 2012). Using a pyrosequencing approach, Zened et al. (2013) evaluated effect of adding 5% sunflower oil in high starch diets. Inclusion of sunflower oil decreased Ruminococcaceae Incertae-Sedis, *Oscillibacter*, *Fastidiosipila*, and *Bifidobacterium*,

but increased *Prevotella* substantially. While *Prevotella* has often been observed as a dominant genus in the rumen (Stevenson and Weimer, 2007), there are likely a diverse array of functions possessed by *Prevotella* species and strains yet to be described in culture. Furthermore, *Prevotella* is not very sensitive to linoleic acid in vitro (Maia et al., 2007) which may allow it to take advantage of a situation where sensitive species are hindered. Others have identified terminal restriction fragments of bacteria associated with biohydrogenation intermediates as *Prevotella* and Lachnospiraceae Incertae-Sedis (Huws et al., 2011). Beyond well-described cultured bacteria, continued elucidation of the rumen microbiome's effect on lipids and unsaturated fatty acids will inform potential effects of diet on the fatty acid composition of food products derived from ruminants as well as microbial metabolism.

Starch and pH effects

Cereal grains are often added to ruminant diets to address increased energy requirements of the animal by shifting fermentation to greater production of propionate, a gluconeogenic precursor. The high starch content in grains is the primary driver of ruminal fermentation changes as their increase is typically coupled with decreased forage in the diet. While greater VFA and propionate production are necessary to help meet additional energy requirements of high-producing and growing ruminants, this also corresponds with a decrease in ruminal pH. The change in substrates provided to the ruminal microbiome and subsequent fermentation end products are linked to changes in the ruminal microbiome composition. There have been consistent observations of changes in the ruminal microbiome with significant additions of grains to the diet. A decrease in alpha diversity, the diversity within a sample, is typically observed with diets higher in energy or starch (Fernando et al., 2010; Pitta et al., 2010; Zened et al., 2013). Alpha diversity is most commonly measured using the Shannon index (Shannon and Weaver,

1949) and takes into account the number of observed species (or OTUs) and the evenness of different bacterial populations. A reduction in the Shannon index for alpha diversity suggests fewer bacteria are adapted to maintain stable population levels when additional grain is included in the diet.

Bacteria with cellulolytic or fibrolytic capabilities are often sensitive to low ruminal pH induced by additional dietary grain (Russell and Dombrowski, 1980). Their decrease under low pH condition is due to inability to regulate internal pH (Nagaraja, 2012) and less available substrate. Classically, this is illustrated by *Fibrobacter succinogenes* as it has lower abundance and activity of the H^+-ATPase transporter in the cell membrane (Miwa et al., 1997). At a pH of < 5.8, *F. succinogenes* was unable to transport its primary substrate cellobiose indicating a pH-sensitive metabolism (Russell, 2002). Conversely, many other bacteria are more acid-tolerant, such as *Streptococcus bovis*. At a pH below 6.0, *S. bovis* and *Megashpaera elsdenii* had a greater amount of H^+-ATPase compared to a pH of 7.0 (Miwa et al., 1997). Thus, acid-tolerant bacteria increase proton export capacity to aid in regulation of intracellular pH. Subsequent research confirmed the importance of the H^+-ATPase to the functional pH tolerance of *S. bovis* using mutant strains with altered transporter function (Miwa et al., 2000). Although differences in acid-tolerance have been studied in several well-described rumen species, there remains many other species within the rumen that their acid-tolerance have not been researched. Furthermore, the abundance and regulation of ATPase in the cell membrane may serve as a proxy for specie acid-sensitivity.

While many studies indicate fibrolytic species decrease with greater energy, this has often been observed in contrasting diets. The trends noted in divergent dietary comparisons may not hold true within a more narrow range of dietary parameters. Therefore, it is important to

keep in mind the dietary context of a given situation. While energy level and rumen pH are believed to be significant factors in determining the ruminal microbiome composition, a variety of other factors remain, including: prior exposure to high energy diets, rate of diet acclimation, diet composition, absorptive capability of rumen epithelium, and feed intake pattern. This variety of factors is important to consider the expected effects on the microbiome when adding starch to the diet as many are related to aspects of beef or dairy cattle production systems.

Within a beef cattle context, populations of *Streptococcus bovis*, *Prevotella bryantii*, *Selenomonas ruminantium*, and *Megasphaera elsdenii* increased during the adaptation to a high-concentrate diet (Fernando et al., 2010). *S. bovis* has been well described in vitro as an amylolytic, facultative anaerobe known to increase with the addition of starch to the diet or when ruminal pH decreases (Slyter, 1976; Owens et al., 1998). Although *S. bovis* increased significantly at the beginning of diet adaptation, populations decreased with subsequent step-up diets suggesting an effective transition to a high-concentrate diet (Fernando et al., 2010). *M. elsdenii* utilizes lactic acid released in the rumen contributing to stabilization of rumen pH and prevention of acidosis (Counotte et al., 1981; Russell et al., 1981). As expected, *Butyrivibrio fibrisolvens* and *Fibrobacter succinogenes* populations decreased with addition of concentrate to the diet. Although both have fibrolytic capabilities, *F. succinogenes* decreased more rapidly during adaptation compared with *B. fibrisolvens*. *B. fibrisolvens* can also utilize maltose and sucrose (Russell and Baldwin, 1978) and significant decreases were not evident until the final step-up diet (Fernando et al., 2010).

In dairy production, the time of the greatest addition of starch to the diet occurs during the transition period as energy requirements dramatically increase with demands of early lactation. Although several studies have evaluated the effects of the transition period on the

microbiome, the changes are affected by dietary components which vary widely between studies. Regardless, understanding the adjustment of the ruminal microbiome may play a role in preventing the various metabolic maladies that may occur during the transition period. Wang et al. (2012) evaluated thirteen ruminal bacteria in seven cows at seven time points from -21 to +21 d relative to parturition. The postpartum diet increased the non-fiber carbohydrates (NFC) from 28 to 40% of the diet and net energy of lactation from 1.36 to 1.60 MCal/kg. After parturition, relative abundance of *Prevotella brevis*, *Prevotella ruminicola*, *Ruminobacter amylophilus*, *Anaerovibrio lipolytica*, *Streptococcus bovis*, and *Lactobacillus* spp. increased as expected (Wang et al., 2012). However, *Fibrobacter succinogenes* and *Butyrivibrio fibrisolvens* actually increased at d 1 and 7 postpartum while *Megasphaera elsdenii* decreased slightly. Overall, results suggest greater concentrate in the diet was not at the expense of fibrolytic bacteria potentially due well-balanced rations that may have mitigated a significant decrease in rumen pH. Others have not observed a change in the overall ruminal microbiome during the transition period using community fingerprinting techniques (Mohammed et al., 2012). Though, it must be noted that the dietary changes were not as drastic with an increase from 39.6 to 43.5% NFC from a prepartum to the lactation diet at most. A recent study implemented 16S sequencing to evaluate the ruminal microbiome during the transition period (Pitta et al., 2014). One sampling time prepartum was compared to 3 time points during the first 8 wks of lactation. Postpartum the increase in Bacteroidetes was due to a greater relative abundance of *Prevotella* while a decrease of *Clostridia, Coriobacteriales*, and *Succiniclasticum* contributed to a reduction of Firmicutes (Pitta et al., 2014). Interestingly, there were significant differences observed between primiparous and multiparous cows at the phylum level suggesting that transition period effected are impacted by parity and should be accounted for in future research.

Corn co-product effects

Increased ethanol production in the United States has resulted in greater utilization in beef cattle rations of the subsequent co-product, distillers grains. Distillers grains are the unfermented grain residue that contains more protein and NDF compared with the unprocessed grain. Callaway et al. (2010) evaluated bacterial diversity in the rumen of cows consuming increasing levels of dried distillers grains (**DDG**) at 0, 25, and 50% replacing a commercial concentrate feed. Pooled samples from two steers on each diet were pyrosequenced to describe the ruminal bacteria populations. Analysis of samples collected prior to the experiment indicated the presence of 74 genera with Prevotella as the most abundant. Averages across three high concentrate diets resulted in detection of more than 400 species. For diets containing 50% DDG, *Prevotella* and *Bacteroides* increased 152 and 276% in relative abundance while *Succinivibrio* populations decreased 406% compared with the 0% DDG diet. Moreover, a trend indicated a decrease of Firmicutes:Bacteroidetes with increasing DDG inclusion due to greater Prevotella and *Bacteroides*. The proteolytic ability of *Prevotella* and *Bacteroides* has been previously reported (Attwood and Reilly, 1995; Reilly et al., 2002), and the response to DDG may be explained by the increase in dietary CP with DDG addition. Reported changes in the rumen microbiome of steers consuming 50% DDG coincided with a decrease in ruminal pH. However, pH values (6.58 - 7.18) were atypical for high-concentrate diets and the unknown composition of the commercial feed prevents accurate diet comparisons.

Corn gluten feed (**CGF**), a co-product of the corn milling, is produced after the germ, starch and gluten have been removed from the corn kernel. Similar to DDG, CGF is higher in protein and fiber than corn. Using a replicated 4 × 4 Latin square, the effect of increasing wet CGF (0, 11, 23, and 34%) was evaluated in lactating dairy cows in diets with equivalent NDF

and CP (Mullins et al., 2013). After 25 d of diet adaptation, ruminal samples were collected 8 times over 3 d and pooled by cow and period. Increasing CGF elicited a change in ruminal fermentation including a linear increase in propionate and valerate in addition to a decrease in acetate, isovalerate, and pH (Mullins et al., 2010). Quantitative PCR was used to determine the response of well-known taxa, but no linear or quadratic effects were observed for the 9 taxon of interest. The sum of the species only represented from 6 - 16% of the total bacteria in the rumen determined using a universal primer. Inter-animal variation accounted for 10 - 55% of the random variance, thus, unexplained variance and the between animal variation were both important contributors to the overall variance (Mullins et al., 2013). Although relative abundance of the evaluated species was stable, changes in bacterial function and many unobserved populations could be related to the documented shifts in fermentation.

Microbiome systems biology

The recent technical advances in the field of rumen microbiology make implementing systems biology approaches possible. Although these approaches have not been applied to the ruminal microbiome, here we provide several examples of how this type of research can be conducted and propose that it would be valuable to apply these techniques to ruminant research. Systems biology concepts can be applied to the host-microbe relationship in several different ways to gain a greater understanding of how the ruminant system is functioning. One way these concepts can be applied is to evaluate the microbiome as its own system. Many studies recently have solely focused on determining the composition in the ruminal microbiome using 16S rRNA sequencing (Pitta et al., 2010; Jami and Mizrahi, 2012; Kittelmann et al., 2013). These approaches can be expanded with additional high-throughput methods by evaluating the metabolic potential (metagenome) and currently expressed genes (metatranscriptome) to model

how the microbiome functions. One of the first successful efforts in this area (Greenblum et al., 2012) used shotgun sequencing metagenomic reads in a systems approach to address the relation of the fecal microbiome to obesity and inflammatory bowel disease (**IBD**) in human. They created simple connectivity-centered networks computationally built from homology-based, large-scale metabolomics databases. While the networks simplify the underlying pathways, topology-based analysis of these networks is very useful for evaluating systems with limited data. Their systems analysis indicated that enzymes located near the periphery of the network were most associated with host states of obesity and IBD and identified potential biomarkers (Greenblum et al., 2012).

Beyond modeling the microbiome itself, other research groups are working on models to describe the interaction between the host and its microbiome. While it is an ambitious goal, progress is being made and it provides another potential avenue for investigating the ruminant system. Recently, these systems techniques were successfully implemented to describe the relationship between the host (mouse) and a single bacterium (*Bacteriodes thetaiotamicron*) (Heinken et al., 2013). They created metabolic reconstructions of the host and the microbe of interest using existing genome assemblies and publically available data. Then metabolite and other high-throughput data were incorporated into the model as well as additional constraints to make it more physiologically accurate. The model between the mouse gut and *B. thetaiotamicron* was able to accurately depict the growth dependencies observed in live animal trials with a variety of dietary conditions. Furthermore, the model identified the microbial origin of metabolites observed in the blood (Heinken et al., 2013). Although current computational tools are not capable of creating a similar model with many additional microbes, it certainly represents a path forward and an area of opportunity in the future. Greater discussion of the

current status of this field can be found in recent reviews by Greenblum et al. (2013) and Thiele et al. (2013).

In microbial ecology, one of the greatest challenges is determining the causative mechanisms leading to observed differences in community composition and function. Overall, our understanding of how microbial communities function collectively is very limited in many instances. By modeling the microbiome as well as its interaction with the host, we will be able to identify essential microbes actuating the observed changes, predict alterations in microbiome functions based on substrates changes, and model effects of loss-of-function or loss-of-microorganisms within a microbiome.

Host-microbiome interface and interaction

Rumen epithelial tissue lies at the crux of the relationship between the host and ruminal microbiome. It has several primary functions including nutrient absorption, metabolism, pH regulation, motility, as well as barrier and immune functions. Unique to other absorptive structures in the gastrointestinal tract, rumen epithelial tissue is comprised of stratified squamous epithelial cells (Dobson et al., 1956). There are four cell layers with coordinated roles. The most apical layer, stratum corneum, is the primary barrier with flat, dead keratinocytes (Graham and Simmons, 2005). The stratum granulosum lies beneath the stratum corneum and has granular cells with tight junctions (Penner et al., 2011). Basolateral of the stratum granulosum, the stratum spinosum is more metabolically active with higher expression of Na^+/K^+-ATPase (Graham and Simmons, 2005). The stratum basale is the most basolateral layer with the greatest ketogenic activity and transport function with the blood (Graham and Simmons, 2005). Volatile fatty acids yielded from microbial fermentation decrease ruminal pH and must be transported to

the blood or metabolized by the epithelium. Relative to other tissues, our understanding of epithelial metabolism is limited beyond ketogenesis and transport functions.

Scanning electron microscopy first revealed populations of ruminal bacteria physically attached to the epithelial surface (Bauchop et al., 1975). These attached bacteria have been referred to as the epimural community (Mead and Jones, 1981). More recent works have observed an epimural community composition distinct from the microbiome associated with rumen contents (Cho et al., 2006; Li et al., 2012). Although their function is not well-described, it seems likely that these bacteria may be key intermediaries in the host-microbe relationship. Epimural bacteria are not as strongly influenced by changes in the diet (Sadet et al., 2007), but others have observed a diet effect (Chen et al., 2011; Petri et al., 2013; Liu et al., 2015). Additionally, epimural bacterial may be host specific and has been related to acidosis susceptibility (Chen et al., 2012) as well as ammonia absorption (Li et al., 2012). Understanding the function of the epimural community could be a key piece to reveal ongoing communication between the host and the microbiome.

Considering the host-microbe relationship, substrate provision and maintenance of the environment itself is the responsibility of the host. The manner the host maintains the environment affects the microbiome by the mixing of the rumen, pattern of intake, and preferential selection of feedstuffs. This concept was classically illustrated in an experiment by Weimer et al. (2010). Although two cows were eating the same diet, they initially had divergent rumen fermentation profiles and pH. They switched nearly the entire rumen contents of each cow and monitored the microbiome composition over time. Almost immediately after switching, the bacterial community began to change back to the original composition. Although they were not able to pinpoint host control points of microbiome composition, it is well established that diet

composition is a key determinant of ruminal microbiome composition (Tajima et al., 2000; Fernando et al., 2010; Pitta et al., 2010). Therefore, the host effect may likely be realized through an individual's feed intake pattern as well as prior conditions affecting the absorptive capacity of rumen epithelium. Observations from the transition period of dairy cows further support this claim; the significant shift in microbiome composition (Wang et al., 2012; Pitta et al., 2014) is directly related to different events occurring during this time such as changes in intake, diet composition, and energy demands (Drackley, 1999).

Rumen epithelium functions

As previously mentioned, ruminal fermentation yields multiple end products critical to meeting the nutrient demands of the host. Specifically, absorption of VFA and ammonia are the principal end products rumen epithelium is tasked with absorbing. Two primary methods of absorption include diffusion and protein-mediated methods (Aschenbach et al., 2011). Absorption by diffusion is controlled by the lipid solubility of specific VFA with butyrate being the most soluble, followed by propionate and acetate. Additionally, lipophilic diffusion favors the undissociated acid form of VFA at lower pH levels. Overall, it has been observed that rate of absorption are similar between the three main VFA (Dijkstra et al., 1993). Protein-mediated transport methods include HCO_3^- exchange proteins, Na^+/HCO_3^- co-transporter 1, Na^+/H^+ exchange proteins, Na^+/K^+ ATPase, and monocarboxylate transporters 1 and 4 (Aschenbach et al., 2011). Collectively, the transporters are responsible for regulating pH in the rumen and epithelial tissue to ensure both are able to function properly. While evaluation of dietary effects on transporter expression is limited, increased barley in the diet has been observed to increase expression of monocarboxylate transporter 1in rumen epithelium (Metzler-Zebeli et al., 2013).

Epithelium Ketogenesis

Butyrate, a major VFA end product of ruminal fermentation, is utilized almost entirely by rumen epithelium in ketogenesis for the formation of β-hydroxybutyrate. In the fed state, rumen epithelium is the greatest maker of ketone bodies (Pennington, 1952). While it is believed to primarily occur in the basolaterial strata (Penner et al., 2011), the rate limiting enzymes of ketogenesis are acetyl-CoA acetyl transferase (ACAT) and 3-hydroxy, 3-methylglutaryl CoA synthase (HMGCS) (Lane et al., 2002). Specifically, the HMGCS2 isoform located in the mitochondria is key to ketone body formation and is known to be regulated by PPAR-α (Meertens et al., 1998; Lane et al., 2002). Nutrient control of regulating transcription factors such as PPARs may exert control of ketogenesis in ruminal epithelium (Penner et al., 2011). A 28 d adaptation to high and low concentrate ration did not affect ketogenic gene expression despite greater plasma β-hydroxybutyrate and butyrate absorption (Penner et al., 2009). However, a grain challenge did downregulate ACAT and HMGCS2 expression in rumen epithelium which paralleled a reduction in plasma β-hydroxybutyrate (Steele et al., 2009). These findings bolster prior supposition that short-term adaptations are accounted for at the cellular level while long-term adaptations occur through increases in surface area (Etschmann et al., 2009).

Barrier Function

Barrier function is a critical role of ruminal epithelium tissue as it divides the "outside" from the inside. A breach of the epithelial barrier in the rumen can facilitate translocation of endotoxins or bacteria which may cause systemic inflammation (Gozho et al., 2007), liver abscesses (Nagaraja and Chengappa, 1998; Kleen et al., 2003), or septic emboli (Krause and Oetzel, 2006). The tight junctions of the stratum granulosum, various proteins that link the granular cells together, serve to maintain the barrier and are found in various epithelial cell types.

The major tight junction proteins observed in rumen epithelium were claudin 1 and zona occludens 1 (ZO-1) which were localized to the stratum granulosum (Graham and Simmons, 2005). Desmosomes are another structural component of the tight junction that add mechanical strength by linking intermediate filaments to intracellular adhesion sites (Green and Simpson, 2007). Although the regulation of these proteins is not well understood especially in the ruminant context, recent efforts have worked to link barrier function with different feeding approaches. In response to high concentrate feeding of sheep for 7 weeks, protein and gene expression of claudin 1 was upregulated while claudin 4, occludin and ZO-1 were down regulated in rumen epithelium compared to sheep on a hay diet (Liu et al., 2013). A breach of barrier function was supported by upregulation of inflammatory cytokines, parakeratosis, and disruption of tight junction proteins localized on the cells membrane. Changing cattle from high forage to a high grain diet for 3 weeks also decreased expression of desmoglein 1 (Steele et al., 2011). Despite some research on physiological response of epithelial tissue to acidotic conditions, much remains unknown about the progression of the loss of barrier function and how the process is mediated.

Fetal Programming of Skeletal Muscle in Ruminants

Introduction

Fetal programming is centered on the idea that non-genotypic factors can alter fetal development and postnatal physiology (Barker, 2007). While currently studied in many species, fetal programming was first described in human epidemiology data after a severe famine in the Netherlands from 1944 - 1945 (Ravelli et al., 1998). Most progress in the fundamental understanding of fetal programming mechanisms has been made in rodents and sheep, but the same strategies may be capable of improving meat quality or the efficiency of cattle production.

However, a multitude of associated factors can influence fetal programming in ruminants such as the genetic diversity within a population, environmental stress, maternal diet, and production system. These factors and the regional specificity of cattle production challenge research findings in their application to other production schemes. Compared with monogastric livestock species, ruminants are often raised in extensive systems such that the implementation of some targeted nutritional treatments may not be feasible. While progress has been observed from a management and nutrition perspective (Funston and Summers, 2013), there remains a dearth of information as to the physiological and molecular mechanisms that cause the phenotypic outcomes of fetal programming.

Mid-gestation effects on myogenesis

In terms of fetal development, mid-gestation represents the peak of secondary myogenesis, the formation of secondary muscle fibers, in ruminants (Bonnet et al., 2010; Du et al., 2010) which develop primarily into fast-twitch muscle fibers (Robelin et al., 1993). Therefore, mid-gestation is a critical time for development of skeletal muscle (Greenwood et al., 2000; Du et al., 2010). Muscle fibers are formed from the fusion of myogenic cells such that a greater abundance of myogenic cells will result in more muscle fiber formation during the fetal stage (Zhu et al., 2004). However, the proliferation of myogenic cells is highly influenced by nutrients and endocrine signaling such that maternal condition has the opportunity to affect proliferation and subsequent muscle fiber formation (Zhu et al., 2004; Tong et al., 2009; Yan et al., 2010). Reduced myofiber formation during the fetal stage due to nutrient availability limits the ability of postnatal compensatory growth of skeletal muscle (Wu et al., 2006). These effects are classically illustrated by the permanently reduced muscle mass in runt piglets (Powell and Aberle, 1980; Handel and Stickland, 1987). Considering that muscle fiber number is believed to

be set at birth (Rehfeldt et al., 2004), postnatal muscle development relies on an increase in cell size, hypertrophy, via recruitment of satellite cells (Rosenblatt 2004). The pool of satellite cells represents a source of replicating cells and additional DNA to support new protein synthesis.

For maternal nutrition to have an effect on skeletal muscle and muscle fiber numbers, the maternal dietary intervention must coincide with key developmental windows. Fahey et al. (2005b) was able to determine the major fiber formation occurred at d 85 of gestation in sheep and was supported by peak mRNA expression of IGF2, myogenin, and histochemical analysis. This would mathematically correspond to d 160 in cattle. A subsequent study evaluated the effect of reducing ewe intake to 50% of requirements for 40 d either before (d 30 - 70), during (d 55 - 95), or after (d 85 - 115) the peak in fiber formation (Fahey et al., 2005a) . Muscle samples from d 14 postnatally indicated that maternal nutrition only had an effect prior to the peak of fiber formation (d 30 - 70) by reducing secondary:primary fiber ratio and myosin heavy chain fast fibers. This timing would correspond to d 57 to 132 in cattle. A similarly design study observed maternal nutrient restriction (d 28 - 78 of gestation) reduced the number of secondary myofibers as wells as phosphorylated mTOR in the fetus at d 78 of gestation (Zhu et al., 2004). A subsequent study observed that when sheep grew to typical slaughter weights (17 wks of age) that differences in muscle fiber type and number were not affected by maternal nutrition with exception of increased fast fibers in the longissimus muscle of lambs out of nutrient-restricted dams (Daniel et al., 2007). In contrast, others have observed decreased myofibers, greater myosin heavy chain 2b (fast), and reduced carnitine palmitoyltransferase-1 activity in skeletal muscle of lambs out of nutrient restricted dams at 8 mo of age (Zhu et al., 2006). An additional proteomic component identified differentially expressed proteins related to mitochondrial function and glucose metabolism being down-regulated in nutrient restricted lambs (Zhu et al.,

2006). While nutrient restriction during early to mid-gestation can alter myosin heavy chain composition in skeletal muscle, the effects are not consistent in the fetal stage, at birth, and during the growing stage later in life.

Fewer fetal programming studies have been conducted in beef cattle relative to sheep, and thus there are more inconsistencies from a design standpoint in the literature. Moreover, the majority of studies in cattle have focused on modifying nutrition during the last trimester (Martin et al., 2007; Larson et al., 2009; Roberts et al., 2009; Funston et al., 2010; Rolfe et al., 2011). Beef cows were nutrient restricted to 70% of requirements from d 45 to d 185 of gestation and although it did not affect birth weight, yield grade and semitendinosus weight were reduced in the progeny after finishing (Long et al., 2012). Adipocyte diameter was also increased in subcutaneous fat as well as fatty acid transporter 1 in nutrient restricted offspring. The authors suggested that effects in fat may have been caused by reduction in skeletal muscle development (Long et al., 2012). Another study investigated the effect of high or low levels of protein (240% and 70% of requirements, respectively) in a factorial arrangement with the initial two trimesters (Micke et al., 2011). Regardless of first trimester protein intake, decreased maternal protein intake during the second trimester resulted in calves with greater rib eye area. They also observed greater plasma IGF-I postnally from d 29 -379 in male calves out of dams with low protein in the first trimester followed by high protein in the second trimester (Micke et al., 2011). Recent work evaluated the effect of a positive or negative energy balance during mid-gestation in beef cows and observed calves out of negative energy status dams tended to have a lower USDA yield grade and a more desirable backfat to marbling ratio (Mohrhauser et al., 2015b). No treatment differences in Warner-Bratzler shear force, L*, a*, or b* were observed in 81 cattle between the 2 treatments. A subsample group of steers were evaluated for gene

expression in the longissimus and semitendinosus muscles (Mohrhauser et al., 2015a). While no effect of mid-gestation energy balance was observed in the slaughter time point on gene expression in the longissimus muscle, the gene for the myosin heavy chain 2a was upregulated in steers from dams with a negative energy balance in the semitendinosus muscle. Considering the limited effects observed in many evaluated variables, surprisingly the humoral antibody response to a novel antigen was dampened in for progeny born to dams in a negative energy balance during mid-gestation (Taylor et al., 2015). Significant variation in production system, cattle genetics, forage quality, supplementation goals, experimental design, and postnatal nutrition all represent contributing factors to major differences in these studies.

Epigenetic mechanisms

At the onset of fetal programming research little was known on how the changes were mediated. Most fetal programming effects have now been linked to a relatively new scientific field called epigenetics. Epigenetics is commonly defined as heritable changes in gene expression without any underlying changes in genetic information (Barker et al., 2002). Literally, epigenetic means "above the genome" and originated from the description of methylated DNA as methyl groups are attached above the cytosine residues. The primary epigenetic mechanisms include DNA methylation, histone modifications, and noncoding RNAs. While many other physiological processes regulate gene expression, these epigenetic mechanisms are heritable from cell to cell and therefore can be transferred through cell lineage or even transgenerationally to grand-offspring (Youngson and Whitelaw, 2008). Although some epigenetic regulatory features may be developmentally malleable, most epigenetic regulation is highly conserved across mammalian and even vertebrate species being critical to normal

development. Therefore, while the majority of fetal programming effects are believe to be epigenetically regulated, most epigenetic regulations cannot be fetally programmed.

DNA Methylation

Within a DNA sequence, methyl groups are bound to the carbon-5 position of cytosines in cytosine-phosphate-guanosine (**CpG**) dinucleotides converting them to 5-methyl cytosines (Cooper and Krawczak, 1989). Approximately 70-80% of the CpG dinucleotides are thought to be methylated (Bird, 2002). However, dense regions of CpG dinucleotides, referred to as CpG islands, are often unmethylated and observed in the promoter region of genes where they cause stable, heritable, transcriptional silencing (Deaton et al., 2011). The presence of methyl groups can prevent binding of key regulatory elements such as transcription factors to their target site, thereby suppressing gene expression (Jones, 1999). DNA methylation is primarily a relatively stable repressive marker but more recent evidence suggests it is regulated more dynamically than first believed (Barrès et al., 2012).

Mammalian genomes contain four DNA methyltransferases (DNMT1, DNMT2, DNMT3A, and DNMT3B) which possess various functions in methylating DNA. While DNMT1 is responsible for maintaining the methylation pattern through cell divisions, DNMT3A and 3B are involved with de novo methylation and demethylation during development (Cedar and Bergman, 2009). However, recent findings suggest DNMT3A and DNMT3B may also have a role in methylation during replication by correcting errors left by DNMT1 (Jones and Liang, 2009). *DNMT2* mRNA is expressed in many bovine tissues and is the most abundant methyltransferase in adult testis and ovary (Golding and Westhusin, 2003), but it does not appear to possess any observable methylating ability despite containing the necessary catalytic domains (Okano et al., 1998).

As only two DNMT's are responsible for all de novo DNA methylation, it is not likely they would be a control point as their regulation should be highly conserved to ensure normal function and development. However, there are several specific examples in livestock research indicating DNMT3B may be connected to nutritionally fetal programming event. Lan et al. (2013) evaluated the effect of three nutritionally divergent diets with varying levels of protein, methionine, and choline fed to ewes from d 67 to 130 of gestation. The dietary treatment based on distillers grains had the greatest amount of rumen undegradable intake protein and corresponded to a four- and nine-fold upregulation of *DNMT3b* in longissimus muscle of fetal tissue compared with dams fed corn and haylage based diets, respectively. Interestingly, an imprinted gene noted for its role in degrading IGF, *IGF2R*, was affected also upregulated in the higher protein diets compared with the corn-based treatment (Lan et al., 2013). This corresponded to greater methylation of intron 2 in *IGF2R* over multiple CpG islands likely being due to greater availability of dietary methyl groups from amino acids. The data suggests intragenic DNA methylation level was positively correlated to *IGF2R* expression which would be the opposite of conventional wisdom regrading greater methylation with lower expression. Some research suggests greater methylation outside of the promoter region in other parts of the gene body may increase expression when an anti-sense non-coding RNA gene may be present within the active gene (Suzuki and Bird, 2008; Langevin and Kelsey, 2013).

Few research efforts in livestock species have studied DNA methylation, but recently the methylation pattern of the entire genome, referred to as the "methylome", was characterized in fetal and adult bovine tissues (Huang et al., 2014a). Subsequent work out of the same group showed greater methylation in a differentially methylated region of the last exon of insulin-like growth factor 2 (*IGF2*) in six adult bovine tissues and which corresponded to lower mRNA

expression of *IGF2* compared with fetal bovine tissues (Huang et al., 2014b). A well-described imprinted gene, IGF2 is integral to muscle development, myoblast proliferation and differentiation (Stewart and Rotwein, 1996). Other research has suggested DNA methylation may be involved in the differentiation of mesenchymal cells within skeletal muscle to myoblasts and adipocytes. After separating the longissimus muscle and intramuscular fat (**IMF**), mRNA expression of adipose associated genes peroxisome proliferator-activated receptor gamma isoform 1 (*PPARG1*) and fatty acid binding protein 4 (*FABP4*) were much greater in IMF (Baik et al., 2014; Hong et al., 2014). Interestingly, they also observed less methylation in the promoter region CpG islands for *PPARG1* and *FABP4* in adipose IMF compared with skeletal muscle suggesting that methylation may be involved in controlling lipogenic gene expression in Korean cattle. One of the major limitations of studying DNA methylation is the vast assortment of methodologies available. As the technology continues to become more accessible to animal scientist, our understanding of DNA methylation to production-relevant traits will increase as well as our understanding of how diet and environmental factors can shape methylation patterns.

Histone modifications

Eukaryotic DNA is organized in chromatin and incorporated into many chromosomes. The chromatin configuration involves a basic nucleosome core units which consists of an octamer of four histones (H2A, H2B, H3, and H4) that among the most evolutionary conserved proteins (Van Holde, 1988). The nucleosome design is intended to facilitate transcription, replication and repair of DNA while still compactly folding the genome to fit into the nucleus. The N-terminal tail of histones can be post-translationally modified by various functional groups including methylation, acetylation, and phosphorylation (Hake et al., 2004; Canani et al., 2011) . The addition or removal of these functional groups change the physical conformation of the

chromatin such that it is relaxed and more accessible for transcription (euchromatin) or more compacted and repressive of gene expression (heterochromatin) (Grewal and Moazed, 2003).

Acetylation of histones at lysine residues is associated with upregulation of gene transcription while deacetylation of histones involves decreasing gene expression and is conducted by histone acetyltransferases (**HATs**) and deacetylases (**HDACs**), respectively (Hake et al., 2004). Acetylated histones typically correspond to transcriptionally competent regions compared with transcriptionally inactive regions with hypoacetylated histones. The effect of methylation on gene transcription is dependent on the position of lysine residues. Methylation of lysine 4 on histone 3 (H3K4Me) will increase gene expression and is typically observed in promoter regions. Alternatively, trimethylation on lysines 9 and 27 of histone 3 (H3K9Me2/3 and H3K27Me3) is associated with silenced expression and is widely observed in heterochromatic regions including centromeres and telomeres (Lachner and Jenuwein, 2002; Maison et al., 2002). In skeletal muscle HDAC1 interacts with myogenic activator, MyoD, to repress gene expression in undifferentiated myoblasts (Puri et al., 2001). Additional HDAC4 and 5 repress MEF2 activity necessary for myoblast differentiation (Lu et al., 2000). A histone methyltransferase is essential for the enforcement of satellite cell commitment by Pax7 as it recruits the complex to regulate *Myf5* transcriptionally (McKinnell et al., 2008). While little is known about the importance of histone modifications relative to skeletal muscle in cattle, their conserved role in regulating gene expression across many tissues is already establish.

Non-coding RNAs

Only about 1.5% of the human genome actually codes for proteins (The ENCODE Project Consortium, 2012). While initially thought to be irrelevant "junk" (Loomis and Gilpin, 1986), the discovery of non-coding RNAs over the last 15 years has changed that perspective.

The most well-known classes of non-coding RNAs include microRNAs (**miRNA**) and long non-coding RNAs (**lncRNA**) which have both been studied in the context of skeletal muscle. miRNAs are short RNAs about 22 nucleotides long that are capable of regulating gene expression post-transcriptionally as part of the RNA-induced silencing complex (Ambros, 2001; Bartel, 2009). Specifically, miRNAs can inhibit translation initiation, translation elongation, as well as co-translational degradation and premature termination of translation (Huntzinger and Izaurralde, 2011). Biogenesis of miRNA involves two key enzymes Drosha and Dicer, and begins as primary miRNA, followed by precursor miRNA, and then mature, active miRNA (Ha and Kim, 2014). The importance of miRNA in muscle development is well-documented by early research that indicated a conditional knockout of Dicer greatly reduced skeletal muscle mass and myofiber formation (Bernstein et al., 2003). There are 793 mature miRNA identified in cattle in miRBase 21 (Kozomara and Griffiths-Jones, 2014), but little experimental validation beyond high-throughput sequencing has been conducted. Three miRNAs (miR-1, miR-133, and miR-206) are specific to muscle and are regulated by myogenic regulator factors such as MyoD, myogenin, MEF2, SRF, YY1, and Twist (Braun and Gautel, 2011; Luo et al., 2013). Furthermore, the effect of miR-1 and miR-206 was evidenced by a single mutation in the myostatin gene of Texel sheep which allowed miR-1 and miR-206 to down-regulate myostatin and increase muscular hypertrophy in the breed (Clop et al., 2006). Recent sequencing efforts identified almost 350 miRNAs in longissimus muscle of beef cattle (Sun et al., 2014) suggesting less than half of all known miRNAs in cattle are expressed in muscle. Results in model species and cattle reveal inconsistent miRNA expression within fat and muscle tissue between specific depots and muscles (Muroya et al., 2013; Meale et al., 2014).

While miRNA directly regulates gene expression posttranscriptionally, they are engaged in epigenetic mechanisms in 2 ways. First, miRNA can target and down regulate enzymes involved in epigenetic regulation such as DNMT, HAT, and HDAC. Greater miR-29b will induce global hypomethylation by decreasing expression of DNMT1, DNMT3A, and DNMT3B which can specifically reactivate tumor suppressing genes (Garzon et al., 2009; Nestal de Moraes et al., 2015; Robaina et al., 2015). Alternatively, epigenetic controls are intertwined with regulating miRNA expression as well. Methylation of CpG dinucleotides in the promoter region of miRNA typically coincides to decreased transcription and subsequent induction of miRNA target genes (Swierczynski et al., 2015; Tu et al., 2015). Moisá et al. (2016) observed miR-34a expression in muscle of the progeny was effected by prior maternal nutrition and may be connected to adipogenesis. Recently, a database of epigenetic modifications and miRNA interactions has been built, EpimiR, from a compilation of experimentally validated results (Dai et al., 2014).

Long non-coding RNA are an assorted collection of non-coding RNA transcripts greater than 200 nucleotides with many newly-appreciated functions in physiological processes (Vance and Ponting, 2014). While they share common biogenesis pathways with other non-coding RNAs, lncRNAs are largest portion of the mammalian non-coding transcriptome (Mercer et al., 2009). Over 100,000 lncRNA have been annotated in the human genome (Volders et al., 2014) but only a few thousand are identified in livestock species in skin, muscle, and mammary gland tissue (Ibeagha-Awemu and Zhao, 2015). Understanding of specific bovine lncRNA is limited, but in humans lncRNA have been linked to multiple disease conditions and immune system development. One of the first lncRNA effects observed in muscle was the linc-MD1 regulation of transcription factors mastermind-like protein 1 (MAML1) and MEF2C (Fatica and Bozzoni,

2014). This lncRNA competitively binds to 2 miRNA to temporally regulate muscle differentiation (Cesana et al., 2011). Research on lncRNA is truly in its infancy and many questions remain on their specific function, localization, and conservation of the varied sizes of lncRNA.

Conclusion

Nutrition remains a key research area in animal science based on associated cost to production and ever expanding feedstuff choices. Consistent improvements in nutrient utilization depend on accurately defining nutrient requirements of individual animals and improving the efficiency of converting nutritional inputs into edible outputs. Compared to other major livestock species in the United States, beef cattle have the greatest obstacles in research implementation with less vertical integration and extreme variety in production environments and genetics. Future efforts to define key physical associative effects related to nutritional effects on skeletal muscle function, rumen microbiota, and epithelium, will be key to enhance nutrient utilization and industry sustainability.

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CHAPTER 2

INCREASING CORN DISTILLERS SOLUBLES ALTERS THE LIQUID FRACTION OF THE RUMINAL MICROBIOME

Abstract

Five ruminally-fistulated steers were used in a 5 × 5 Latin square design to determine the effects of increasing dietary fat and sulfur from corn distillers solubles (**CDS**) on the ruminal microbiome. Treatments included a corn-based control (**CON**) and 4 levels of CDS (0, 10, 19, and 27%) in a coproduct-based (corn gluten feed and soybean hulls) diet. Fat concentrations were 1.79, 4.43, 6.80, and 8.91%, respectively, for diets containing 0, 10, 19, and 27% CDS. Steers were fed for ad libitum intake once daily. After 18 d of adaptation to the diet, ruminal samples were collected 3 h post-feeding. Samples were separated into solid and liquid fractions. Microbial DNA was extracted for bacterial analysis using paired-end sequencing of the V4 region of the 16S rRNA gene on the MiSeq Illumina platform and quantitative PCR (**qPCR**) of selected species. Orthogonal contrasts were used to determine linear and quadratic effects of CDS inclusion. Increasing CDS inclusion decreased (linear; $P < 0.05$) alpha-diversity and species richness in the liquid fraction. Analysis of Bray-Curtis similarity indicated a treatment effect ($P = 0.01$) in the liquid fraction. At the phyla level, relative abundance of Bacteroidetes decreased in steers fed increasing dietary inclusion of CDS as Firmicutes increased to 82% of sequences for the 27% CDS treatment. Family Ruminococcaceae increased (linear; $P < 0.01$) 2-fold in the liquid fraction when feeding CDS increased from 0 to 27% CDS, yet genera *Ruminococcus* tended ($P = 0.09$) to decrease in steers fed greater CDS. The most abundant

family of sulfate-reducing bacteria, Desulfovibrionaceae, increased ($P < 0.03$) in the solid and liquid fraction in steers fed additional dietary CDS and sulfur. Relative abundance of family Veillonellaceae and *Selenomonas ruminantium* was increased (linear; $P \leq 0.02$) in the solid fraction as steers were fed increasing CDS. There were no effects ($P > 0.10$) of feeding increasing dietary fat from CDS on fibroylytic genus *Fibrobacter* in either fraction. Results demonstrate increasing fat and sulfur from CDS in a coproduct-based diet markedly alters the liquid fraction ruminal microbiome, but does not elicit negative effects on relative abundance of identified fiber-fermenting bacteria.

Keywords: distillers solubles, rumen, microbiome, bacteria

Introduction

Dietary fat is a concentrated energy source and may be fed to growing or lactating cattle. Recent studies suggest feedings coproduct-based diets to cattle during the growing phase results in similar marbling scores compared with those fed starch-based diets (Retallick et al., 2010; Meteer et al., 2011; Segers et al., 2012). Data suggest increased fat concentrations may be responsible for maintaining intramuscular fat deposition when cattle are fed coproducts, such as distillers grains with solubles. Lipids can affect ruminal fermentation by decreasing the acetate:propionate ratio and methane (Chalupa et al., 1984; Boggs et al., 1987), but are also capable of reducing VFA production and ruminal digestion of structural carbohydrates (Ikwuegbu and Sutton, 1982; Jenkins and Palmquist, 1984). Significant variation exists in the fatty acid profile of various feedstuffs and corresponds to known toxic effects of particular unsaturated fatty acids on specific rumen bacteria (Maczulak et al., 1981). Thus, the source of the dietary fat can greatly affect the aforementioned effects on ruminal digestion.

Condensed distillers solubles (**CDS**) is the most common nonfat liquid feed used to provide supplemental fat in feedlot cattle diets (Samuelson et al., 2016), and CDS typically ranges from 9 to 25% fat on a DM basis (Lardy, 2009). Including CDS up to 30% of the diet, without other coproducts improved performance but data indicated less CDS should be used in coproduct-based finishing diets (Pesta et al., 2015). High inclusion rates of distillers coproducts and specifically CDS may result in suitable growth performance, but the understanding of the effect of CDS on ruminal fermentation and the corresponding microbiome composition is limited. Given the relevance of CDS in the beef industry as an individual feedstuff and component of distillers grains with solubles, the objective was to determine changes in the ruminal microbiome associated with increasing inclusion of CDS in coproduct-based diets.

Materials and Methods

Experiment Design

The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana Champaign. Five ruminally-fistulated Angus-Simmental steers (BW = 335 $\pm$ 56 kg) were used in a 5 × 5 Latin square design to determine effects of increasing dietary CDS on digestion and ruminal fermentation (Segers et al., 2015). Dietary treatments included a corn-based control (CON) and 4 coproduct diets (corn-gluten and soy hulls) with increasing levels of CDS (0, 10, 19, and 27%). Fat concentrations were 1.79, 4.43, 6.80, and 8.91% for diets containing 0, 10, 19, and 27% CDS, respectively. Animals were fed once daily for ad libitum intakes and allowed ad libitum access to water. The 5 experimental periods consisted of 21 d with 18 d for adaption. Ruminal samples were collected 3 h post-feeding on d 19 via ruminal cannula from 3 locations in the rumen and separated into the liquid and solid fractions. Samples were immediately put on ice and kept at -20° C prior to extraction.

Feed samples were composited across period and 50 mg of each dried feedstuff (20 mg for CDS) was analyzed for fatty acid composition (Table 2.1) as previously described (Masood et al., 2005). C17:0 triacylglycerol was added as an internal standard at the extraction step and later used to quantify peak areas.

Bacterial DNA Extraction and qPCR Analysis

The solid fraction samples (25 g) were used for DNA extraction by first homogenizing the digesta followed by phenol/chloroform extraction as described by Stevenson and Weimer (2007). Liquid fraction samples (50 mL) were extracted using the ZR-96 Fecal DNA Kit (ZYMO Research, Irvine, CA). Extracted DNA was standardized to 8 ng/μL concentration for quantitative PCR and 20 ng/ μL for 16S rRNA sequencing. Extracted DNA was stored at -80° C for later use.

Bacterial quantitative PCR (qPCR) primers utilized are listed in Table 2.2 and were validated using gel electrophoresis and Sanger sequencing. Each 10 μL reaction consisted of 4 μL sample DNA, 5 μL 1× SYBR Green with ROX (Quanta BioSciences, Gaithersburg, MD), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL DNase/RNase free water in a MicroAmp^{TM} Optical 384-Well Reaction Plate (Applied Biosystems, Foster City, CA). All reactions were performed using an ABI Prism 7900 HT (Applied Biosystems, Foster City, CA) with the following conditions: 5 min at 95° C, 40 cycles of 1 s at 95° C and 30 sec at 60° C. The presence of a single PCR product was verified with an additional dissociation stage. All reactions were run in triplicate. Relative abundance of bacterial species was calculated using the geometrical mean of 2 universal primers with the efficiency-corrected Δ^{-CT} method (Ramirez-Farias et al., 2009). The portion of the 16S rRNA gene corresponding to the target of the eubacterial primer 3 (Muyzer et al., 1993) was commercially synthesized (IDT, Coralville, IA).

A standard curve from 9.5×10^7 to 3.0×10^4 molecules per µL was used to obtain the 16S copy number from each sample. Samples were diluted to 1 ng/µL for suitable qPCR performance of eubacterial primer 3.

Library Construction and 16S rRNA Sequencing

Amplification of the V4 region of the 16S rRNA gene used modified F515/R806 primers as described by Caporaso et al. (2012). The reverse PCR primer was indexed with 12-base Golay barcodes to facilitate multiplexing of samples. The PCR and sequencing protocol has been previously described in detail (Derakhshani et al., 2016). The 150 paired-end sequencing reaction was performed on a MiSeq platform (Illumina, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Canada.

16S rRNA Read Analysis

The PANDAseq assembler was implemented to merge overlapping paired-end Illumina fastq files (Masella et al., 2012). All the sequences with low quality base calling scores and uncalled bases (N) in the overlapping region were discarded. The subsequent fastq file was processed using the QIIME pipeline v1.8 (Caporaso et al., 2010b). Assembled reads were demultiplexed and quality filtered; reads were truncated after 3 consecutive bases with a quality score below 1e-5 and discarded if shorter than 75 bases. Chimeric reads were filtered using UCHIME (Edgar et al., 2011), and reads were clustered into OTU (Operational Taxonomic Units) based on 97% similarity with UCLUST (Edgar, 2010). Representative sequences from each OTU were assigned a taxonomy using RDP Classifier (Wang et al., 2007) and aligned to the Greengenes 13_5 reference database (McDonald et al., 2012) using PyNAST (Caporaso et al., 2010a).

After sample size standardization to the smallest sample library size (23,000 sequences), OTU richness, and alpha- and beta-diversity metrics were estimated. Alpha rarefaction curves were generated using the Chao1 metric (Chao, 1984). Between sample comparisons of diversity (beta-diversity) were calculated using the Bray-Curtis metric (Beals, 1984). Bray-Curtis distance matrices were utilized in principal coordinate analysis (PCoA) to generate two-dimensional plots in PRIMER v6 software (Clarke and Gorley, 2006). Permutational multivariate analysis of variance (PERMANOVA) was implemented to test differences in beta-diversity among treatments.

Statistical Analysis

Relative abundance of bacteria present at > 0.1% at the phyla and family and genus level were evaluated and logit transformed ($z = \log[p/(1-p)]$) if necessary to ensure normal distribution of the residuals, where p represents the relative abundance of a bacterial taxa. Bacterial relative abundance was analyzed using the MIXED procedure of SAS 9.3 (SAS Inst. Inc., Cary, NC). Terms in the model included treatment and period as fixed effects, and steer as a random effect. Treatment means were calculated using the LSMEANS option. Linear and quadratic contrasts evaluated level of CDS inclusion. The IML procedure was used to determine the coefficients for the nonlinear inclusion of CDS in the diets. Significance was declared at $P \leq 0.05$ while tendencies are discussed at $0.05 < P \leq 0.10$.

Results

A total of 1,617,146 quality-filtered reads were generated with an average of about 33,000 reads per sample. Sequencing depth ranged from 23,229 to 122,894. An average of 1,483 OTUs based on 97% similarity were obtained for each sample. Within the Greengenes database, 89.9 and 53.5% of sequences were identified at the family and genera taxonomic level,

respectively. At the community level, the largest effects were observed in the liquid fraction. The Chao1 index indicated a linear decrease ($P = 0.01$) in species richness when cattle were fed increasing concentrations of CDS in the diet (Table 2.3). Similarly, alpha-diversity decreased (linear; $P \leq 0.02$) with increased CDS inclusion as observed in the Shannon and Simpson's indices. Species richness and alpha-diversity for CON was the lowest of treatments in the liquid fraction and most similar to 27% CDS. In the solid fraction, no effect of CDS inclusion was observed on species richness. Analysis of beta-diversity, a comparative measure of diversity between samples, in the liquid fraction revealed a separation by treatment primarily by the second principal coordinate (Figure 2.1; $P = 0.01$). The first two principal coordinates collectively accounted for 64% of the observed variation between samples. A Spearman correlation greater than 0.8 indicated *Prevotella* was associated with the separation of 0% CDS samples (data not shown). However, there was no treatment effect ($P = 0.2$; data not shown) using the Bray-Curtis similarity of beta-diversity observed in the solid fraction by PERMANOVA analysis.

Liquid Fraction Microbiome Effects

Firmicutes was the most abundant phylum in the liquid fraction representing more than 70% of all sequences (Table 2.4). A linear increase ($P < 0.01$) in relative abundance of Firmicutes was observed in steers fed increasing concentrations of CDS. This increase in Firmicutes corresponded with a decrease (linear; $P \leq 0.01$) in Bacteroidetes, Cyanobacteria, and Spirochaetes. Within the phylum Firmicutes, the linear increase, when greater CDS was fed, was primarily driven by family Ruminococcaceae as its increase (linear; $P < 0.01$; Table 2.5) represented 75% of the increase in relative abundance at the phylum level. Phyla level effects of Bacteroidetes, Cyanobacteria, and Spirochaetes were observed at the family level ($P \leq 0.01$) in

Paraprevotellaceae, order Bacteroidales, Spirochaetaceae, and order YS2, respectively. Phylum Fibrobacteres was not affected ($P = 0.41$) by dietary treatment fed to cattle or to a linear increase in CDS ($P = 0.81$) despite a weak tendency ($P = 0.07$) for a quadratic response. A quadratic increase (P = 0.03) in relative abundance of Desulfovibrionaceae was observed with the greatest abundance detected at 19% CDS. At the genus level (Table 2.6), *Prevotella* was most abundant and tended (linear; $P = 0.08$) to decrease with greater CDS. Relative abundance of *Ruminococcus* and *Oscillospira* tended (linear; $P = 0.09$) to decrease with increasing CDS which was the opposite response observed for all reads assigned to the family Ruminococcaceae. Although the majority (~87%) of reads assigned to Ruminococcaceae were unassigned at the genus taxonomic level, the percentage of reads that did assign to *Ruminococcus* ranged from 36% for 0% CDS, to 4% for 27% CDS. *Bifidobacteria* and *Treponema* also decreased (linear; $P \leq 0.02$) with greater CDS inclusion. A quadratic response ($P = 0.03$) in *Coprococcus* relative abundance was observed and peaked at 19% CDS.

Solid Fraction Microbiome Effects

In the solid fraction, Firmicutes and Bacteroidetes comprised 82 to 90% percent of reads in a treatment (Table 2.7). Overall, few dietary effects were observed at the phyla level. Cyanobacteria was affected by dietary treatment ($P = 0.02$) with the lowest relative abundance observed for 27% CDS. The relative abundance of Firmicutes tended ($P = 0.10$) to increase with greater CDS inclusion. At the family level (Table 2.8), Veillonellaceae and Ruminococcaceae, members of the Firmicutes phyla, increased linearly ($P \leq 0.04$) with greater CDS inclusion. Veillonellaceae linear effects were primarily driven by the genus *Succiniclasticum* (Table 2.9) where more than 75% of the Veillonellaceae sequences classified at the genus level. Within Bacteroidetes, family Paraprevotellaceae and unidentified sequences in order Bacteroidales

decreased (linear; $P \leq 0.01$) with additional CDS. Cattle fed CON had a 4-fold increase in phyla Fibrobacteres, but no overall effect ($P = 0.11$) of dietary treatment or increasing CDS in the diet was detected ($P > 0.58$). Desulfovibrionaceae was affected by treatment ($P = 0.01$) with the lowest relative abundance observed for CON and the greatest for 19% CDS. A quadratic response ($P \leq 0.01$) was observed for *Moryella* with the lowest relative abundances observed for 10 and 19% CDS, while *Mitsuokella* and *Coprococcus* increased with greater CDS (linear; $P \leq 0.04$). A main effect of treatment ($P = 0.03$) was observed for *Corynbacterium* with the greatest relative abundance observed for 19% CDS which was 2-fold greater than any other treatment.

The relative abundance of bacterial species measured using qPCR in the solid fraction revealed a linear increase ($P = 0.02$) of *Selenomonas ruminantium* with increasing CDS inclusion (Table 2.10). In contrast, a decrease (linear; $P = 0.01$) in relative abundance of *Streptococcus bovis* occurred with greater CDS primarily driven by the 4-fold higher values observed for 0% CDS. Moreover, *S. bovis* populations in the CON diet were nearly 26-fold greater than the 0% CDS. Although no effect of CDS inclusion was observed for *Megasphaera elsdenii*, there was a tendency ($P = 0.09$) for a 45-fold reduction for cattle fed CON compared with those fed any of the coproduct-based diets. Variation observed in relative abundance of *Anaerovibrio lipolytica* led to no differences ($P > 0.11$) despite a nearly 9-fold increase for 0% CDS. A trend ($P = 0.06$) for an increase in 16S log copy number was observed with increasing CDS.

Discussion

Many studies have evaluated the effects of supplemental fat on ruminal fermentation and biohydrogenation (Sackmann et al., 2003; Atkinson et al., 2006; Hess et al., 2008). However, the variation in basal diet composition, saturation of the supplemented fatty acids, and the amount of additional fat provided all contribute to differences observed for fermentation and

bacterial effects. The fatty acid content of the CDS used in this experiment was similar to a previous report by Sasikala-Appukuttan et al. (2008). The data from Sasikala-Appukuttan et al. (2008) revealed that the addition of CDS from 10 to 20% of the diet increased ruminal ammonia and the molar proportion of butyrate, but propionate and acetate concentrations were not affected. Similarly, the corresponding ruminal fermentation results for the present study reported by Segers et al. (2015) indicated neither acetate, propionate, and ruminal pH nor total tract NDF digestion were affected by dietary treatment. However, greater NDF digestion has been observed when cattle fed diets containing wet distillers grains with solubles were compared with cattle fed a corn bran and gluten meal diet with corn oil at similar levels of ether extract (Vander Pol et al., 2009). Although dietary fatty acid composition was not reported, the fatty acid profile of corn oil (Gillis et al., 2004) is similar to CDS with C18:2 representing more than 50% of fatty acids. Cattle fed the diet containing wet distillers grains with solubles had a greater proportion of unsaturated fatty acids (18:1 trans, 18:1, 18:2, and 18:3) flowing to the duodenum compared with cattle fed the corn oil diet suggesting differential levels of biohydrogenation (Vander Pol et al., 2009). Variation in biohydrogenation of feedstuffs with similar fatty acid content is supported by the difference in biohydrogenation observed between corn and corn oil (Duckett et al., 2002). Collectively, the data suggest reduced biohydrogenation and increased lipid digestibility likely contribute to positive animal responses to wet distillers grains with solubles compared with corn oil (Klopfenstein et al., 2008). Considering the varied effects of different fat sources with a similar fatty acid profile, the effect of CDS on the ruminal microbiome is an important piece to understand the effects of high levels of coproduct inclusion in beef cattle diets.

The first evaluation of CDS effects on rumen bacteria in vivo revealed a tendency to increase counts of total culturable, amylolytic, and lactilytic bacteria (Fron et al., 1996). Despite the increased inclusion of CDS in ruminant diets with greater ethanol production, this is the first study since Fron et al. (1996) to evaluate the effect of CDS on ruminal bacteria. Our 16S rRNA $\log_{10}$ copy number results support their findings suggesting an increase in bacteria in the liquid fraction with greater CDS. Compared with other fat sources, the sulfur and phosphorus concentrations and low pH of CDS make it a unique supplemental fat source among those fed to ruminants. While most of the lipids in CDS are incorporated into triacylglycerol, it does contain much greater concentrations of free fatty acids compared with corn oil (Moreau et al., 2011).

The effect of CDS inclusion was greater in the liquid fraction due to observed changes in community level measures of alpha-diversity, species richness, and beta-diversity. While unsaturated fatty acids have long been known to inhibit fiber-degrading bacteria (Henderson, 1973), recent studies have observed no effect on community alpha-diversity with additional dietary lipids in rumen fluid (Zened et al., 2013; Huws et al., 2015). However, Huws et al. (2010) observed decreased denaturing gradient gel electrophoresis (DGGE) band numbers in liquid-associated bacteria, but not solid-associated bacteria, when cows fed a red clover silage diet were supplemented with fish oil. Supplemental fish oil also reduced DGGE band number in the liquid fraction when cattle were fed a grass silage diet, but alpha-diversity was not affected (Kim et al., 2008).

Within the liquid fraction, greater CDS inclusion increased relative abundance of Firmicutes and decreased Bacteroidetes primarily driven by corresponding changes in Ruminococcaceae and *Prevotella*, respectively. Adding sunflower oil to a silage-based diet fed to cattle caused similar numerical effects as relative abundance of Firmicutes increased while

Bacteroidetes decreased (Zened et al., 2013); however, authors suggested large variation within these low starch diets prevented detection of statistical differences. A comparison of the data suggests more than 12 d may be needed for some animals to fully adapt to dietary changes as our samples were collected on d 19 of each period. Furthermore, despite the fact that the diets had similar NDF concentrations, sources of NDF varied significantly from silage and alfalfa hay in diet of Zened et al. (2013) compared with a mixture of silage, soy hulls, and corn gluten feed in this experiment. Our results agree with previous findings for *Treponema*, as it was decreased by the addition of fat as sunflower oil (Zened et al., 2013) and CDS in our experiment. Although cultured strains are not cellulolytic, *Treponema bryantii* increased fiber degradation in co-culture with *Fibrobacter succinogenes* (Stanton and Canale-Parola, 1980).

At the family taxonomic level, Ruminococcaceae increased with CDS inclusion, but the opposite tendency occurred within the genus *Ruminococcus* as it decreased with greater CDS. Ruminococcus assigned reads likely correspond to a greater proportion of cultured Ruminococci with cellulolytic capabilities and known sensitivities to unsaturated fatty acids (Maczulak et al., 1981; Maia et al., 2007). The fact that a large proportion of Ruminococcaceae reads unidentified at the genus level have been commonly observed in 16S rRNA sequencing studies (Zened et al., 2013; McCann et al., 2014) indicates many Ruminococcaceae members remain uncultured.

The phylum Cyanobacteria increased significantly in the 0% CDS diet with nearly all reads assigned to the order YS2. Although prior work on the ruminal microbiome has identified 16S rRNA reads as Cyanobacteria (Mao et al., 2013; Zhao et al., 2015), typically the reported relative abundances are under 1% compared with the 7% we observed in the liquid of fraction ruminal fluid from cattle fed the 0% CDS diet. Classically considered to be photosynthetic organisms, a new lineage within Cyanobacteria, Melainabacteria, has recently been observed in

human fecal samples and is nonphotosynthetic (Di Rienzi et al., 2013; Soo et al., 2014). Previous studies have been able to assemble draft genomes from metagenomic DNA extracted from koala feces with a high prevalence of Melainabacteria (Soo et al., 2014). FeFe hydrogenases observed in the gut associated genomes suggest Melainabacteria may produce hydrogen and interact with hydrogenotrophic methanogens or acetogens (Di Rienzi et al., 2013). In addition, the Melainabacteria genomes encoded for the complete biosynthesis pathways of 4 B vitamins and may indicate a mutualistic relationship with the host (Di Rienzi et al., 2013).

Overall effects of CDS inclusion in the solid fraction were more modest. Little change was observed in community-level measures of diversity and phylum-level relative abundance. Similar to the liquid fraction, Ruminococcaceae and Veillonellaceae increased slightly in the solid fraction with greater CDS. Corresponding increases in *Succiniclasticum* and *Mitsuokella* with increasing CDS fed in the diet agree with the description of cultured species in vitro. Out of 22 rumen bacteria cultures, *Mitsuokella multiacidus* was able to form oleic acid from linoleic acid and ranked second in terms of membrane stability in presence of linoleic acid (Maia et al., 2007). Another Veillonellaceae family member, *Selenomonas ruminantium* was detected using qPCR and also increased linearly with CDS inclusion in the diet. While unaffected by polyunsaturated fatty acids in vitro (Maia et al., 2007), the addition of oleic acid increases growth of *S. ruminantium* in vitro (Maczulak et al., 1981).

Within the solid fraction, there was no effect of additional CDS on relative abundance of *Fibrobacter succinogenes*, as indicated by qPCR and supported by 16S rRNA sequencing results at the phyla level. While Huws et al. (2010) observed no effect on *F. succinogenes* in the rumen by the addition of fish oil to the diet, genus Fibrobacter decreased in cattle fed a diet supplemented with flax oil (Huws et al., 2015). In vitro, *F. succinogenes* is very sensitive to

unsaturated fatty acids as C18:2 slowed growth and C18:3 inhibited growth completely (Maia et al., 2007). Despite the increase in C18:2 and C18:3 with greater CDS inclusion in the diet, no effect of diet on Fibrobacter was observed in the present study. In a 60% brome hay-based diet, total Fibrobacteres increased in cattle fed greater dietary distillers grains with solubles, but a specific OTU classified as *F. succinogenes* remained unaffected by diet (Castillo-Lopez et al., 2014). Overall, these findings suggest Fibrobacteres may occupy a niche within the rumen that offers some protection from unsaturated fatty acids in CDS.

The most well-known rumen bacteria with lipolytic capabilities is *Anaerovibrio lipolytica*. Interestingly, *A. lipolytica* increased sharply on the 0% CDS diet which contained the least amount of fat. Early research on *A. lipolytica* indicated sensitivity to low pH for growth and lipase activity (Hobson, 1965; Henderson, 1971) and is supported by recent work in dual-flow fermenters (Fuentes et al., 2009). While Segers et al. (2015) reported no dietary effect on ruminal pH, results from 0% CDS revealed that it was the most stable throughout the day in addition to being the only diet without a distillers coproduct.

As the inclusion of CDS increased, dietary S increased and the relative abundance of sulfate-reducing family Desulfovibrionaceae increased in both the solid and liquid fractions. Recommendations for minimum S for growing beef cattle are 0.15% to meet the requirements of cellulolytic bacteria, while 0.3% has been suggested as a maximum to avoid reducing the risk of limiting DMI and occurrence of S-induced polioencephalomalacia (S-PEM). Loerch et al. (2012) observed a 15 d adaptation period for ruminal H_2S to increase after starting lambs on a diet with added sodium sulfate, thus suggesting our sampling on d 19 was sufficient time for Desulfovibrionaceae to respond. Although high dietary S has been shown to limit intake (Sarturi et al., 2013) which could affect the ruminal microbiome, a reduction in DMI was not observed

(Segers et al., 2015) within the experimental period of 21 d. Overall, our data support preliminary results described by Drewnoski et al. (2014) that Desulfovibrionaceae is the most abundant sulfate-reducing bacterial family in the rumen and it responds to greater dietary S by increasing in relative abundance.

Implications

Addition of CDS to a coproduct-based diet up to 27% caused the greatest change within the liquid fraction of the ruminal microbiome. Specifically, greater CDS inclusion reduced species richness, alpha-diversity, and relative abundance of Bacteroidetes while increasing Ruminococcaceae. Overall alterations in the solid fraction microbiome were modest, but notable increases in *Succiniclasticum*, *Mitsuokella,* and *S. ruminantium* were observed with greater CDS. Desulfovibrionaceae increased with greater dietary S from CDS in both fractions with greatest relative abundance observed at 19% CDS. An unusually large proportion of Cyanobacteria were observed on the 0% CDS diet and suggest non-photosynthetic Cyanobacteria may have a niche in the rumen. Overall, results indicate important alterations to the liquid fraction ruminal microbiome when increasing dietary inclusions of CDS are fed in a coproduct based diet without significant alterations to fiber-fermenting bacteria.

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Table and Figures

Table 2.1. Dietary fatty acid composition

		CDS Inclusion[1]				CDS[2]
Item	CON	0%	10%	19%	27%	100%
Ether extract, % DM	5.53	1.79	4.43	6.80	8.91	27.94
Fatty acids, g/100 g of total fatty acids						
C16:0	15.73	21.79	16.48	15.29	14.70	13.39
C18:0	2.35	4.47	2.71	2.35	2.13	1.69
C18:1 n-9	24.59	20.67	23.48	24.26	24.58	25.27
C18:2 n-6	48.46	44.13	52.14	54.12	54.99	57.38
C18:3 n-3	1.78	5.49	2.84	2.22	1.94	1.18
C20:0	0.42	0.69	0.46	0.40	0.38	0.32
C20:1 n-9	0.62	0.57	0.39	0.35	0.33	0.27
C22:0	0.40	0.71	0.40	0.32	0.29	0.20

[1]CDS= condensed distillers solubles.

[2]Dietary fatty acid composition of the ingredient.

Table 2.2. Primers utilized for quantitative PCR of ruminal bacteria.

Bacteria species		Primers (5` - 3`)	Source
Anaerovibrio lipolytica	F	GAAATGGATTCTAGTGGCAAACG	(Minuti et al., 2015)
	R	ACATCGGTCATGCGACCAA	
Butyrivibrio proteoclasticus	F	GGGCTTGCTTTGGAAACTGTT	(Minuti et al., 2015)
	R	CCCACCGATGTTCCTCCTAA	
Eubacterium ruminantium	F	CTCCCGAGACTGAGGAAGCTTG	(Stevenson and Weimer, 2007)
	R	GTCCATCTCACACCACCGGA	
Fibrobacter succinogenes	F	GCGGGTAGCAAACAGGATTAGA	(Stevenson and Weimer, 2007)
	R	CCCCCGGACACCCAGTAT	
Megaspheara elsdenii	F	AGATGGGGACAACAGCTGGA	(Stevenson and Weimer, 2007)
	R	CGAAAGCTCCGAAGAGCCT	
Prevotella bryantii	F	AGCGCAGGCCGTTTGG	(Stevenson and Weimer, 2007)
	R	GCTTCCTGTGCACTCAAGTCTGAC	
Selenomonas ruminantium	F	CAATAAGCATTCCGCCTGGG	(Stevenson and Weimer, 2007)
	R	TTCACTCAATGTCAAGCCCTGG	
Streptococcus bovis	F	TTCCTAGAGATAGGAAGTTTCTTCGG	(Stevenson and Weimer, 2007)
	R	ATGATGGCAACTAACAATAGGGGT	
Eubacterial primer 1	F	GGATTAGATACCCTGGTAGT	(Fliegerova et al., 2014)
	R	CACGACACGAGCTGACG	
Eubacterial primer 2	F	GTGSTGCAYGGYTGTCGTCA	(Maeda et al., 2003)
	R	ACGTCRTCCMCACCTTCCTC	
Eubacterial primer 3	F	CCTACGGGAGGCAGCAG	(Muyzer et al., 1993)
	R	ATTACCGCGGCTGCTGG	

Table 2.3. Effect of increasing corn distillers solubles (CDS) on alpha diversity in the liquid and solid fraction of the ruminal microbiome[1]

		CDS Inclusion				*P*-values[2]		
Item[3]	CON	0%	10%	19%	27%	Trt	L	Q
Liquid fraction								
Chao1	1407	2188	2013	1729	1463	0.03	0.01	0.67
Shannon	5.19	6.58	5.90	5.57	5.26	<0.01	<0.01	0.47
Simpson's	0.880	0.950	0.898	0.907	0.893	0.04	0.02	0.18
Solid fraction								
Chao1	2428	2476	2692	2454	2494	0.89	0.86	0.63
Shannon	7.24	7.19	7.46	7.29	7.48	0.64	0.37	0.79
Simpson's	0.98	0.96	0.97	0.97	0.98	0.37	0.07	0.80

[1]Number of observations: CON (n = 5), 0% (n = 4), 10% (n = 5), 19% (n = 5), 27% (n = 5).
[2]Trt = main effect of dietary treatment; L = linear contrast of CDS inclusion; Q = quadratic contrast of CDS inclusion.
[3]Chao1 index describes species richness in a community. Shannon and Simpson's indices describe alpha diversity of a community that represent a combination of species richness and species evenness.

Table 2.4. Effect of increasing corn distillers solubles (CDS) on relative abundance of bacterial phyla in the liquid fraction using 16S rRNA sequencing[1]

		CDS Inclusion				*P*-values[2]		
Item	CON	0%	10%	19%	27%	Trt	L	Q
Firmicutes	78.4	53.8	73.6	78.4	82.4	<0.01	<0.01	0.09
Bacteroidetes[3]	12.6	25.3	13.6	8.3	9.2	0.08	0.01	0.22
Actinobacteria	2.41	3.27	5.41	5.16	4.11	0.17	0.56	0.11
TM7	1.92	1.81	1.62	1.70	1.02	0.66	0.31	0.60
Cyanobacteria[3]	0.61	7.17	1.26	0.42	0.53	<0.01	<0.01	0.04
Proteobacteria	1.31	1.39	1.32	1.0	0.79	0.53	0.14	0.68
Spirochaetes[3]	0.17	1.23	0.37	0.09	0.24	0.01	<0.01	0.03
Fibrobacteres[3]	0.04	0.04	0.02	0.02	0.04	0.41	0.81	0.07

[1]Phyla listed were detected at greater than 0.1% relative abundance averaged across all liquid fraction samples.

[2]Trt = main effect of dietary treatment; L = linear contrast of CDS inclusion; Q = quadratic contrast of CDS inclusion.

[3]Data were logit transformed to ensure normality of residuals.

Table 2.5. Effect of increasing corn distillers solubles (CDS) on relative abundance of bacterial families in the liquid fraction using 16S rRNA sequencing[1]

		CDS Inclusion				*P*-value[2]		
Item	CON	0%	10%	19%	27%	Trt	L	Q
Firmicutes								
Ruminococcaceae	44.5	16.3	32.5	31.6	37.8	<0.01	<0.01	0.20
Mogibacteriaceae	1.35	0.79	1.04	1.03	1.51	0.35	0.09	0.63
Erysipelotrichaceae[3]	0.60	0.99	0.70	0.51	0.37	0.50	0.09	0.97
Clostridiaceae[3]	0.27	0.51	0.31	0.26	0.25	0.34	0.06	0.39
Bacteroidetes								
Prevotellaceae[3]	9.82	15.91	9.44	6.96	8.14	0.37	0.08	0.28
Bacteroidales[3,4]	1.14	4.59	1.61	0.40	0.39	<0.01	<0.01	0.34
Paraprevotellaceae[3]	0.66	1.42	0.82	0.15	0.38	0.01	<0.01	0.13
Proteobacteria								
Succinovibrionaceae	0.91	0.27	0.23	0.32	0.14	<0.01	0.57	0.53
RF-32[3,4]	0.10	0.16	0.11	0.05	0.04	0.11	0.01	0.91
Desulfovibrionaceae	0.014	0.0001	0.063	0.077	0.034	0.12	0.21	0.03
Other								
YS2[3,4]	0.53	7.19	1.24	0.40	0.51	<0.01	0.01	0.04
Bifidobacteriaceae	0.08	1.98	1.71	0.83	0.50	0.04	0.02	0.80
Spirochaetaceae	0.22	1.93	0.42	0.11	0.40	<0.01	<0.01	0.01
Corynebacteriaceae	0.11	0.18	0.12	0.26	0.32	0.09	0.07	0.25
Fibrobacteriaceae[3]	0.039	0.048	0.020	0.018	0.045	0.41	0.28	0.07

[1]Families listed were detected at greater than 0.1% relative abundance averaged across all liquid fraction samples and were affected by dietary treatment ($P < 0.1$).

[2]Trt = main effect of dietary treatment; L = linear contrast of CDS inclusion; Q = quadratic contrast of CDS inclusion.

[3]Data were logit transformed to ensure normality of residuals.

[4]Unidentifed sequences listed at the lowest level of taxonomic assignment (order).

Table 2.6. Effect of increasing corn distillers solubles (CDS) on relative abundance of bacterial genera in the liquid fraction using 16S rRNA sequencing[1]

		CDS Inclusion				*P*-value[2]		
Item	CON	0%	10%	19%	27%	Trt	L	Q
Prevotella[3]	9.82	15.9	9.44	6.96	8.14	0.37	0.08	0.28
Ruminococcus	8.23	5.97	3.82	1.79	1.55	0.09	0.09	0.69
Bifidobacterium	0.08	1.94	1.67	0.82	0.50	0.04	0.02	0.81
Treponema[3]	0.17	1.27	0.36	0.09	0.24	0.01	<0.01	0.02
CF231[3]	0.23	0.94	0.47	0.04	0.22	0.03	0.02	0.12
Oscillospira	0.17	0.60	0.33	0.26	0.10	0.45	0.09	0.80
RFN-20	0.09	0.88	0.14	0.06	0.02	0.01	<0.01	0.62
Coprococcus[3]	0.10	0.16	0.26	0.44	0.10	0.07	0.73	0.03
Corynebacterium	0.11	0.18	0.12	0.26	0.32	0.09	0.07	0.25
Clostridium	0.03	0.36	0.11	0.08	0.08	0.03	0.02	0.21
Shuttleworthia[3]	0.18	0.04	0.05	0.27	0.03	0.03	0.62	0.05

[1]Genera listed were detected at greater than 0.1% relative abundance averaged across all liquid fraction samples and were affected by dietary treatment ($P < 0.1$).
[2]Trt = main effect of dietary treatment; L = linear contrast of CDS inclusion; Q = quadratic contrast of CDS inclusion.
[3]Data were logit transformed to ensure normality of residuals.

Table 2.7. Effect of increasing corn distillers solubles (CDS) on relative abundance of bacterial phyla in the solid fraction using 16S rRNA sequencing[1]

		CDS Inclusion				*P*-value[2]		
Item	CON	0%	10%	19%	27%	Trt	L	Q
Firmicutes	52.6	55.0	65.1	67.2	69.2	0.18	0.10	0.52
Bacteroidetes[3]	37.7	32.4	16.7	17.0	20.5	0.28	0.35	0.24
Actinobacteria	5.29	5.55	6.72	6.69	4.96	0.86	0.84	0.36
TM7[3]	0.48	1.32	0.58	0.75	0.41	0.42	0.12	0.81
Cyanobacteria[3]	0.08	0.11	0.07	0.11	0.02	0.02	0.01	0.09
Proteobacteria[3]	0.24	0.25	0.21	0.58	0.20	0.25	0.81	0.33
Spirochaetes[3]	0.10	0.19	0.06	0.08	0.04	0.36	0.10	0.57
Fibrobacteres[3]	0.23	0.04	0.06	0.04	0.04	0.11	0.97	0.58

[1]Phyla listed were detected at greater than 0.1% relative abundance averaged across all solid fraction samples.

[2]Trt = main effect of dietary treatment; L = linear contrast of CDS inclusion; Q = quadratic contrast of CDS inclusion.

[3]Data were logit transformed to ensure normality of residuals.

Table 2.8. Effect of increasing corn distillers solubles (CDS) on relative abundance of bacterial families in the solid fraction using 16S rRNA sequencing[1]

		CDS Inclusion				*P*-value[2]		
Item	CON	0%	10%	19%	27%	Trt	L	Q
Firmicutes								
Veillonellaceae	10.5	7.0	10.4	11.4	14.9	0.10	0.01	0.88
Ruminococcaceae	11.3	8.1	9.8	11.8	12.3	0.27	0.04	0.79
Bacteroidetes								
Paraprevotellaceae	1.69	2.90	0.71	0.94	0.39	0.05	0.01	0.17
Bacteroidales[4]	0.96	2.30	0.40	1.10	0.47	0.01	<0.01	0.05
S24-7[3]	0.89	0.21	0.56	0.48	0.57	0.09	0.07	0.24
Other								
Corynebacteriaceae	0.07	0.14	0.14	0.32	0.06	0.03	0.93	0.05
Succinivibrionaceae[3]	0.08	0.02	0.03	0.07	0.02	0.08	0.89	0.11
Desulfovibrionaceae[3]	0.008	0.013	0.052	0.214	0.093	0.01	0.01	0.09

[1]Families listed were detected at greater than 0.1% relative abundance averaged across all solid fraction samples and were affected by dietary treatment ($P < 0.1$).
[2]Trt = main effect of dietary treatment; L = linear contrast of CDS inclusion; Q = quadratic contrast of CDS inclusion.
[3]Data were logit transformed to ensure normality of residuals.
[4]Unidentifed sequences listed at the lowest level of taxonomic assignment (order).

Table 2.9. Effect of increasing corn distillers solubles (CDS) on relative abundance of bacterial genera in the solid fraction using 16S rRNA sequencing[1]

		CDS Inclusion				*P*-value[2]		
Item	CON	0%	10%	19%	27%	Trt	L	Q
Succiniclasticum	9.4	5.2	8.4	9.3	11.5	0.16	0.02	0.86
Moryella	1.7	1.9	0.9	0.9	1.5	0.03	0.18	<0.01
Coprococcus[3]	0.23	0.40	0.71	0.97	0.81	<0.01	0.04	0.16
Shuttleworthia[3]	1.01	0.32	0.26	0.77	0.48	0.09	0.20	0.89
Mitsuokella	0.04	0.05	0.72	0.47	0.98	0.02	0.01	0.72
Corynebacterium	0.07	0.14	0.14	0.32	0.06	0.03	0.93	0.05

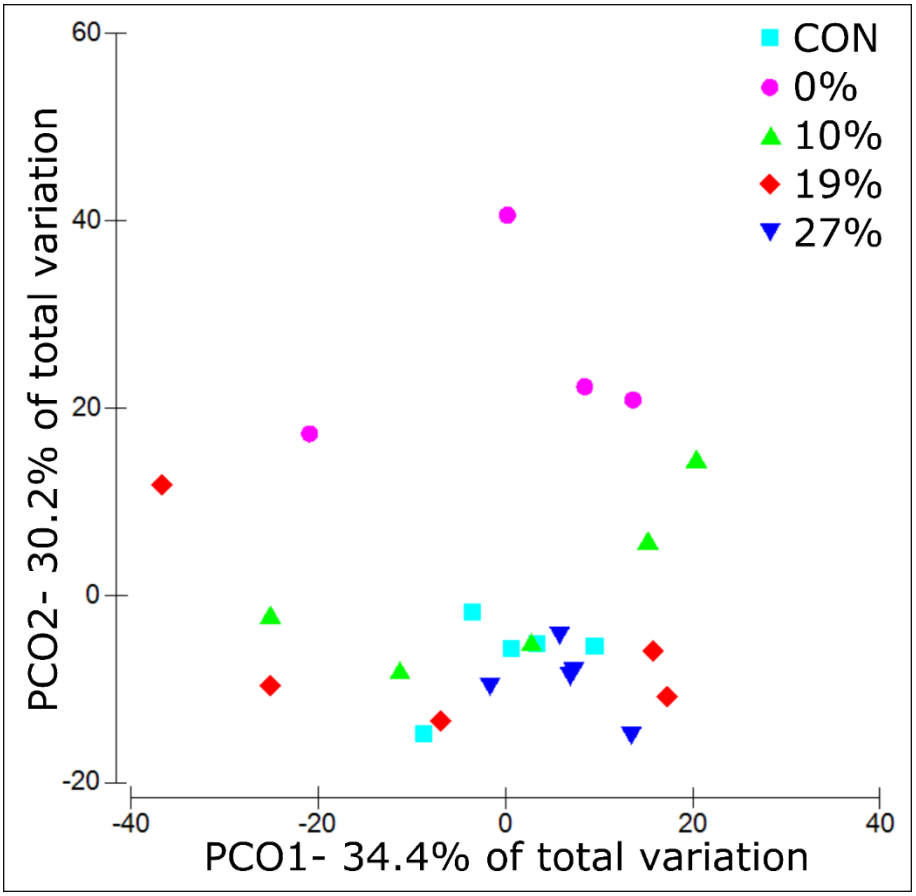
[1]Genera listed were detected at greater than 0.1% relative abundance averaged across all solid fraction samples and were significantly affected by dietary treatment ($P < 0.1$).
[2]Trt = main effect of dietary treatment; L = linear contrast of CDS inclusion; Q = quadratic contrast of CDS inclusion.
[3]Data were logit transformed to ensure normality of residuals.

Table 2.10. Effect of increasing corn distillers solubles (CDS) on relative abundance of bacterial genera in the solid fraction using qPCR.

		CDS Inclusion				*P*-values[1,2]		
Item	CON	0%	10%	19%	27%	Trt	L	Q
A. lipolytica[3]	0.0001	0.0044	0.0002	0.0002	0.0005	0.27	0.21	0.11
B. proteoclasticus[3]	0.0158	0.0747	0.0381	0.0226	0.0537	0.36	0.54	0.23
E. ruminantium	0.2871	0.2089	0.2322	0.2455	0.1814	0.96	0.90	0.69
F. succinogenes[3]	0.0065	0.0058	0.0079	0.0026	0.0027	0.47	0.21	0.70
M. elsdenii[3]	2.8×10^{-5}	1.7×10^{-3}	1.3×10^{-3}	3.7×10^{-3}	1.3×10^{-3}	0.09	0.96	0.79
P. bryantii[3]	2.3×10^{-5}	2.5×10^{-5}	5.2×10^{-5}	1.1×10^{-5}	2.4×10^{-5}	0.69	0.64	0.95
S. ruminantium	0.54	0.83	1.56	1.20	2.18	0.02	0.02	0.69
S. bovis[3]	0.0907	0.0035	0.0006	0.0008	0.0005	<0.01	0.01	0.12
16S rRNA copy no.[4]	7.41	7.30	7.35	7.36	7.40	0.21	0.06	0.94

[1]No period effects were observed ($P < 0.05$).
[2]Trt = main effect of dietary treatment; L = linear contrast of CDS inclusion; Q = quadratic contrast of CDS inclusion.
[3]Data were logit transformed to ensure normality of residuals.
[4]16S rRNA $\log_{10}$ copy number/ng DNA.

Figure 2.1. Principal coordinate analysis (PCoA) of beta-diversity in the liquid fraction using Bray-Curtis similarity. Analysis by PERMANOVA revealed a treatment effect (P = 0.01).



CHAPTER 3

INDUCTION OF SUBACUTE RUMINAL ACIDOSIS AFFECTS THE RUMINAL MICROBIOME AND EPITHELIUM

Abstract

Subacute ruminal acidosis (SARA) negatively impacts the dairy industry by decreasing dry matter intake, milk production, profitability, and increasing culling rate and death loss. Six ruminally-cannulated, lactating Holstein cows were used in a replicated incomplete Latin square design to determine the effects of SARA induction on the ruminal microbiome and epithelium. Experimental periods were 10 d with d 1 - 3 for ad libitum intake of control diet, followed by 50% feed restriction on d 4, and ad libitum access on d 5 to the basal diet or the basal diet with an additional 10% of a 50:50 wheat/barley pellet. Based on subsequent ruminal pH, cows were grouped (SARA grouping; SG) as Non-SARA or SARA based on time < 5.6 pH (0 and 3.4 h, respectively). Ruminal samples were collected on d 1 and 6 of each period prior to feeding and separated into liquid and solid fractions. Microbial DNA was extracted for bacterial analysis using 16S rRNA gene paired-end sequencing on the MiSeq Illumina platform and quantitative PCR (qPCR). Ruminal epithelium biopsies were taken on d 1 and 6 before feeding. Quantitative RT-PCR was used to determine gene expression in rumen epithelium. Bray-Curtis similarity indicated samples within the liquid fraction separated by day and coincided with an increased relative abundance of genera *Prevotella*, *Ruminococcus*, *Streptococcus*, and *Lactobacillus* on d 6 ($P < 0.06$). Although Firmicutes was the predominant phyla in the solid fraction, a SG × day interaction ($P < 0.01$) indicated a decrease on d 6 for SARA cows. In contrast, phylum

Bacteroidetes increased on d 6 ($P < 0.01$) for SARA cows driven by greater genera *Prevotella* and YRC22 ($P < 0.01$). *Streptococcus bovis* and *Succinivibrio dextrinosolvens* populations tended to increase on d 6 but were not affected by SG. In ruminal epithelium, *CLDN1* and *CLDN4* expression increased on d 6 ($P < 0.03$) 24 h after SARA induction and a tendency for a SG × day interaction ($P < 0.10$) was observed for *CLDN4*. Overall, results indicate more rapid adaptation to an induced bout of SARA in the solid fraction ruminal microbiome compared with ruminal epithelium.

Keywords: subacute ruminal acidosis, rumen, microbiome, ruminal epithelium, bacteria

Introduction

The nutrient density of dairy cattle diets has increased to maintain consistent improvements in milk yield (Plaizier et al., 2008). These dietary shifts, primarily achieved via greater concentrate inclusion relative to forage, can lead to an accumulation of volatile fatty acids in the rumen and reduced buffering capacity (Kleen et al., 2003; Stone, 2004). An overall reduction in ruminal pH such that it remains < 5.6 for more than 3 hours per day has been defined as subacute ruminal acidosis (**SARA**) (Gozho et al., 2005). Compared with acute ruminal acidosis, SARA is not associated with accumulation of lactic acid in the rumen (Oetzel et al., 1999). The effects of SARA extend beyond ruminal pH and include rumen epithelial damage (Steele et al., 2011), laminitis (Cook et al., 2004), inflammation (Khafipour et al., 2009b), decreased dry matter intake (Stock and Smith, 2000; Kleen et al., 2003), lower milk yield (Stone, 1999), reduced in situ fiber degradation (Plaizier et al., 2001), and liver abscesses (Dirksen et al., 1985). Prevalence of SARA has been documented from 19 - 26% in early to mid-lactation cows (Garrett et al., 1997; Oetzel et al., 1999) and thus represents a significant concern for the dairy industry.

Changes in ruminal fermentation and function are the source of the multi-faceted and unfavorable consequences of SARA. Although typically described by ruminal pH, multiple reports indicate SARA effects are caused by a combination of ruminal pH and diet type (Mould and Ørskov, 1983; Russell, 1998; Calsamiglia et al., 2008; Khafipour et al., 2009a). Altering the timing and availability of dietary substrate composition may modify the bacterial community function and composition within the rumen. Understanding the shifts in the ruminal microbiome related to the observed changes in ruminal pH may uncover bacteria critical to the onset of SARA. Furthermore, effects on the microbiome may provide a more suitable definition of SARA. Advancements in high-throughput sequencing have facilitated description of bacterial communities at unprecedented detail.

Rumen epithelial tissue has many functions including nutrient absorption, metabolism, pH regulation, as well as immune and barrier functions. Impairment of barrier function has been classically linked to a decreased pH associated with periods of rapid fermentation (Gäbel et al., 1987; Aschenbach et al., 2011). The primary proteins identified in rumen epithelial tissue associated with barrier function include claudin-1 and zona occludin-1 both of which are localized in the stratum granulosum (Graham and Simmons, 2005). The molecular changes in rumen epithelium after a mild SARA bout are not well defined. Therefore, the objectives of this experiment were to determine the effect of SARA induction on the rumen microbiome composition and predicted function in the solid and liquid fraction, describe this effect on gene expression in rumen epithelial tissue, and to link these effects with the severity of an acidotic bout.

Materials and Methods

Experiment Design

The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana Champaign. Six ruminally-cannulated Holstein cows were used in a replicated incomplete Latin square design. Three experimental periods consisted of 10 d with all animals receiving the same basal diet (Table A.1). Ad libitum intake was maintained for the initial 3 d of each period. On d 4, intake was reduced to 50% based on average intake from the previous 3 days. Subsequently, on d 5 all animals were given ad libitum access to the basal diet or the basal diet topdressed with a wheat/barley pellet at 10% of prior dry matter intake. Ruminal pH measurements were taken hourly from -2 to 22 h relative to SARA induction. Using the pH response data on d 5, cows were grouped (SARA grouping; **SG**) as Non-SARA (n = 7) or SARA (n = 5) if ruminal pH was < 5.6 for more than three hours (Table A.2) regardless of pellet inclusion on d 5. Data for ruminal pH, feed intake, urine pH, fecal pH, milk production have been reported previously (Luan et al., 2016). In this article, we reinterpreted the pH data in the context of effects on the ruminal microbiome and epithelium.

Rumen Sampling and Nucleic Acid Extraction

Prior to morning feeding on d 1 and 6, ruminal contents were sampled via the ruminal cannula from the ventral sac of the rumen after mixing of the contents. Ruminal contents were squeezed through three layers of cheesecloth to separate into liquid and solid fractions. Samples were immediately put on ice and stored at -20° C prior to extraction.

DNA from the solid fraction (25 g) was extracted by homogenization followed by phenol/chloroform protocol as described by Stevenson and Weimer (2007). DNA from the liquid fraction (50 mL) was extracted using the ZR-96 Fecal DNA Kit (ZYMO Research, Irvine, CA), which included a bead-beating step for mechanical lysis of bacterial cell walls. Extracted

DNA from the solid fraction was standardized to 8 ng/μL for quantitative PCR (qPCR) and 20 ng/μL for Illumina sequencing. Extracted DNA was stored at -80° C for later use.

Rumen epithelium biopsies were taken on d 1 and 6 of the study prior to morning feeding. Ruminal contents were evacuated from the ventral sac allowing retraction of the epithelium approximately 6-9 inches below the ruminal cannula (Kelly et al., 1995). Papillae biopsies were excised, washed with PBS, immediately frozen in liquid nitrogen, and stored at -20° C until extraction. Rumen epithelium tissue samples were weighed and 0.4 - 0.6 g were subjected to RNA extraction using ice-cold QIAzol Lysis Reagent and the miRNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. All samples were treated with DNaseI (Qiagen, Valencia, CA) to remove genomic DNA and quantification was determined using a Nanodrop ND-1000 (Nanodrop Technologies, Rockland, DE). The quality of extracted RNA was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) with an average RNA integrity number = 8.3 (minimum RIN = 7.4). Complementary DNA was synthesized using 100 ng RNA, 1 μg dT18 , 1μL 10 mmol/L dNTP mix (Invitrogen Corp., Carlsbad, CA), 1 μL random primers (Invitrogen Corp., Carlsbad, CA), and 10 μL DNase/RNase free water. The mixture was incubated at 65 °C for 5 min and kept on ice for 3 min. A total of 6 μL of master mix composed of 5.5 μL 5X Reaction Buffer, 0.25 μL (50 U) of RevertAidTM Reverse Transcriptase (Fermentas Inc., Hanover, MD), and 0.25 μL of RNase Inhibitor (10 U, Promega, Fitchburg, WI) was added. The reaction was performed in an Eppendorf Mastercycler® Gradient using the following temperature program: 25 °C for 5 min, 42 °C for 120 min and 70 °C for 15 min.

Bacterial Quantitative PCR

Primers utilized for bacterial qPCR are listed in Table A.3 and were validated using gel electrophoresis and Sanger sequencing. Each 10 μL reaction consisted of 4 μL sample DNA, 5 μL 1× SYBR Green with ROX (Quanta BioSciences, Gaithersburg, MD), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL DNase/RNase free water in a MicroAmp^{TM} Optical 384-Well Reaction Plate (Applied Biosystems, Foster City, CA). All reactions were performed using an ABI Prism 7900 HT (Applied Biosystems, Foster City, CA) using the following conditions: 5 min at 95° C, 40 cycles of 1 s at 95° C and 30 sec at 60° C except an annealing temperature of 56° C used for eubacterial primer 3. The presence of a single PCR product was verified with an additional dissociation stage. All reactions were run in triplicate. Relative abundance of bacterial species was calculated using the geometric mean of two universal primers (Maeda et al., 2003; Fliegerova et al., 2014) with the efficiency-corrected Δ^{-CT} method (Ramirez-Farias et al., 2009). A portion of the 16S gene corresponding to the target of the eubacterial primer 3 (Muyzer et al., 1993) was commercially synthesized (IDT, Coralville, IA). A standard curve from 9.5×10^7 to 3.0×10^4 molecules per μL was used to obtain the 16S copy number from each sample. Samples were diluted to 1 ng/μL for suitable qPCR performance.

Library Construction and 16S rRNA Gene Sequencing

Amplification of the V4 region of the 16S rRNA gene used modified F515/R806 primers as described by Caporaso et al. (2012). The reverse PCR primer was indexed with 12-base Golay barcodes to facilitate multiplexing of samples. The PCR and sequencing protocol has been previously described in detail (Derakhshani et al., 2016). The 150 bp paired-end sequencing reaction was performed on a MiSeq platform (Illumina, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Canada. The sequencing data were deposited into the Sequence Read

Archive (SRA) of NCBI (http://www.ncbi.nlm.nih.gov/sra) and can be accessed via accession number SRR3271885.

16S rRNA Read Analysis

Overlapping paired-end Illumina fastq files were merged using the PANDAseq assembler (Masella et al., 2012). All the sequences with low quality base calling scores as well as those containing uncalled bases (N) in the overlapping region were discarded. The subsequent fastq file was processed using the QIIME pipeline v1.8 (Caporaso et al., 2010b). Assembled reads were demultiplexed according to the barcode sequences, chimeric reads were filtered using UCHIME (Edgar et al., 2011), and reads were clustered into OTU (Operational Taxonomic Units) de novo based on 97% similarity with UCLUST (Edgar, 2010). Representative sequences from each OTU were assigned a taxonomy using RDP Classifier (Wang et al., 2007) and aligned to the Greengenes reference database (McDonald et al., 2012) using PyNAST (Caporaso et al., 2010a).

After sample size standardization to the smallest library size (23,000 sequences/sample), OTU richness, and alpha- and beta-diversity metrics were estimated. Alpha rarefaction curves were generated with ten sampling iterations using the Chao1 index (Chao, 1984). Between sample comparisons of diversity (beta-diversity) were calculated using the Bray-Curtis metric (Beals, 1984). Bray-Curtis distance matrices were utilized in principal coordinate analysis (PCoA) to generate two-dimensional plots in PRIMER v6 software (Clarke and Gorley, 2006). Permutational multivariate analysis of variance (PERMANOVA) was implemented to test differences in beta-diversity among SG and time.

Functional metagenomic predictions were made using the bioinformatics tool PICRUSt (Langille et al., 2013). Quality-filtered, paired-end reads were used for closed-reference OTU

picking in QIIME. The resulting OTU table was used in PICRUSt version 1.0.0 and functional predictions were made to the KEGG Ontology Pathways (Kanehisa and Goto, 2000). Within PICRUSt, the 16S copy number was normalized, molecular functions were predicted, and all results were summarized into KEGG pathways.

Rumen Epithelium Quantitative Reverse Transcription-PCR

Primers utilized for rumen epithelium quantitative reverse transcription-PCR (qRT-PCR) are listed in Table A.4. The primer for *IGFBP5* was designed using Primer3 (Untergasser et al., 2012) and verified using gel electrophoresis and sequencing. The reaction components, real-time machine, and conditions were the same as described for bacterial qPCR. The presence of a single PCR product was verified with an additional dissociation stage. All reactions were run in triplicate. A six point relative standard curve was used to determine gene expression. Relative quantities were normalized using the geometric mean of genes *CMTM6*, *MRPL39*, and *ERC1* (Naeem et al., 2012; Minuti et al., 2015)

Statistical Analysis

Partial least square discriminant analysis (PLS-DA) was performed on genus level assignments to identify the effect of SG and day using SIMCA P+ 13.0 (Umetrics, Umea, Sweden). In the analysis, the X variables were bacterial genera, Y variables were either SG or day comparisons, and the data were scaled using Unit Variance. Permutation was conducted to validate the models and genera with variable influence projection values below 0.5 were removed from the final model (Li et al., 2012). The R^2 and Q^2 estimates were used to evaluate goodness of fit and the predictive value of the model, respectively. The PLS regression coefficients were used to identify genera significantly correlated with Y variables and used to label loading scatter plots.

Relative abundance of bacteria present at > 0.1% at the phyla, family, and genus taxonomic level were evaluated and logit transformed ($z = \log[p/(1-p)]$) if necessary to ensure normal distribution of the residuals, where p represents the relative abundance of a bacterial taxa. Bacterial relative abundance and normalized epithelial gene expression data were analyzed using the MIXED procedure of SAS 9.3 (SAS Inst. Inc., Cary, NC). Terms in the model included SG, day, SG × day, and period with cow nested within square as a random effect. SARA grouping means were calculated using the LSMEANS option. Additionally, bacterial relative abundance change from d 6 to d 1 was correlated with measures of pH previously reported by Luan et al. (2016) using Pearson correlations within the CORR procedure of SAS and visualized in custom heat maps. Time < 5.8 was used for correlation analysis as pH data < 5.6 in Non-SARA cows was zero-inflated. Significance was declared at $P < 0.05$ while tendencies are discussed at $P < 0.10$.

All predicted KEGG pathways by PICRUSt were subjected to a Welch's t-test in STAMP 2.1.3 (Parks et al., 2014) using a Storey false discovery rate (FDR) correction (Storey and Tibshirani, 2003). After correcting for multiple tests, 63 pathways were different ($P < 0.05$) between Non-SARA and SARA cows on d 6. These pathways were then analyzed in SAS 9.4 using the MIXED procedure with the aforementioned model. All pathways with a SG × day interaction ($P < 0.05$) are shown in the results and supplement.

Results

A total of 1,677,722 reads were generated after quality control and chimera removal resulting in an average of about 35,000 reads per sample. Sequencing depth was not affected ($P > 0.1$) by any main effect and ranged from 23,621 to 110,941. After clustering reads at 97% similarity, an average of 2,094 OTUs were obtained for each sample. At the family and genus

taxonomic levels, 81.1% and 54.9% of reads were identified within the Greengenes database, respectively. Within the liquid fraction, a SG × day interaction ($P = 0.03$) was observed for the Chao1 index as community richness was higher for SARA cows on d 1 and decreased to similar levels to Non-SARA cows on d 6 (Table 1.1). The Shannon and Simpson's indices indicated that overall alpha-diversity decreased ($P \leq 0.07$) on d 6 in the liquid fraction. At the community level, effects of SARA induction on the microbiome were not as strongly evidenced in the solid fraction with no change in richness (Chao1) and Simpson's index. A SG × day interaction ($P = 0.06$) was observed for the Shannon index as alpha-diversity decreased on d 6 for Non-SARA cows but increased for SARA cows.

Beta-diversity, measured by Bray-Curtis similarity, was visualized in principal coordinates and separated liquid fraction samples by collection day (Figure 3.1A; $P = 0.003$). Spearman correlations greater than 0.85 indicated unclassified sequences within Clostridiales and *Prevotella* were associated with the separation between d 1 and 6, respectively (data not shown). Liquid fraction samples did not cluster by SG ($P = 0.60$) and solid fraction samples did not cluster by SG or day using Bray-Curtis similarity ($P \geq 0.19$; Figure 3.1B).

Solid Fraction qPCR

The relative abundance of targeted bacteria species is presented in Table 3.2. A SG × day interaction ($P < 0.04$) was observed for *Anaerovibrio lipolytica*, *Prevotella bryantii*, and *Succinimonas amylolytica*. These bacteria increased on d 6 in SARA cows while no change or a decrease was observed on d 6 in Non-SARA cows regardless of day. The increase in relative abundance for *S. amylolytica* and *P. bryantii* was more than 6- and 4-fold, respectively. A SG effect ($P = 0.01$) was observed for *Eubacterium ruminantium* as it was greater in SARA cows. *Streptococcus bovis*, and *Succinivibrio dextrinosolvens* tended to be greater ($P = 0.10$) on d 6.

The greatest value for each of these bacteria was observed on SARA d 6, but no SG effect or interaction ($P > 0.16$) was detected for *S. bovis* and *S. dextrinosolvens*. While there was no effect of SARA induction on relative abundance of *Megasphaera elsdenii* and *Selenomonas ruminantium*, *Fibrobacter succinogenes* tended to be greater ($P = 0.08$) on d 1.

Solid Fraction Microbiome Effects

Firmicutes was the most abundant phyla in the solid fraction representing 80% of all sequences while Bacteroidetes relative abundance averaged 10% (Table 3.3). Both phyla had a SG × day interaction ($P < 0.01$) as Firmicutes on d 6 decreased for SARA and Bacteroidetes increased to 23%. The effects observed within the phylum Bacteroidetes were driven by the genus *Prevotella* which averaged 77% of the sequences in the phylum. Within Firmicutes, no effects were observed for the predominant families Lachnospiraceae, Ruminococcaceae, and order Clostridiales sequences not identified at the family level (Table 3.4). Lactobacillaceae increased on Non-SARA d 6 resulting in a SG × day interaction ($P = 0.06$). Genera *Streptococcus* and *Succiniclasticum* increased on d 6 ($P = 0.03$) but were not affected by SG (Table 3.6). A SG × day interaction ($P \leq 0.03$) with a slight decrease on Non-SARA d 6 and a larger increase in relative abundance on SARA d 6 was observed for *Clostridium*, YRC22, *Psuedobutyrivibrio*, *Anaerostipes*, and *Shuttleworthia*.

The association heat map (Figure 3.2A) supports the 16S results and also indicates the change in bacterial relative abundance from d 1 to d 6 was proportional to the severity of the acidotic bout on d 5. The strongest observed relationships are positive correlations among bacteria that increased on d 6 and greater area under the curve (AUC) below a 5.8 pH. Of the measured pH parameters, AUC < 5.8 may be the most sui indicator of SARA effects on the microbiome within our experimental pH range.

Liquid Fraction Microbiome Effects

At the phyla level (Table 3.3), Bacteroidetes, representing more than 60% of the sequences, tended to increase in relative abundance on d 6 ($P = 0.06$). Within Bacteroidetes, genera *Prevotella* (Table 3.7) and YCR22, and family S24-7 (Table 3.5) increased ($P < 0.04$) on d 6, but unidentified sequences from order Bacteroidales decreased on d 6 ($P < 0.01$). While a SG × day interaction ($P = 0.11$) was not observed for Firmicutes, numerical trends indicated a slight decrease on d 6 for Non-SARA cows while SARA increased on d 6. This effect was realized at the family level in Lachnospiraceae ($P = 0.01$) and at the genus level in *Butyrivibrio* ($P < 0.01$). As expected, the relative abundance of *Streptococcus* and *Lactobacillus* increased on d 6 ($P < 0.06$). Collectively, in the liquid fraction many day effects ($P < 0.05$) were observed for bacterial families suggesting the impact of feed restriction and subsequent refeeding had a greater effect on microbiome composition than an acidotic bout. Correlations between the change in liquid fraction taxa and d 5 pH parameters are shown in Figure 3.2B. Relative to the solid fraction, fewer bacteria had strong correlations in the liquid fraction. Bacteria with greater correlations were also identified in the mixed model analysis with SG × day effects.

Multivariate Analysis

A partial least squares-discriminant analysis (PLS-DA) was used to identify bacteria related to day and SG. Liquid fraction samples separated based on sampling day in the score plot as a three component model explained 97.1% (R^2Y) and predicted 66.2% (Q^2Y) of the data (Figure 3.3A). A loading score scatter plot was used to visualize specific groups of bacteria with significant coefficients in the model (Figure 3.3B). Eight bacteria had coefficients significantly different from zero that were responsible for day differences in the model; genera *Bulleida*, BF311, p-75-a5, and order Bacteroidales were enriched on d 1 while *Clostridium*, *Lactobacillus*,

Pediococcus, and order Lactobacillales were increased on d 6. No model could be validated for an effect of SG within the liquid fraction.

Within the solid fraction, a three component model separated the samples based on SARA SG (Figure 3.4A). The model explained 93.9% (R^2Y) and predicted 62% (Q^2Y) of the data. The loading score scatter plots revealed five bacteria with significant coefficients related to SARA including *Prevotella*, p-75-a5, *Lachnospira*, family S24-7, and phylum SR1 (Figure 3.4B). Three taxa were associated with Non-SARA including genus *Anaerovorax*, family BS11, and unidentified sequences from the order Clostridiales.

Predicted Metagenome

The functional capability of the ruminal microbiome was predicted using PICRUSt to connect community composition changes in the functional profile. In the solid fraction, there were 43 affected level 3 KEGG pathways with a SG × day interaction ($P < 0.05$). The relative abundance of genes associated with the energy metabolism, oxidative phosphorylation, starch and sucrose metabolism, and sphingolipid metabolism KEGG pathways increased on d 6 for SARA compared to Non-SARA (Figure 3.5A). Pathways for bacterial invasion of epithelial cells, lipopolysaccharide (LPS) biosynthesis and proteins were also increased in SARA on d6 (Figure 3.5B). Conversely, bacterial pathways for glycolysis/gluconeogenesis, pyruvate metabolism, propanoate metabolism, and fatty acid biosynthesis were enriched on d 6 for Non-SARA cows compared to SARA. Additional significant affected pathways are listed in the supplemental materials (Figure S1). Analysis of liquid fraction samples did not elucidate any differences with the predicted metagenome with no difference between SG on either day (data not shown).

Ruminal Epithelium Gene Expression

Expression of genes related to barrier function in ruminal epithelium was affected by SARA induction. Claudin 4 (*CLDN4*) expression was upregulated ($P = 0.01$) on d 6 and a tendency for a SG × day interaction ($P = 0.08$) was observed with a greater increase for SARA cows on d 6 (Figure 3.6). Claudin 1 (*CLDN1*) was also upregulated ($P = 0.03$) on d 6 but the SG × day interaction ($P = 0.10$) indicated only SARA cows had greater expression on d 6. A tendency for a SG × day interaction ($P = 0.10$) was observed for Tight junction protein 1 (*TJP1*) as no change in expression was detected for SARA cows while it was down-regulated in Non-SARA cows on d 6. Relative expression of Toll-like receptor 2 (*TLR2*) decreased on d 6 ($P = 0.05$; Figure 3.7), but no day effect was detected for *TLR4* ($P = 0.18$). A day effect ($P = 0.02$) for *DSG1* indicated a decrease in expression on d 6 which was due the marked decrease for Non-SARA cows. Although there was a tendency for Coxsackie virus and adenovirus receptor (*CXADR*) expression to be increased on d 6 ($P = 0.10$), no main effects or interactions were observed for *JAM2*, *OCLN*, *TLR4*, *IGFBP3*, and *IGFBP5*. The change in rumen epithelium gene expression from d 1 to d 6 was correlated with pH response parameters (Figure 3.8). A strong association ($R^2 > 0.5$) was revealed between expression of *CLDN1* and *DSG1* to the pH nadir, AUC < 5.8 and time < 5.8. An increased expression of *CLDN1* and *DSG1* on d 6 (relative to d 1) positively corresponded to a proportional increase in AUC < 5.8 and time < 5.8 as well as a negative correlation to the pH nadir.

Discussion

While current best management practices strive to minimize SARA occurrence, the continued relevance of SARA in the dairy industry is reflected in ongoing academic research. Because of the debate over the definition of SARA (Plaizier et al., 2008), the understanding of its etiology needs to be strengthened. Our objective was to elucidate effects of SARA induction

using a feed restriction model on the solid and liquid fractions of the ruminal microbiome in addition to the ruminal epithelium. We defined SARA with a pH threshold of 5.6 (Gozho et al., 2005) understanding that pH is an important but not the only factor driving the onset of SARA (Calsamiglia et al., 2008). Using a post-hoc grouping, we were able to ensure SARA and Non-SARA cows were on the same basal diet with a goal of understanding key mediators in the ruminal microbiome and epithelium 24 h after a single bout of SARA. Although there was slight dietary variation based on the provision of a wheat/barley pellet to some cows on d 5, the inclusion of the pellet was ineffective at inducing SARA for some cows. While dietary composition and intake are primary causative agents of SARA, this study set out to understand the role of observed effects on the ruminal microbiome and epithelium during the onset of SARA.

We observed a reduction in richness for SARA cows on d 6 in the liquid fraction and a tendency for a decrease in alpha-diversity on d 6 overall. These findings correspond well with decreases in richness and diversity associated with SARA induction (Mao et al., 2013) as well as with grain feeding in general (Fernando et al., 2010). Moreover, the slight increase in richness on d 6 for Non-SARA cows suggests greater resilience in the community may be important to prevent the onset of SARA. While our results in the solid fraction were surprising as alpha-diversity increased on d 6 for SARA cows, additional evidence of greater richness post SARA induction has been observed using DGGE banding of whole rumen content samples (Lettat et al., 2012). Beta-diversity results suggest there was more variation among liquid samples compared with the solid fraction. Whereas day had a major effect characterizing the differences within the liquid fraction, more of the variation observed in the solid fraction was accounted for by the SG × day interaction. Others have reported greater variation in liquid samples when evaluating

SARA microbiome changes using DGGE (Huo et al., 2014) as well as other diet types (McCann et al., 2014).

As noted by the increase in Bacteroidetes on d 6, the taxonomic evaluation of changes in the microbiome suggested the greatest effects of SARA were induced in the solid fraction. Relative abundance of Bacteroidetes was even greater on d 1 and then was only accentuated on d 6 after SARA induction. This may indicate greater levels of Bacteroidetes or *Prevotella* may predispose the rumen to the onset of SARA. Golder et al. observed cows consuming higher levels of crushed wheat and ryegrass silage with greater SARA eigenvalues had greater Prevotellaceae in a predominantly liquid sample (Golder et al., 2014). In contrast, a longer-term induction model over 21 d with greater dietary differences resulted in lower *Prevotella* in whole rumen contents of cows with SARA (Mao et al., 2013). Evaluating the severity of acidosis during the transition period revealed a relationship between *Prevotella* in the liquid fraction and severity of acidosis (Mohammed et al., 2012), and agrees with our findings in the liquid fraction. Variation with the genus *Prevotella* has also been observed in response to SARA induction (Khafipour et al., 2009c). Understanding the undescribed diversity in the *Prevotella* genus (Bekele et al., 2010) and the limitations of taxonomic identifications with current 16S rRNA sequencing technology underscore the challenge comparing across studies given the vast differences in diet, experimental design, sampling, and methodology.

Based on pH response (Table A.2), the level of SARA experienced by cows in our study most closely matches the mild grain-induced SARA described by Khafipour et al. (2009c). Despite not having a group similar to our Non-SARA cows, the collection time at h 0 and control vs induction period coincide well with d 1 and d 6 in our study. Similarly to these findings, *Anaerovibrio lipolytica* and *Prevotella bryantii* increased on d 6 relative to d 1 while *Fibrobacter*

succinogenes levels were not affected. The greater abundance of *F. succinogenes* observed for SARA cows was surprising given its pH sensitive metabolism (Chow and Russell, 1992) and lower abundance and activity of the cell membrane H^+-ATPase transporter (Miwa et al., 1997). However, a similar trend for greater abundance of Fibrobacteraceae was observed in cows with greater SARA eigenvalues (Golder et al., 2014). Relative abundance of *Streptococcus bovis*, a well-described lactate producer, increased on d 6 but no effect of SG was detected despite numerical trends for a greater increase for SARA cows. Similarly, only day effects were observed at the genus level in the liquid and solid fraction. Overall increases in *S. bovis* were not related with the severity of acidotic bout within this experimental pH range which is consistent with descriptions of SARA being unassociated with lactic acid accumulation (Nagaraja and Titgemeyer, 2007). The 2.7 fold increase in *S. bovis* for SARA cows on d 6 coincided with a 2.3 fold increase in *M. elsdenii* supporting a level of synchrony between lactate producers and utilizers that may have prevented a more severe bout of SARA from developing after an abrupt feed restriction (Oetzel, 2003).

The functional capability of the rumen is more static than community composition due to functional redundancy across many community members (Weimer, 2015). In ruminants, metagenomic predictions using 16S rRNA data are comparable with shotgun sequencing data (Lopes et al., 2015). Despite a similar basal diet, energy metabolism and starch and sucrose metabolism pathways were enriched under SARA conditions which is consistent with greater glucose levels observed on higher concentrate diets with a lower ruminal pH (Ametaj et al., 2010). Significant increases in sphingolipid metabolism on d 6 in SARA cows are linked to greater relative abundance of *Prevotella*. While many gram-negative bacteria possess lipopolysaccharide on their cell membrane, a limited number of bacteria and fungi contain

sphingolipids in their cell membrane including *Bacteroides* and *Prevotella* (Kato et al., 1995). Recent research has indicated bacterial sphingolipids are critical for survival during stressful oxidative conditions in *Bacteroides fragilis* (An et al., 2011). Although not tested under pH related stress, this mechanism may be key to the increase of *Prevotella* observed after the SARA bout. Increased LPS biosynthesis and proteins for SARA cows on d 6 corresponded well with greater levels of LPS observed with SARA induction and higher grain feeding (Khafipour et al., 2009b; Saleem et al., 2012; Mao et al., 2015) and is due to the increase of gram-negative phyla (primarily Bacteroidetes). Release of LPS from the outer membrane of gram-negative bacteria occurs during growth and stationary phases as well as during cell lysis (Wells and Russell, 1996). Pathways related to bacterial invasion of epithelial cells further suggest an increased presence of bacteria poised to take advantage of compromised barrier function in rumen epithelium. Enriched pathways related to cyanoamino acid metabolism were observed in SARA cows on d 6. This pathway is linked to beta-alanine metabolism via aspartate which connects to pantothenate and CoA biosynthesis. The coordinated enrichment of these pathways in SARA cows is supported by previous work reporting increased aspartate and beta-alanine in the rumen fluid with increased grain feeding (Saleem et al., 2012; Mao et al., 2015).

While long-term feeding of high grain diets is known to disrupt barrier function proteins (Liu et al., 2013), a single mild SARA induction did not affect epithelial barrier function determined in Ussing chambers in vitro (Penner et al., 2010). Claudins are tight junction proteins primarily located in the membrane of stratum granulosum cells (Graham and Simmons, 2005). Increased expression of *CLDN1* and *CLDN4* most closely coincided with a lower pH observed during SARA induction. Acidotic conditions increased expression of multiple claudins in the duodenum of rodents (Charoenphandhu et al., 2007). Although claudins can be

downregulated by the transcription factor SNAI1(Ikenouchi et al., 2003), a mechanistic link with a low pH has not been elucidated. Desmosomes are a multi-protein complex responsible for intercellular adhesion (Holthöfer et al., 2007). Desmoglein (*DSG1*), a component of desmosomes, is highly upregulated during the recovery from an acidotic bout (Steele et al., 2011). Similarly, we observed the greatest expression levels of *DSG1* on d 1 which may represent a carry-over effect from the prior period. Toll-like receptors initiate the inflammatory response by binding to pathogen-associated molecules (Akira and Takeda, 2004). While increased expression of *TLR2* and *TLR4* has been associated with resistance to acidosis (Chen et al., 2012), this response observed in a subsequent acidosis induction following feeding a high concentrate diet for 58 d. Our results did not suggest that these adaptations occur within 24 h of a single bout of SARA. Feeding high grain diets at SARA levels over multiple weeks has been shown to increase epithelial proliferation by IGF-1 via upregulation of *IGFBP5* and downregulation of *IGFBP3* (Steele et al., 2011; Steele et al., 2012). The fact no effect on *IGFBP5* and *IGFBP3* was observed suggests factors unrelated to a short-term SARA induction are responsible for their regulation.

In conclusion, these data indicate that feed restriction and subsequent SARA induction cause alterations in the ruminal microbiome and epithelium not observed in Non-SARA cows. More specifically, SARA cows had increased relative abundance of *Prevotella* and *Eubacterium ruminantium* in the solid fraction. Ruminal microbiome beta-diversity results suggest the effect of feed restriction was greater than pH differences in the liquid fraction. Predicted functional profile of the ruminal microbiome corresponded to known metabolites impacted by high concentrate feeding. Ruminal epithelium made minor adaptations 24 h after SARA including upregulation of *CLDN1* and *CLDN4*. Overall, these results extend our understanding of the

rumen microbiome's dynamic response to acidotic conditions and may facilitate targeted mediation of these events to prevent SARA.

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Tables and Figures

Table 3.1. Effect of SARA induction on alpha-diversity in the liquid and solid fraction of the ruminal microbiome.[1]

	Non-SARA		SARA			*P*-value[2]		
	d 1	d 6	d 1	d 6	SEM	SG	Day	SG × Day
Liquid fraction								
Chao1[3]	4030	4499	5788	5019	461	0.10	0.56	0.03
Shannon[4]	9.56	9.47	9.65	9.39	0.16	0.96	0.07	0.36
Simpson's[4]	0.996	0.995	0.996	0.995	0.0006	0.82	0.03	0.78
Solid fraction								
Chao1	2777	2555	3080	3375	359	0.29	0.86	0.24
Shannon	8.35	7.91	8.43	9.20	0.44	0.27	0.59	0.06
Simpson's	0.979	0.969	0.983	0.998	0.014	0.38	0.76	0.21

[1]Non-SARA = cows (n = 7) in which ruminal pH was not < 5.6 for 3 h on d 5. SARA = cows (n = 5) in which ruminal pH was < 5.6 for 3 h on d 5.
[2]SG = SARA grouping of cows based on ruminal pH as Non-SARA or SARA.
[3]An alpha-diversity index that estimates the number of undiscovered species within a sample as a measure of richness.
[4]Alpha-diversity measures that take into account richness and evenness of the community within a sample.

Table 3.2. Effect of SARA induction on relative abundances of bacterial genera in the solid fraction using qPCR.[1]

	Non-SARA		SARA		*P*-value[2]		
	d 1	d 6	d 1	d 6	SG	Day	SG × Day
A. lipolytica	0.0024	0.0023	0.0008	0.0053	0.76	0.02	0.02
B. proteoclasticus	0.29	0.19	0.47	0.63	0.07	0.70	0.13
E. ruminantium	0.07	0.03	0.16	0.23	0.01	0.53	0.06
F. succinogenes[3]	0.0053	0.0010	0.0087	0.0076	0.20	0.08	0.13
M. elsdenii	8.1×10^{-4}	7.2×10^{-4}	4.3×10^{-4}	1.0×10^{-3}	0.94	0.41	0.27
P. bryantii[3]	3.1×10^{-4}	2.1×10^{-4}	7.6×10^{-4}	3.4×10^{-3}	0.09	0.22	0.04
S. ruminantium	0.08	0.05	0.04	0.05	0.59	0.47	0.14
S. amylolytica[3]	6.5×10^{-4}	2.8×10^{-4}	3.6×10^{-4}	3.9×10^{-3}	0.24	0.09	<0.01
S. bovis[3]	1.6×10^{-4}	2.3×10^{-4}	4.5×10^{-4}	1.2×10^{-3}	0.17	0.10	0.41
S. dextrinosolvens[3]	3.3×10^{-5}	4.8×10^{-5}	9.5×10^{-5}	2.8×10^{-4}	0.18	0.10	0.42
16S rRNA copy number[4]	4.6×10^{6}	4.9×10^{6}	4.2×10^{6}	4.6×10^{6}	0.75	0.39	0.97

[1]Non-SARA = cows (n = 7) in which ruminal pH was not < 5.6 for 3 h on d 5. SARA = cows (n = 5) in which ruminal pH was < 5.6 for 3 h on d 5.

[2]SG = SARA grouping of cows based on ruminal pH as Non-SARA or SARA.

[3]Data were logit transformed to ensure normality of residuals.

[4]16S copy number/ng DNA

Table 3.3. Effect of SARA induction on relative abundances of bacterial phyla in the solid fraction using 16S rRNA sequencing.[1]

	Non-SARA		SARA		*P*-value[2]		
	d 1	d 6	d 1	d 6	SG	Day	SG × Day
Liquid fraction							
Firmicutes	31.4	28.1	27.7	29.9	0.84	0.73	0.11
Bacteroidetes	59.2	64.8	63.5	64.4	0.67	0.06	0.16
Cyanobacteria	1.35	0.55	0.65	0.25	0.31	0.02	0.43
TM-7	1.01	0.46	0.51	0.42	0.32	0.02	0.10
Actinobacteria	0.52	0.39	0.32	0.34	0.43	0.34	0.15
Proteobacteria[3]	0.18	0.10	0.12	0.13	0.95	0.22	0.16
SR-1	0.015	0.013	0.007	0.032	0.59	0.04	0.02
Solid fraction							
Firmicutes[4]	85.5	87.9	79.9	69.0	<0.01	0.01	<0.01
Bacteroidetes	4.8	3.1	9.9	23.0	<0.01	<0.01	<0.01
Actinobacteria	6.6	6.1	7.4	6.4	0.76	0.48	0.79
TM-7	2.0	1.5	2.0	0.9	0.73	0.05	0.49
SR-1	0.05	0.03	0.03	0.20	0.09	<0.01	<0.01
Proteobacteria[3]	0.02	0.01	0.05	0.04	0.18	0.64	0.95
Cyanobacteria[3]	0.05	0.03	0.02	0.01	0.23	0..14	0.93

[1]Non-SARA = cows (n = 7) in which ruminal pH was not < 5.6 for 3 h on d 5. SARA = cows (n = 5) in which ruminal pH was < 5.6 for 3 h on d 5.
[2]SG = SARA grouping of cows based on ruminal pH as Non-SARA or SARA.
[3]Data were logit transformed to ensure normality of residuals.
[4]Period effect $P < 0.05$.

Table 3.4. Effect of SARA induction on relative abundances of bacterial families in the solid fraction using 16S rRNA sequencing.[1,2]

	Non-SARA		SARA		*P*-value[3]		
	d 1	d 6	d 1	d 6	SG	Day	SG × Day
Bacteroidetes							
Prevotellaceae	3.29	2.42	8.13	18.32	<0.01	<0.01	<0.01
S24-7	0.63	0.33	1.12	2.01	<0.01	0.14	<0.01
Paraprevotellaceae[4]	0.18	0.06	0.32	2.35	0.02	0.34	<0.01
Bacteroidales[5]	0.39	0.22	0.37	0.73	0.45	0.44	0.03
Firmicutes							
Lactobacillaceae[4]	0.29	2.91	0.61	0.38	0.67	0.19	0.06
Streptococcaceae	0.38	1.19	0.71	0.98	0.91	0.04	0.27
Leuconostocaceae[4]	0.02	0.38	0.01	0.02	0.26	0.07	0.11
Other							
F-16	1.99	1.45	1.99	0.90	0.72	0.05	0.49

[1]Families listed were affected by SARA induction ($P < 0.10$). Additional families unaffected by SARA induction are listed in Table A.5.
[2]Non-SARA = cows (n = 7) in which ruminal pH was not < 5.6 for 3 h on d 5. SARA = cows (n = 5) in which ruminal pH was < 5.6 for 3 h on d 5.
[3]SG = SARA grouping of cows based on ruminal pH as Non-SARA or SARA.
[4]Data were logit transformed to ensure normality of residuals.
[5]Unidentifed sequences listed at the lowest level of taxonomic assignment (order).

Table 3.5. Effect of SARA induction on relative abundances of bacterial families in the liquid fraction using 16S rRNA sequencing.[1,2]

	Non-SARA		SARA		*P*-value[3]		
	d 1	d 6	d 1	d 6	SG	Day	SG × Day
Bacteroidetes							
Prevotellaceae	40.1	49.0	43.5	46.6	0.93	0.01	0.17
S24-7	0.57	0.94	0.72	2.26	0.18	<0.01	0.04
Bacteroidales[5]	11.5	8.7	11.4	7.7	0.74	<0.01	0.56
Firmicutes							
Lachnospiraceae[4]	8.86	6.64	8.15	10.23	0.49	0.73	<0.01
Clostridiales[5]	5.62	4.27	4.75	3.09	0.33	<0.01	0.72
Christensenellaceae	1.31	0.69	0.59	0.12	0.16	<0.01	0.66
Erysipelotrichaceae	0.63	0.34	0.57	0.37	0.88	<0.01	0.20
Lactobacillales[5]	0.06	0.82	0.05	0.14	0.47	0.09	0.17
Streptococcaceae[4]	0.011	0.031	0.011	0.023	0.89	0.02	0.62
Lactobacillaceae[4]	0.008	0.075	0.018	0.026	0.94	0.09	0.20
Other							
Coriobacteriaceae	0.47	0.33	0.30	0.27	0.35	0.06	0.23
F-16	1.01	0.46	0.51	0.42	0.31	0.02	0.10

[1]Families listed were affected by SARA induction ($P < 0.10$). Additional families unaffected by SARA induction are listed in Table A.6.
[2]Non-SARA = cows (n = 7) in which ruminal pH was not < 5.6 for 3 h on d 5. SARA = cows (n = 5) in which ruminal pH was < 5.6 for 3 h on d 5.
[3]SG = SARA grouping of cows based on ruminal pH as Non-SARA or SARA.
[4]Data were logit transformed to ensure normality of residuals.
[5]Unidentifed sequences listed at the lowest level of taxonomic assignment (order).

Table 3.6. Effect of SARA induction on relative abundances of bacterial genera in the solid fraction using 16S rRNA sequencing.[1,2]

	Non-SARA		SARA		*P*-value[3]		
	d 1	d 6	d 1	d 6	SG	Day	SG × Day
Bacteroidetes							
Prevotella	3.29	2.42	8.13	18.32	<0.01	<0.01	<0.01
YRC22[4]	0.15	0.04	0.30	2.42	0.02	0.39	<0.01
Firmicutes							
Butyrivibrio	16.66	12.14	12.45	12.01	0.52	0.16	0.24
Ruminococcus	4.92	7.37	7.27	8.09	0.54	0.11	0.41
Lactobacillus[4]	0.23	1.55	0.58	0.38	0.87	0.28	0.10
Streptococcus	0.30	1.15	0.73	1.00	0.80	0.03	0.23
Coprococcus	0.62	0.72	0.33	1.26	0.69	<0.01	0.01
Moryella	1.08	0.88	1.26	0.94	0.76	0.07	0.68
Clostridium	0.48	0.35	0.31	0.68	0.71	0.20	0.02
Blautia	0.30	0.20	0.21	0.14	0.27	0.02	0.64
Pseudobutyrivibrio[4]	0.07	0.02	0.07	0.42	0.18	0.57	0.01
Anaerostipes	0.27	0.17	0.10	0.27	0.74	0.55	0.03
Shuttleworthia	0.22	0.19	0.07	0.33	0.94	0..04	0.01
Other							
Succiniclasticum[4]	0.70	0.92	0.27	1.24	0.66	0.03	0.11

[1]Genera listed were observed at greater than 0.1% of all solid samples.
[2]Non-SARA = cows (n = 7) in which ruminal pH was not < 5.6 for 3 h on d 5. SARA = cows (n = 5) in which ruminal pH was < 5.6 for 3 h on d 5.
[3]SG = SARA grouping of cows based on ruminal pH as Non-SARA or SARA.
[4]Data were logit transformed to ensure normality of residuals.

Table 3.7. Effect of SARA induction on relative abundances of bacterial genera in the liquid fraction using 16S rRNA sequencing.[1,2]

	Non-SARA		SARA		*P*-value[3]		
	d 1	d 6	d 1	d 6	SG	Day	SG × Day
Bacteroidetes							
Prevotella	40.13	48.94	43.51	46.56	0.93	0.01	0.17
YRC22	1.45	1.60	1.90	2.72	0.24	0.04	0.13
Firmicutes							
Butyrivibrio[4]	6.27	4.23	4.99	6.62	0.71	0.58	<0.01
Ruminococcus	5.61	7.31	6.56	9.12	0.48	0.04	0.66
Coprococcus[5]	0.11	0.20	0.16	0.23	0.40	0.01	0.49
Streptococcus	0.011	0.017	0.014	0.025	0.66	0.06	0.55
Lactobacillus[5]	0.005	0.070	0.013	0.021	0.89	0.03	0.11
Moryella	0.008	0.008	0.014	0.009	0.65	0.56	0.61
Anaerostipes	0.048	0.029	0.065	0.107	0.24	0.50	0.09
Clostridium	0.090	0.096	0.089	0.170	0.54	0.13	0.21
Blautia[4]	0.049	0.037	0.047	0.044	0.88	0.29	0.46
Pseudobutyrivibrio	0.004	0.006	0.002	0.011	0.79	0.05	0.27
Shuttleworthia	0.007	0.008	0.035	0.012	0.16	0.08	0.04
Other							
Succiniclasticum[5]	0.143	0.189	0.222	0.342	0.40	0.09	0.69

[1]Genera listed were observed at greater than 0.1% of all liquid samples.
[2]Non-SARA = cows (n = 7) in which ruminal pH was not < 5.6 for 3 h on d 5. SARA = cows (n = 5) in which ruminal pH was < 5.6 for 3 h on d 5.
[3]SG = SARA grouping of cows based on ruminal pH as Non-SARA or SARA.
[4]Period effect $P < 0.05$.
[5]Data were logit transformed to ensure normality of residuals.

Figure 3.1. Principal coordinate analysis (PCoA) of beta-diversity in the liquid (A) and solid fraction (B) using Bray-Curtis similarity. Analysis by PERMANOVA revealed a day effect (P = 0.003), but no effect of SG (P = 0.60) and SG × day (P = 0.18) in the liquid fraction. In the solid fraction, PERMANOVA analysis indicated no effect of day (P = 0.19), SG (P = 0.83), or SG × day (P = 0.43).

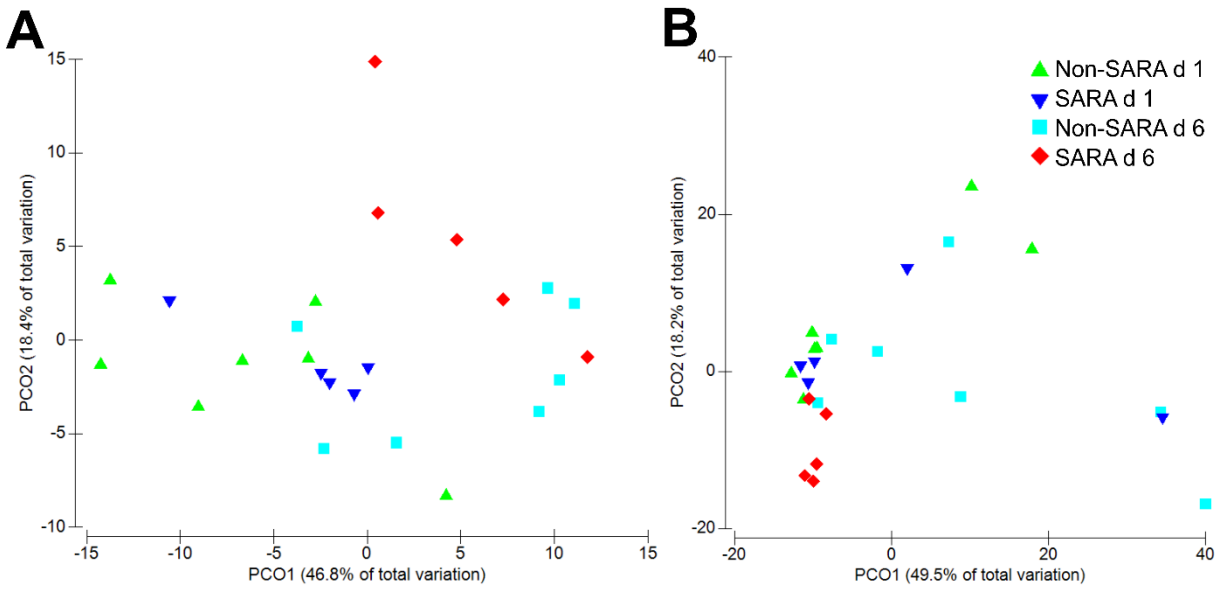


Figure 3.2. Association heat map between the change in bacterial relative abundance over time (d 6 - d 1) and ruminal pH response on d 5 in the solid (A) and liquid (B) fractions using Pearson correlations.

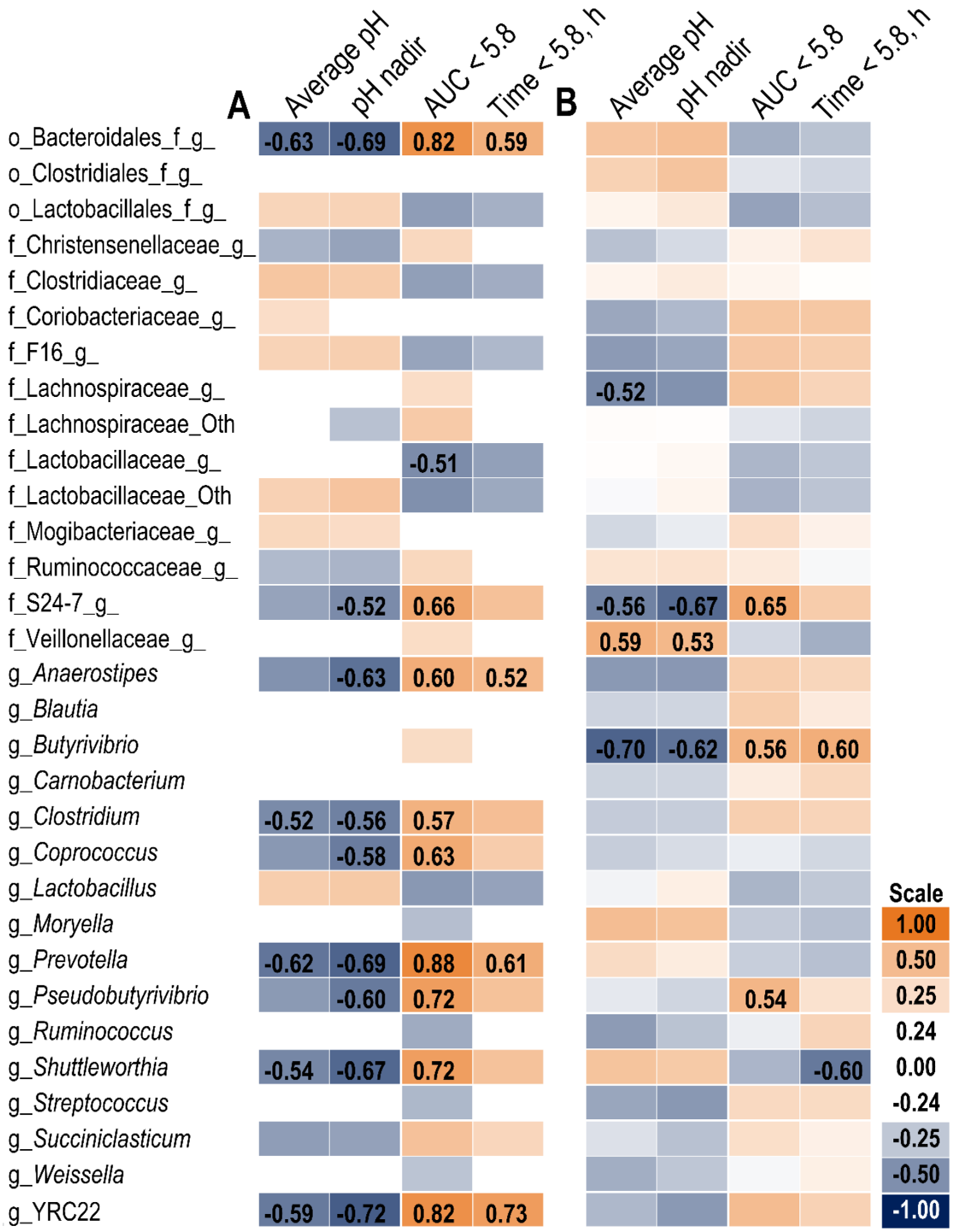


Figure 3.2 (cont.). All correlation coefficients greater than 0.5 or less than -0.5 are listed. The scale bar colors denote the correlation coefficients with 1 indicating a perfect positive correlation (orange) and -1 indicating a perfect negative correlation. Letter prefix denotes the lowest level of taxonomic identification [genus (g); family (f); and order (o)].

Figure 3.3. Partial least squares discriminant analysis (PLS-DA) model of the liquid fraction bacterial communities with 3 components.

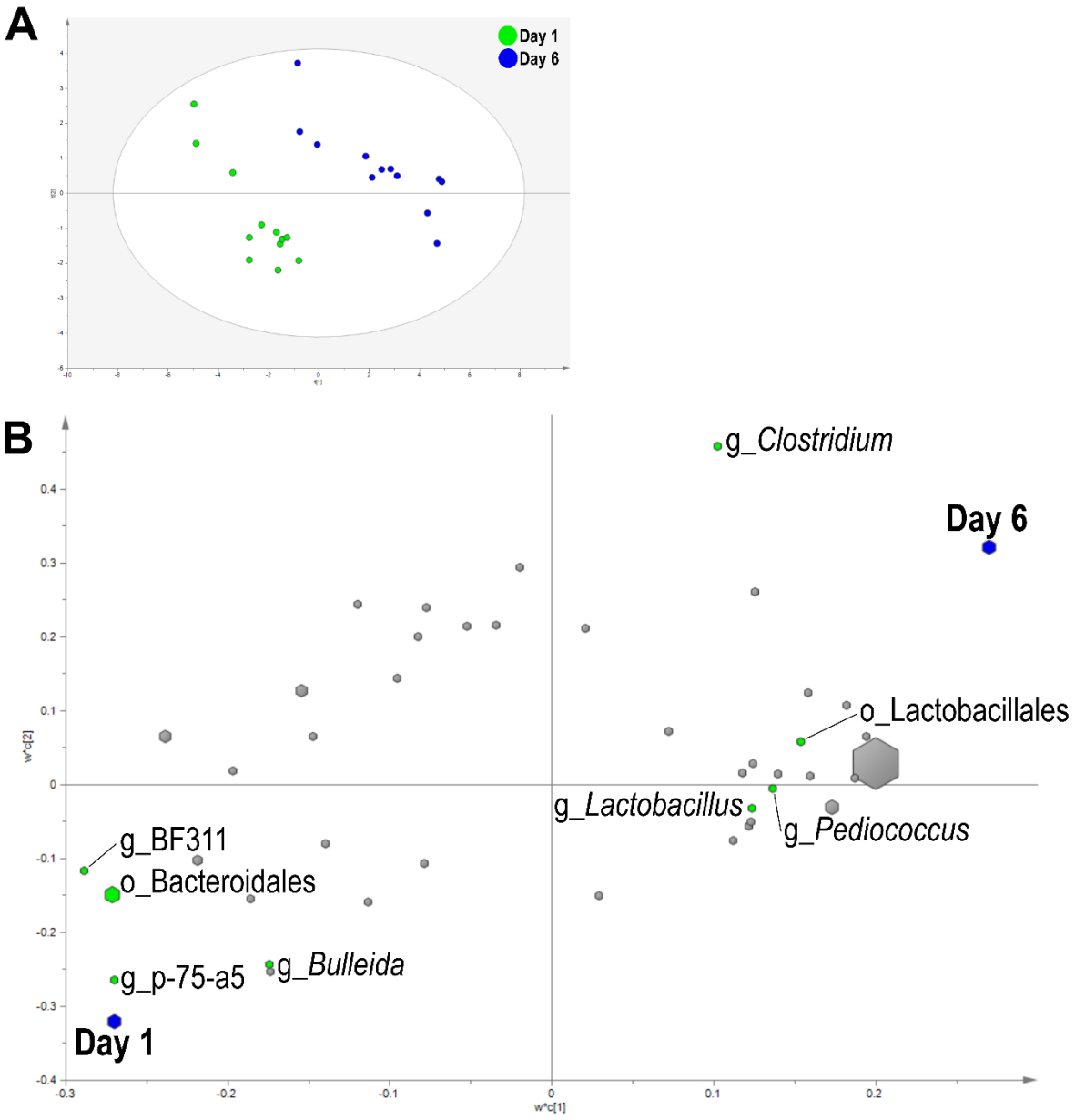


Figure 3.3. (cont.). (A) PLS-DA score scatter plot discriminating between d 1 and d 6 in the liquid fraction (goodness-of-fit parameter (R^2) (Y) = 0.97; predictive ability parameter (Q^2) (cum) = 0.66) with each point representing a single sample. **(B)** PLS-DA loading scatter plot of bacteria classified to the lowest taxonomic level. Taxa with significant coefficient values (relationship between X and Y variables) are labeled. The size of each point corresponds to the average relative abundance of the taxa. Letter prefix denotes the lowest level of taxonomic identification [genus (g) and order (o)].

Figure 3.4. Partial least squares discriminant analysis (PLS-DA) model of the solid fraction bacterial communities with 3 components.

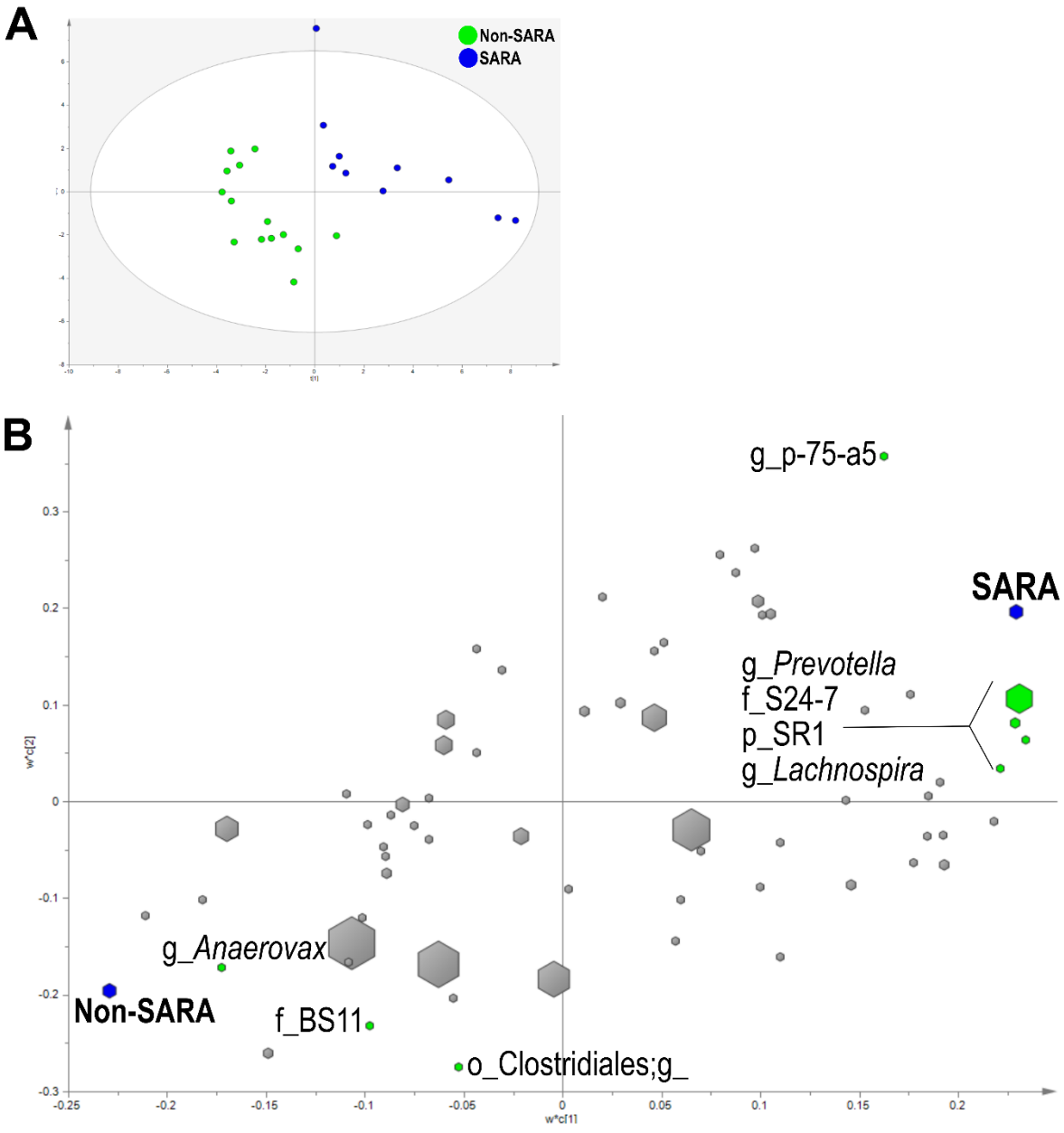


Figure 3.4. (cont.). (A) PLS-DA score scatter plot discriminating between Non-SARA and SARA cows in the solid fraction (goodness-of-fit parameter (R^2) (Y) = 0.94; predictive ability parameter (Q^2) (cum) = 0.62) with each point representing a single sample. **(B)** PLS-DA loading scatter plot of bacteria classified to the lowest taxonomic level. Taxa with significant coefficient values (relationship between X and Y variables) are labeled. The size of each point corresponds to the average relative abundance of the taxa. Letter prefix denotes the lowest level of taxonomic identification [genus (g); family (f); order (o); and phylum (p)].

Figure 3.5. Effect of SARA induction on the predicted metagenome pathways related to energy metabolism (A) and epithelial barrier function (B) in the solid fraction. Values represent the percentage change in expression of a given pathway from d 1 to d 6. Positive values indicate an increased representation on d 6 compared with d 1 of a given pathway in the predicted metagenome, while negative values describe a percent decrease on d 6 of a predicted pathway.

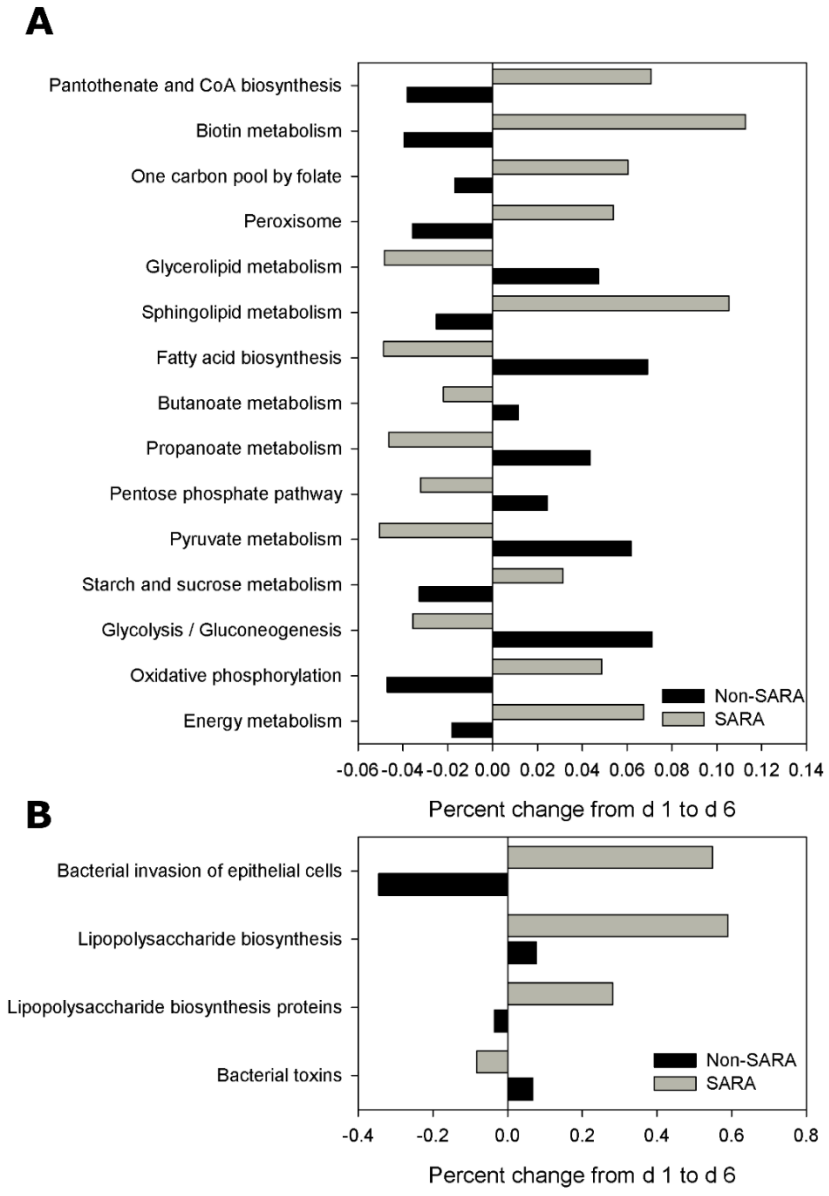


Figure 3.6. Effect of SARA induction on barrier function gene expression in rumen epithelium tissue. Effects ($P \leq 0.05$) are indicated by symbols: day effect (*), treatment effect (#), treatment × day effect (§), and period effect (‡). Tendencies ($P \leq 0.1$) are indicated by symbols: day effect (**), treatment effect (##), treatment × day effect (§§), and period effect (‡‡). Subscripts indicate pairwise differences of $P < 0.05$.

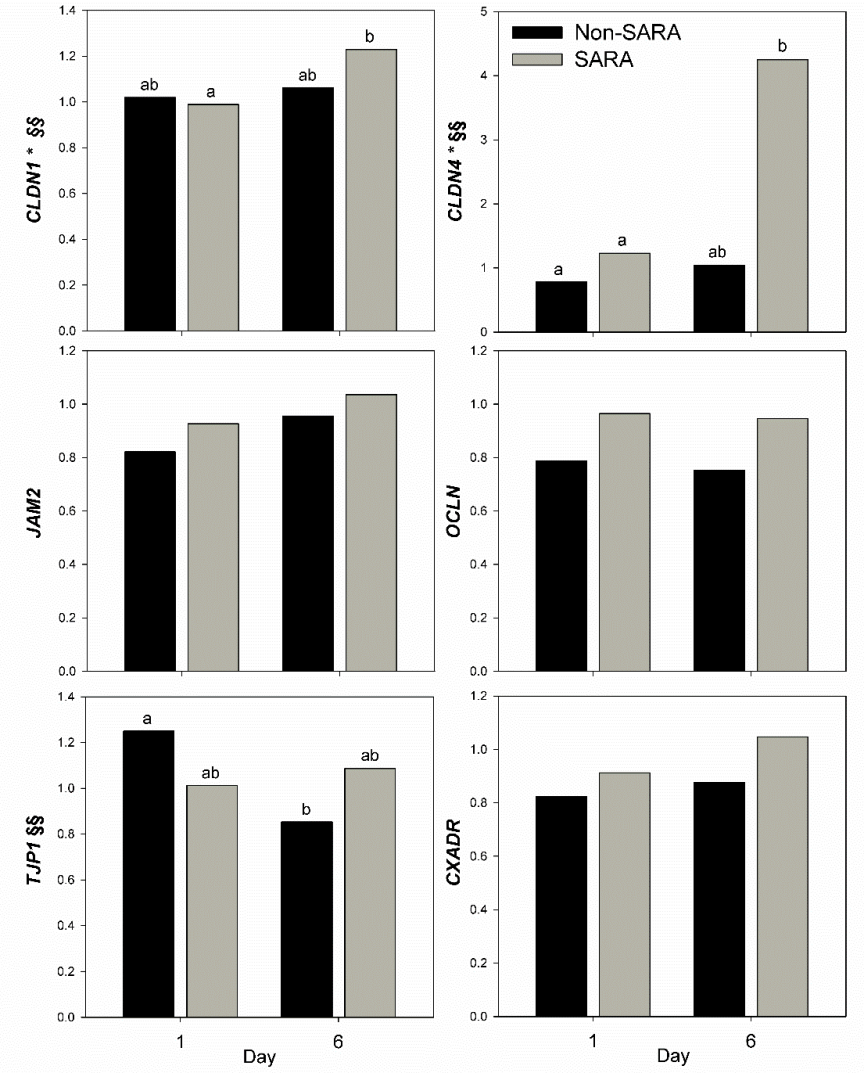


Figure 3.7. Effect of SARA induction on gene expression in rumen epithelium tissue.

Effects ($P \leq 0.05$) are indicated by symbols: day effect (*), treatment effect (#), treatment × day effect (§), and period effect (‡). Tendencies ($P \leq 0.1$) are indicated by symbols: day effect (**), treatment effect (##), treatment × day effect (§§), and period effect (‡‡). Subscripts indicate pairwise differences of $P < 0.05$.

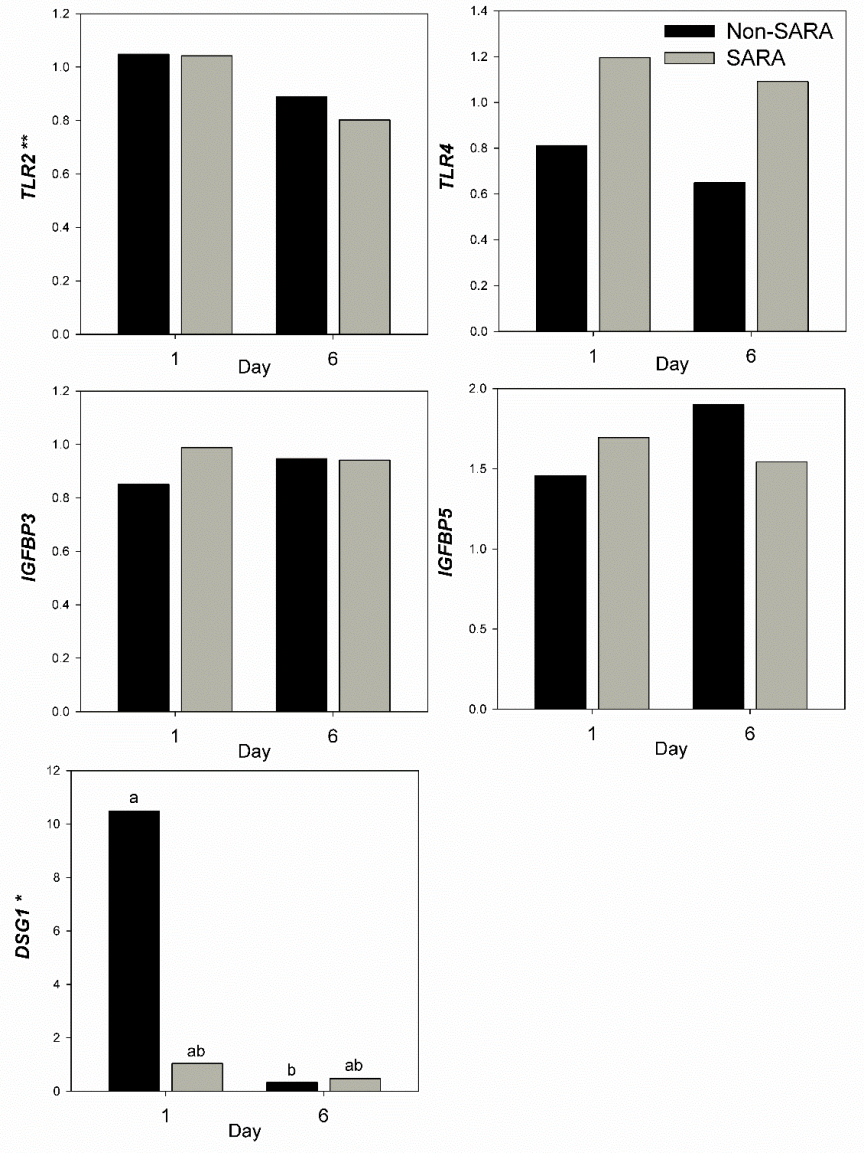
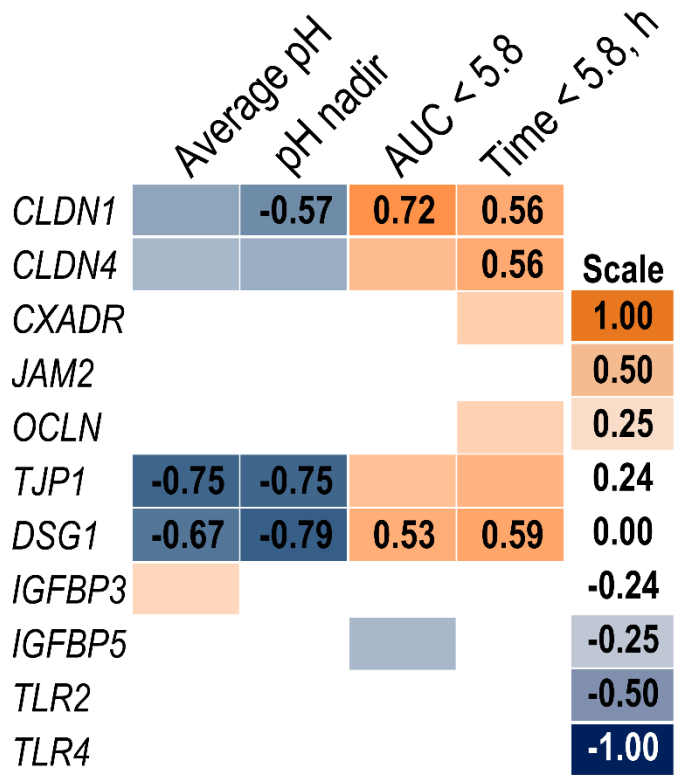


Figure 3.8. Association heat map between the change in rumen epithelium gene expression over time (d 6 - d 1) and ruminal pH response on d 5 using Pearson correlations. All correlation coefficients greater than 0.5 or less than -0.5 are listed. The scale bar colors denote the correlation coefficients with 1 indicating a perfect positive correlation (orange) and -1 indicating a perfect negative correlation.



CHAPTER 4

MATERNAL PLANE OF NUTRITION DURING MID-GESTATION AFFECTS THE SKELETAL MUSCLE TRANSCRIPTOME AND MICRORNA EXPRESSION IN BEEF CATTLE PROGENY

Abstract

The objectives were to determine the effect of maternal nutrition management during mid-gestation on the skeletal muscle transcriptome in progeny using 3 dietary treatments. Spring-calving, multiparous cows (n = 25) were limit-fed a common diet at three levels of intake to achieve 70% NRC requirement (70%REQ; 5.2. kg DMI), 100% NRC energy and protein requirements (REQ; 7.4 kg DMI), and 130% NRC requirements (130%REQ; 9.7 kg DMI). Diet composition included soy hulls, corn silage, and alfalfa haylage. Treatment diets were fed during mid-gestation (d 88 - 171 of gestation), and a common diet formulated to meet 100% NRC requirements was fed during the remainder of gestation. After calving, all cows calves were managed similarly as a single contemporary group. Longissimus muscles biopsies were taken on 99, 197, and 392 d of age. Extracted RNA was used in paired-end RNA sequencing on the Illumina HISeq2500 platform to analyze the transcriptome at d 392 while targeted mature microRNA (miRNA) expression was determined by quantitative RT-PCR. Transcriptomic data were mapped to the UMD3.1.1 bovine reference genome and analyzed using a mixed model approach within the R-package limma. Over 7,000 expressed genes were included in weighted gene co-expression network analysis (WGCNA) which clustered genes into 11 modules including 4 that correlated to phenotypic measures. The largest module included over 2,000 co-

expressed genes and was downregulated ($P < 0.01$) in progeny born to 130%REQ-fed compared with REQ-fed dams. The Dynamic Impact Approach annotated genes within this module to KEGG pathways. The pathways most impacted by maternal plane of nutrition were mostly lipid-associated including steroid and steroid hormone biosynthesis, sulfur metabolism, retinol metabolism, ketone synthesis and degradation, fat digestion and absorption, and PPAR signaling pathways. Module 7 (342 genes) was correlated positively with Warner-Bratzler shear force and negatively with marbling score. Major pathways in module 7 centered on glycolysis/gluconeogenesis, energy metabolism, and calcium signaling. These genes and associated pathways support increased glycolytic muscle fibers (type 2x) in progeny born to 130%REQ-fed compared with 70%REQ-fed dams. miRNA were robustly regulated over time suggesting an important role in muscle hypertrophy during the growing phase. Maternal nutrition effects ($P < 0.1$) were observed for miR-376d and miR-381. Results indicate the skeletal muscle transcriptome and associated metabolic functions prior to slaughter are affected by mid-gestation maternal plane of nutrition and may be regulated by epigenetic factors.

Keywords: fetal programming, skeletal muscle, transcriptome, microRNA

Introduction

Secondary myogenesis occurs during mid-gestation and altered nutritional status of the dam during this time can have long-term effects on skeletal muscle in progeny (Rehfeldt et al., 2011). In sheep, nutrient restriction of dams during mid-gestation resulted in reduced myofiber number and increased myosin 2b fiber type in skeletal muscle of 8 mo old progeny (Zhu et al., 2006). Restricting cows to 70% of nutrient requirements during early to mid-gestation decreased semitendinosus weight and increased subcutaneous adipocyte diameter (Long et al., 2012). For spring-calving beef herds, mid-gestation may coincide with limited forage availability and

quality. Improved nutritional management of the cowherd during mid-gestation may translate to more desirable progeny phenotypes and increase production of high-quality beef. However, variation in the observed effects of cow maternal nutrition underscores the need to elucidate mechanisms causing phenotypic effects.

In non-ruminant species, alterations in the epigenome have been identified as the method of transferring effects of maternal nutrition to the offspring (Seki et al., 2012). Epigenetics can be defined as the study of heritable alterations in gene expression patterns not caused by changes in the genomic DNA sequence. RNA sequencing (**RNA-Seq**) approaches allow high-throughput coverage of the transcriptome and have been successfully used to uncover wide spread metabolic changes in multiple tissues (Akula et al., 2014; Fok et al., 2014; Ayuso et al., 2015). microRNA (**miRNA**) are short, non-coding RNA about 22 nt long that downregulate gene expression pre- and post-transcriptionally (Ambros, 2001; Guo et al., 2010). miRNA are incorporated in the RNA-induced silencing complexes (**RISC**) that target specific mRNAs and typically bind to the 3` untranslated region (Bartel, 2004). The importance of miRNA in muscle development is well established by research on muscle-specific miRNAs: miR-1, miR-133, and miR-206 (van Rooij et al., 2008). We hypothesized maternal plane of nutrition would alter skeletal muscle metabolism driven by effects on the transcriptome and miRNA expression and coincide with phenotypic differences. Therefore, our objective was to determine the effect of over- or under-nutrition during mid-gestation on skeletal muscle transcriptome and miRNA expression in progeny.

Materials and Methods

Experimental Design and Animal Management

All animal procedures were approved by the University of Illinois Institute of Animal Care and Use Committee. Spring-calving, multiparous Angus and Angus Simmental crossbred cows (n = 25) at the University of Illinois Beef Cattle and Sheep Field Research Laboratory were used in the experiment. Cows were bred to one of 6 Simmental or Simmental × Angus bulls and fetuses were sexed via rectal ultrasonography 83 d after breeding. Cows were stratified by calf sex, calf sire, and BW, and then allotted to 9 pens. Pens were randomly assigned to 1 of 3 planes of nutrition formulated to address 130% (**130%REQ**; n = 8), 100% (**REQ**; n = 9), or 70% (**70%REQ**; n = 8) of NRC (1996) requirements for energy and protein. For 82 d during mid-gestation (d 88 - 171 of gestation), cows were limit fed a common diet (52% corn silage, 24% soy hulls, and 24% alfalfa haylage) at different DMI levels to meet plane of nutrition treatments. On d 172 of gestation, cows were fed a common ration to meet 100% of energy and protein requirement. Cows were kept in three sided barns open to the south with rubber matting and partially slotted floors. After calving, calves had exclusive access to the portion of the pen with solid flooring. Additional experimental procedure details are reported by Wilson (2015) and include results on calf performance, carcass characteristics, methane production, glucose tolerance, and targeted gene expression.

After parturition, all calves were managed alike as a single contemporary group. Calves were weaned on d 198 ± 14. Longissimus muscle (**LM**) biopsies were collected on d 97 ± 14, 197 ± 14, and 392 ± 14 postpartum by needle biopsy (12 gauge core biopsy needle; Bard Magnum, C. R. Bard, Covington, GA). The biopsy area was anesthetized using an intramuscular injection of 10 ml of Lidocaine-HCl. More than 0.5 g of tissue was collected from each animal and stored in liquid nitrogen until RNA extraction.

Molecular methods

Total RNA was extracted from muscle samples using QIAzol Lysis Reagent (Qiagen, Valencia, CA) and miRNAeasy Mini Kit (Qiagen, Valencia, CA). All samples were treated with DNaseI (Qiagen, Valencia, CA) on-column, and quantification was determined using a Nanodrop ND-1000 (Nanodrop Technologies, Rockland, DE).

The qScript microRNA cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD) was utilized for cDNA synthesis. Each reaction had 1 μg of total RNA mixed with 2 μL of Poly (A) Tailing Buffer (5x), 1 μL of Poly (A) Polymerase, and 7 μL of RNase/DNase free water. The mixture was incubated for 60 min at 37° C and 5 min at 70° C. The second mix contained 9 μL of microRNA cDNA Reaction Mix and 1 μL qScript Reverse Transcriptase, and it was incubated for 20 min at 42° C followed by 5 min at 85° C. A pool of cDNA for all samples was used to prepare a 6-point standard curve (1:4 dilution), and the cDNA samples were also diluted at 1:4 with DNase/RNase free water. Each reaction consisted of a 1 μL of diluted cDNA combine with 10 μL of the mix containing 5.25 μL of Perfecta SYBR Green Fast Mix (Quanta Biosciences, Gaithersburg, MD), 0.45 μL of 10 μM sequence-specific forward primer, 0.45 μL of Universal PCR Primer (Quanta Biosciences, Gaithersburg, MD), and 3.85 μL of DNase/RNase free water, and was added to each well of a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems, Grand Island, NY). The reactions were performed using the ABI Prism 7900 HT SDS instrument (Applied Biosystems, Grand Island, NY) using the following conditions: 95°C for 2 min, and 40 cycles of 95°C for 5 s and 60°C for 30 s, followed by a dissociation curve step. All miRNA forward primer sequences were obtained from miRBase (Kozomara and Griffiths-Jones, 2014) for selected miRNA. As internal controls, the geometric mean of miR-let7a, miR-103, and miR-191 were used to normalize the micoRNA expression data.

RNA-Seq cDNA libraries were constructed using total RNA isolated from d 392 samples based on greater divergence between treatments observed by Wilson (2015). The Illumina TruSeq v2 RNA Sample Prep kit was used for paired-end read library construction following the manufacturer's instructions with mRNA enrichment. Libraries were multiplexed across flow cell lanes of the Illumina HiSeq2500 (Illumina Inc., San Diego, CA) platform and generated 19,700,616 paired-end 100 bp reads per sample.

Bioinformatic and Statistical Analysis

Paired-end reads were first filtered using Trimmomatic 0.33 (Bolger et al., 2014) using a minimum quality score of 28 leading and trailing with a minimum length of 30 bp long and subsequently checked using FastQC 0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were then mapped to the Bos taurus UMD 3.1.1 reference genome (1/29/16 NCBI release) using default settings of STAR 2.5.1b (Dobin et al., 2013) with the quantMode option for gene counts. Over 76% of reads mapped to intragenic regions (within introns and exons) and read fates for each sample are described in Table 4.1. Based on clustering analysis of samples by read fates, one sample was excluded due to irregularities unrelated to treatment (data not shown). Further data analysis was conducted on the paired-end reads using R. 3.2.4 (R Core Team, 2016) and the noted packages. Reads uniquely assigned to a gene were used for subsequent analysis. After accounting for high expression genes and library size differences using trimmed mean of M-values (TMM) normalization in edgeR (Zhou et al., 2014), genes were filtered if 2 samples did not have > 1 count per million (**CPM**) mapped reads. Normalization of reads was conducted using the voom variance stabilization function in limma (Ritchie et al., 2015). Differential expression analysis was conducted in limma using a mixed model analysis including fixed effects of treatment (3 levels), sex (2 levels), sire (6 levels), and

pen (9 levels) as a random effect. Raw *P*-values were adjusted using the false discovery rate (**FDR**) method (Storey and Tibshirani, 2003). Principal component analysis (**PCA**) was conducted on voom-transformed values using the R-package affycoretools (MacDonald, 2008) to determine the effect of maternal nutrition after removing sex, sire, and pen effects with the removeBatchEffect() function in limma.

To further understand treatment effects, a weighted gene co-expression network analysis (**WGCNA**; WGCNA package) was conducted using genes with an overall treatment effect FDR *P*-value < 0.5 to include more true positives (Langfelder and Horvath, 2008). Importantly, the effect of sex, sire, and pen were removed from gene expression values used in WGCNA analysis. The primary advantage of this network analysis is the ability to overcome multiple testing correction factors on a large number of genes and correlate to phenotypes of interest (Langfelder and Horvath, 2008). A signed hybrid analysis was conducted using a soft power threshold of 7, a bicor correlation type, a minimum of 30 genes per module, and modules merging at 0.2 dissimilarity. A total of 7,031 genes were assigned to 11 modules with 315 genes left unassigned. The expression pattern of all genes in a given module was summarized by the first principal component score for each sample referred to as the module eigengene value (**ME**). The eigengene values were subjected to ANOVA contrasts to identify modules affected by maternal plane of nutrition.

To select biologically relevant modules for further analysis, module-trait relationships were determined by correlating eigengene values for each model with phenotypic traits of interest (Wilson, 2015) using Pearson correlations in the CORR procedure of SAS 9.4 (SAS Institute Inc., Cary, NC) and visualized in custom heat maps. Genes from modules 1 and 7 were selected based on module-trait correlations ($P < 0.05$) and a suitable number of genes (> 100) for

further pathway annotation. The connectivity of a gene to a module parameter (k_{ME}) was used to remove genes ($k_{ME} < 0.5$) not well-described by the module. The curated gene list for module 1 and 7 was annotated and analyzed using the Dynamic Impact Approach (**DIA**) (Bionaz et al., 2012). An overview of the complete workflow used for transcriptome data is visualized in Figure 4.1.

miRNA expression data were analyzed with the MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). Terms in the model included treatment, biopsy time, treatment × biopsy time, sex, and sire with calf within treatment as a random effect. Repeated measures analysis of time utilized a compound symmetry covariance structure. Data were log-scale transformed if necessary for normal distribution of residuals. All reported means were calculated using the LSMEANS option and mean separation was conducted using the PDIFF statement.

Results and Discussion

Principal component analysis indicated the skeletal muscle transcriptome on d 392 was affected by maternal plane of nutrition (Figure 4.2). The WCGNA approach was implemented to evaluate voom-normalized reads from high-throughput RNA sequencing on 25 samples from the LM at d 397 of age. This analysis depends on the assumption that strongly correlated expression levels of multiple genes are functioning together in related pathways and contribute to a phenotype of interest (Langfelder and Horvath, 2008; Kogelman et al., 2014). The approach is unsupervised because genes are assigned to a given module without knowledge of their biological connections to other genes within the module. The constructed co-expression network consisted of 7,346 genes which were assigned by hierarchal clustering to 11 modules with the largest and smallest containing 2,087 and 47 genes, respectively (Figure 4.3). A total of 315 genes were not assigned to a network module.

Within each module, the eigengene value is the first principal component and explains the expression variation accounted for by genes assigned to the module. Pairwise contrasts of the eigengene values indicated differences between progeny born to differing planes of maternal treatments for all 11 modules ($P < 0.01$; Figures 4.4 and 4.5). The eigengene values were correlated with animal phenotypes previously reported by Wilson (2015) to determine module-trait relationships. The correlation matrix heat map indicated modules 1 (2,807 genes), 7 (342 genes), 10 (54 genes), and 11 (47 genes) were correlated ($P < 0.05$) with one or more phenotypic traits (Figure 4.6). Module 1 and 7 were chosen for annotation and impact analysis using the DIA based on number of genes needed for an effective pathway analysis. Genes within each selected module were analyzed using the DIA to determine impact and flux of annotated KEGG pathways. Given that all genes with a module shared a similar expression pattern, the flux of all affected pathways within a module followed the same trend of the module eigengene for each comparison and is not shown.

Weighted Gene Co-expression Network Module 1

In module 1, the most impacted pathways included steroid biosynthesis, sulfur metabolism, retinol metabolism, and steroid hormone biosynthesis as well as many other affected pathways (Figure 4.7). Each of these pathways was downregulated in progeny born to 130%REQ-fed dams compared with REQ-fed dams. Affected pathways were evaluated using the impact determined by DIA in conjunction with relevance to skeletal muscle physiology, fold change of genes in the pathway, module eigengene-based connectivity (k_{ME}), inclusion of a gene in multiple pathways, and *P*-values of for the most affected genes in the pathway.

The greatest differences in the LM transcriptome were observed in module 1 between progeny born to 130%REQ-fed vs. REQ-fed dams at 397 d of age ($P = 0.001$). Many lipid

metabolism pathways were downregulated in progeny born to 130%REQ -fed dams and included in module 1. Specifically, steroid biosynthesis and steroid hormone biosynthesis pathways were reduced with notable downregulation of squalene epoxidase (*SQLE*), lanosterol synthase (*LSS*), and multiple genes in the cytochrome P450 family (*CYP3A4*, *CYP2B6*, *CYP2D14*, and C*YP51A1*). While the majority of steroid hormones are produced in sex organs, adipose tissue expresses all necessary enzymes for activation, interconversion, and inactivation of steroid hormones (Bélanger et al., 2002). Recent work confirmed adipocytes are able to synthesize an oxysterol, pregnenolone, de novo from cholesterol (Li et al., 2014). Adipose-derived steroid hormones produced or converted locally serve to regulate adipose tissue metabolism and contribute quantitatively to the steroid levels in the body (Li et al., 2015), but there is not a confirmed role in intramuscular fat (**IMF**). *CYP3A4* was the most downregulated gene in the top 15 impacted pathways of module 1. It is a major contributor of all-trans retinoic acid hydroxylation in the liver and can be activated by retinoic acids (Wang et al., 2008). Activation of the steroid hormone biosynthesis pathway has been observed in the liver of obese swine (Ponsuksili et al., 2008) and dairy cattle mobilizing fat during severe negative energy balance (McCabe et al., 2012). Alterations in many adipose tissue-related pathways is commonly observed in LM later in the finishing period with the development of IMF (Graugnard et al., 2009; Moisá et al., 2013).

Within the metabolism of cofactors and vitamins sub-pathway, retinol metabolism was reduced in progeny born to 130%REQ-fed dams compared with dams fed REQ. While several cytochrome P450-related genes also have a role in this pathway (*CYP3A4* and *CYP2B6*), lecithin:retinol acyltransferase (*LRAT*) and diacylglycerol O-acyltransferase (*DGAT1*) were also downregulated (fold change < 0.6). The esterification of retinol to retinyl esters by LRAT is

required for activating the STRA6/JAK2/STAT5 cascade via holo-RBP (Marwarha et al., 2014). Retinyl esters are also the major form of retinoids with more than 80% being stored in liver, but retinol was the primary storage form in adipose tissue of mice (Tsutsumi et al., 1992). Important to adipose tissue, retinoids regulate metabolism by binding to nuclear receptors such as retinoid X receptor (RXR) which links to peroxisome proliferator-activated receptor gamma (PPARG) to control adipogenic transcription (Ziouzenkova and Plutzky, 2008). Accordingly, RXR gamma (*RXRG*) was included within the PPAR signaling pathway in module 1.

Other adipose-related pathways were similarly downregulated in cattle born to 130%REQ-fed dams including synthesis and degradation of ketone bodies, glycosphingolipid biosynthesis, biosynthesis of unsaturated fatty acids, fat digestion and absorption, and glycerolipid metabolism. Also, PPAR signaling pathway was accordingly reduced as there was a two-fold reduction in *PPARG* and a corresponding target gene, phosphoenolpyruvate carboxykinase 1 (*PCK1*). A high fat maternal diet fed to mice downregulated liver *PCK1* in offspring due to histone modifications and caused increased plasma glucose (Strakovsky et al., 2011). Twenty-four genes in the PPAR signaling pathway were included in module 1 with very few outlying genes assigned to other modules. Known as a master regulator of adipogenesis, PPARG targets genes involved in glyceroneogenesis, adipocyte differentiation, fatty acid transport and oxidation (Evans et al., 2004). Different PPAR isoforms are cell-type specific and PPAR alpha (*PPARA*) was included in module 2 with two additional genes (*UBC* and *ACOX1*) which were only slightly activated in progeny born to 130%REQ-fed compared with REQ-fed dams.

Highly impacted pathways, drug metabolism- cytochrome P450 and metabolism of xenobiotics by cytochrome P450, have 7 overlapping genes in module 1. These pathways are

connected to sulfur metabolism and allow adipose to have a protective or detoxifying function (Forner 2009). Consistent with other adipogenic functions, these pathways were downregulated in progeny born to 130%REQ-fed dams. Many of the affected genes such as thiosulfate sulfurtransferase (*TST*), sulfide quinone reductase-like (*SQRDL*), glutathione S-transferases (*MGST1*, *GSTM2*, and *GSTA2*), and aldehyde dehydrogenases (*ALDH1A3*, *ALDH9A1*, and *ALDH3A2*) were detected in a proteomic evaluation of white adipose tissue mitochondria (Forner et al., 2009). Across multiple proteomic studies of liver mitochondria, TST and ALDH2 were the most consistently observed in obese or insulin resistant conditions (Peinado et al., 2014). In sheep, a glutathione S-transferase and aldehyde dehydrogenase protein were downregulated in progeny LM by a mid-gestation nutrient restriction to their dams (Zhu et al., 2006). However, there is a limited understanding of sulfur metabolism and cytochrome P450 pathways in adipose tissue of model species and no research on a role specific to IMF.

The primary effect of a higher plane of nutrition to cows during mid-gestation on the progeny skeletal muscle transcriptome was downregulation of lipid related pathways likely associated with IMF. It is noteworthy no differences in IMF percentage or marbling score were observed between progeny born to 130%REQ-fed dams compared with REQ-fed dams (Wilson, 2015). However, progeny born to 130%REQ-fed dams had less backfat measured by ultrasound at 298 d of age but only a 12% numerical reduction was detected for carcass backfat compared with progeny born to REQ-fed dams (Wilson, 2015). Considering there were not differences in lipid content of LM at d 392 biopsy, altered lipid-related pathways indicate maternal over-nutrition during mid-gestation can affect IMF metabolism in LM. These findings may also be related to greater insulin AUC values observed during a glucose tolerance test conducted at 318 d of age. Progeny born to 130%REQ-fed dams had numerically the greatest insulin AUC values

while maintaining more similar blood glucose to other treatments (Wilson, 2015). Downregulated lipogenic pathways in IMF or other adipose depots may have elicited a greater insulin response to clear the excess glucose administered during the test. During the last 60 – 90 d of the finishing period, cattle rapidly deposit more adipose tissue as they reach the end of their growth curve. A glucose tolerance test closer to the slaughter date may have detected greater differences in insulin signaling and glucose clearance rates. Altered insulin signaling has been observed in progeny born to dams receiving excess nutrients during gestation. Long et al. (2015) observed greater glucose dysregulation progressively during the finishing phase for progeny born to obese ewes and hypoinsulinemic during a glucose tolerance test. Maternal obesity effects may impact multiple generations as recently demonstrated in granddaughters of obese ewes with elevated blood glucose and insulin at birth (Shasa et al., 2014). It should be noted that in the obese ewe model employed in many fetal programming studies the animals received 150% of nutrient requirements during mid- and late gestation, but would rarely apply to production practices in the beef cattle industry.

Weighted Gene Co-expression Network Module 7

Module 7 eigengene values were positively correlated with an increase in Warner-Bratzler shear force and were negatively associated with greater marbling scores and IMF percentage ($P < 0.05$). The antagonistic relationship between Warner-Bratzler shear force and marbling is well established in beef (Savell et al., 1987; Clark et al., 2011). Fewer pathways were identified in module 7 (> 4 genes) using the DIA analysis since it was a smaller module containing 347 genes (Fig4.re 7). Energy metabolism pathways were the most highly impacted being upregulated in progeny born to 130%REQ-fed dams compared with REQ-fed dams. Glycolysis/gluconeogenesis was the most impacted pathway in module 7 with an average fold

change increase of 1.39 for the 14 included genes in addition to having the greatest module connectedness of any pathway (average k_{ME} = 0.94). Phosphofructokinase, muscle (*PFKM*), lactate dehydrogenase (*LDHA*), and enolase 3 (*ENO3*) were the most activated genes in progeny born to 130% REQ-fed dams. Zhu (2006) also observed greater expression of an enolase protein in LM of progeny born to control vs. nutrient restricted dams. Lactate dehydrogenase staining is a common enzymatic method to determine muscle fiber type. Greater activity of lactate dehydrogenase is found in glycolytic/fast-twitch fiber types including 2x and 2b to facilitate rapid conversion of pyruvate to lactate and regeneration of NAD^+.

Additional important genes were identified in the top 15 module connectedness values (k_{ME}) of module 7 including phosphorylase kinase, alpha 1 (*PYGM*), phosphoglycerate mutase 2 (*PGAM2*), adenylate kinase 1 (*AK1*), and myozenin 3 (*MYOZ3*). While not incorporated into KEGG pathways with greater than 3 genes in module 7, myosin heavy chain 1 (*MYH1*), myosin light chain, phosphorylatable fast (*MYLPF*), and myosin light chain kinase 2 (*MYLK2*) were all upregulated with an average fold change of 1.45 in progeny born to 130%REQ-fed dams compared with 70%REQ-fed dams. *MYH1* corresponds to the type 2x fibers in cattle which is considered an intermediate fast twitch muscle fiber type compared with other species (Lefaucheur and Gerrard, 2000). Similarly, *MYH1* mRNA expression determined by qPCR was decreased in progeny born to 70%REQ-fed dams at d 392 (Wilson, 2015). Comparisons of type 2b and 1 myofiber transcriptomes confirmed the main KEGG pathways over-expressed in type 2b fibers included glycosis/gluconeogenesis, insulin signaling, Wnt signaling, and lysosome (Chemello et al., 2011).

Collectively, the activation of the glycolysis/gluconeogenesis pathway supports greater fast-twitch muscle fiber type in the LM of progeny born to 130%REQ-fed dams. Although a

variety of methods exist for fiber typing muscle, mRNA abundance of myosin heavy chains aligns with observed protein expression (Park et al., 2009). *MYH1* expression was also downregulated in LM of Wagyu × Hereford cattle with greater marbling compared with Piedmontese × Hereford cattle (Wang et al., 2009). While mRNA expression of myosin heavy chains were regulated over time with the greatest change occurring from d 197 to 392 (Wilson, 2015), immunostaining of LM in cattle suggest relative proportion of fiber types remains consistent from weaning to 200 d later (Lehnert et al., 2006). In contrast, muscle fiber type differences observed at birth due to maternal nutrition were not present at weaning (Greenwood et al., 2004; Greenwood and Cafe, 2007).

Long-term effects of maternal nutrition on progeny muscle fiber type have been most clearly documented in mice (da Silva Aragão et al., 2014), pigs (Bee, 2004), and sheep (Zhu et al., 2006; Daniel et al., 2007). Nutrient restriction during mid-gestation across multiple species supports negative effects on muscle development, but the results are more divergent in this area of cattle research. Cows in a negative energy balance during mid-gestation lost 1 body condition score, but no effects on progeny LM gene expression were observed (Mohrhauser et al., 2015a). However, a tendency to shift expression from type 2x to 2a fibers was observed in progeny born to nutrient restricted dams. This same subsample of cattle from a larger study also had lower WBSF after 21 d aging which was attributed to less total collagen (Mohrhauser et al., 2015b). In this study, genetic differences in the cattle were not accounted for in the statistical model and may have limited detection of treatment differences. Cattle born to nutrient restricted cows grazing native range were unaffected in LM fiber type 1:2 (Underwood et al., 2010). Glycolytic fibers have a greater cross-sectional area and may contribute to additional muscle mass, but links to growth performance and muscle growth capacity have been inconclusive (Lefaucheur and

Gerrard, 2000). Muscle fiber type is directly related to meat quality and increased glycolytic fibers can improve color stability in beef (Renerre, 1990) and may increase tenderness via more rapid post mortem maturation (Seideman, 1986).

microRNA Expression

Almost all miRNA evaluated in skeletal muscle were tightly regulated over time suggesting a coordinated role in postnatal skeletal muscle development (Figures 4.8 and 4.9). Muscle-specific miRNA, miR-1 and miR-133a, both increased from d 100 to 200 and treatments appeared to diverge at 400 d of age. The similar pattern of miR-1 and miR-133a is expected as they are bicistronic, being cotranscribed in skeletal muscle (Rosenberg et al., 2006). Their effects are mediated by targeting myogenic factors including mef2, SRF, and myostatin (Chen et al., 2009). A treatment × d interaction ($P = 0.04$) was observed for cattle born to 130%REQ-fed dams with greater expression of miR-26a at d 392. Experimentally-validated miR-26a targets in muscle include Smad1 and Smad4 (Dey et al., 2012), Ezh2 (Wong and Tellam, 2008), and GSK3B (Mohamed et al., 2010). Although CCAAT enhancer-binding protein a (C/EBPa) is known to activate miR-26a expression (Mohamed et al., 2010), it was included in module 1 and downregulated in progeny born to 130%REQ-fed dams suggesting other regulators may be capable of inducing miR-26a.

Expression of miR-181a decreased with successive biopsy times ($P < 0.001$), but was more activated at d 392 in progeny born to 130%REQ-fed dams ($P = 0.04$). Hamrick et al. (2010) also observed miR-181a downregulation with increasing age of mice in addition to its computationally predicted target, Acvr2a. In cardiac muscle, miR-181a targets homeobox protein A11 (Hox-A11) and facilitates terminal differentiation of myoblasts (Li et al., 2009). Expression of miR-376d tended to be affected by maternal plane of nutrition ($P = 0.09$) as it was

activated in progeny born to 130%REQ-fed dams compared with 70%REQ-fed dams ($P = 0.04$). While miR-376d was differentially expressed in fetal skeletal muscle in obese ewes during gestation, it was decreased compared with control sheep (Yan et al., 2013). No other studies have evaluated miR-376d because it is not conserved across mammalian species. Progeny born to 70%REQ-fed dams also had decreased expression of miR-381 compared with 130%REQ-fed ($P = 0.02$) and REQ-fed dams ($P = 0.08$). Yan et al. (2013) observed greater miR-381 expression in fetal muscle of obese ewes. Computation predictions and experimental data suggest miR-381 may target secrete Frizzled-related protein 2 (sRFP2), an inhibitor of the Wnt signaling pathway (Snyder et al., 2013). Additional miRNA evaluated included miR-1, miR-133a, miR-206, miR-29a, miR-23a, let-7b, and let-7g; all were unaffected in progeny skeletal muscle by maternal plane of nutrition.

Implications

Plane of maternal nutrition during mid-gestation altered the skeletal muscle transcriptome in progeny at 392 d of age. Cattle born to 130%REQ-fed dams had decreased lipid metabolism associated pathways including steroid biosynthesis, steroid hormone biosynthesis, synthesis and degradation of ketone bodies, and PPAR signaling compared to REQ-fed dams despite no differences in IMF. Glycolytic pathways associated with fast twitch muscle fiber type and myosin heavy chain type 2x in cattle were upregulated in progeny born to 130%REQ-fed dams compared with 70%REQ-fed dams. miRNA likely have a role in mediating these changes in the skeletal muscle transcriptome, but a more high-throughput approach is needed to link their varied functions and targets to the LM transcriptome. Practical levels of over- and undernutrition occurring in mid-gestation in beef cattle can have long-term consequences on metabolism within the skeletal muscle of progeny at the end of the finishing period.

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Tables and Figures

Table 4.1. Paired-end read fates from RNA sequencing of d 392 longissimus muscle samples after trimming, quality filtering, and alignment.

			Percentage of total reads					
Animal	Treatment	Total reads	Quality filtered	Intragenic	Not in a gene	Not aligned	Not unique	Ambiguous
A454	130%REQ	21,541,659	1.7	77.9	15.4	2.5	2.0	0.6
A456	130%REQ	19,133,210	1.8	77.3	15.9	2.5	1.8	0.6
A458	130%REQ	20,666,854	1.5	76.9	16.8	2.4	1.8	0.6
A460	130%REQ	18,426,757	2.0	76.2	16.1	3.0	2.1	0.6
A103	130%REQ	20,160,459	1.8	75.6	17.8	2.5	1.7	0.7
A108	130%REQ	21,068,774	1.8	77.2	16.1	2.5	1.8	0.6
A109	130%REQ	13,482,841	1.9	76.8	16.3	2.6	1.8	0.6
A463	130%REQ	19,872,268	1.8	77.8	14.6	3.0	2.2	0.6
A100	70%REQ	19,385,785	1.9	68.3	15.7	7.6	5.8	0.7
A453	70%REQ	17,754,667	1.7	76.1	17.3	2.4	1.8	0.6
A105	70%REQ	21,310,865	1.7	77.2	16.2	2.6	1.7	0.6
A107	70%REQ	19,202,049	1.8	77.1	16.0	2.7	1.8	0.6
A455	70%REQ	21,126,585	1.9	76.3	17.0	2.4	1.7	0.6
A461	70%REQ	19,768,462	2.0	78.5	14.3	2.6	2.1	0.6
A462	70%REQ	20,483,609	1.8	76.1	16.9	2.7	1.9	0.6
A472	70%REQ	18,938,360	1.7	79.1	14.3	2.4	1.8	0.6
A452	REQ	18,333,381	1.9	78.0	15.2	2.6	1.8	0.6
A459	REQ	19,764,562	2.1	76.4	16.5	2.5	1.9	0.7
A464	REQ	19,219,384	1.8	78.5	14.5	2.8	1.8	0.6
A465	REQ	19,486,033	1.9	78.3	14.7	2.7	1.9	0.6
A467	REQ	22,114,688	1.9	77.4	15.7	2.5	1.9	0.6
A470	REQ	19,449,294	1.6	77.0	16.5	2.5	1.8	0.6
A471	REQ	20,008,166	1.8	74.6	17.9	3.2	1.8	0.7
A468	REQ	21,418,028	1.6	76.7	17.1	2.2	1.8	0.6
A469	REQ	20,398,659	1.9	78.1	14.8	2.8	1.9	0.6

Figure 4.1. Overview of RNA-Seq analysis workflow.

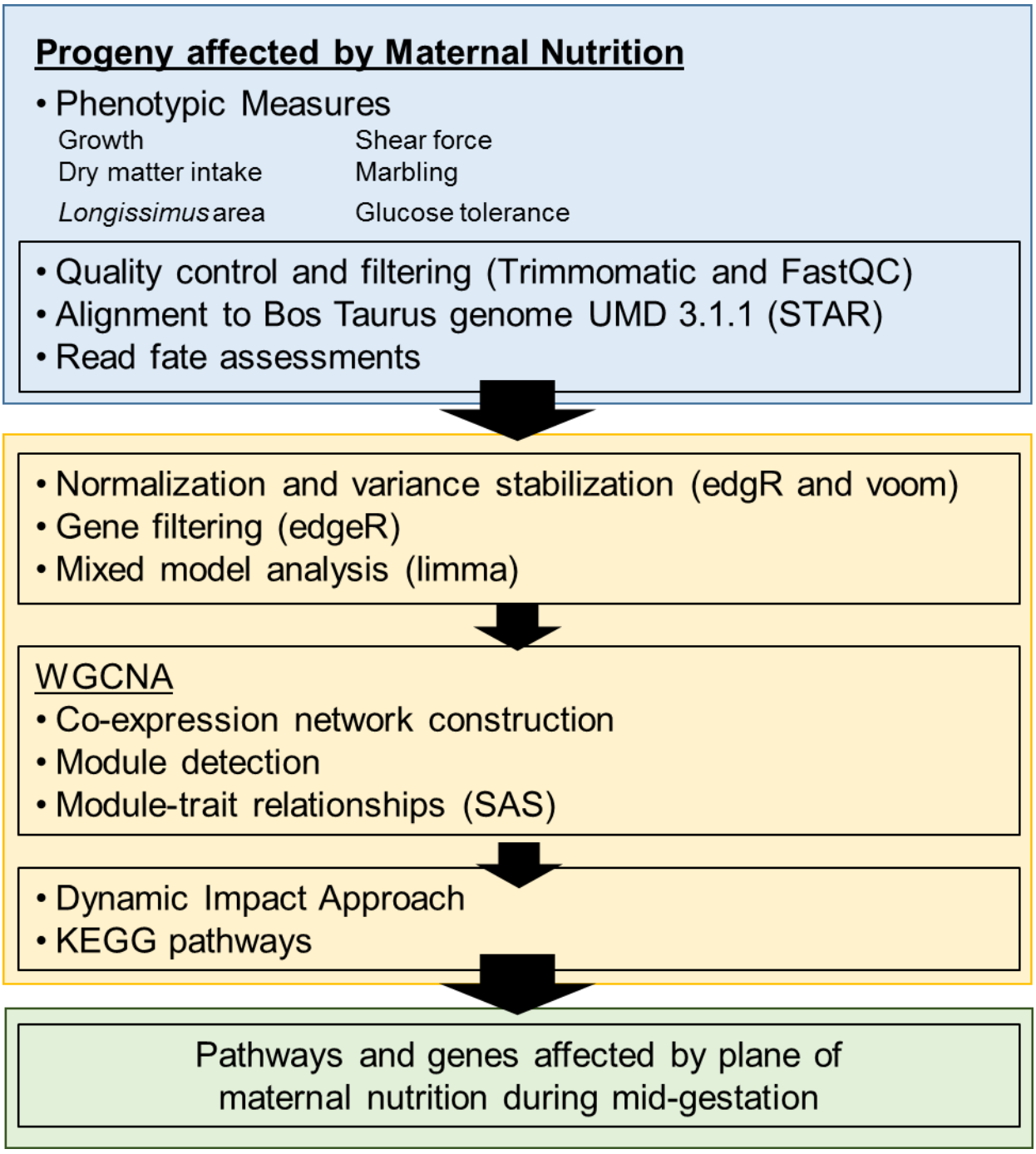


Figure 4.2. Effect of maternal plane of nutrition during mid-gestation on principal component analysis of progeny longissimus muscle transcriptome at 392 d of age.

H = heifer; S = steer; PC1 = principal component; PC2 = principal component 2.

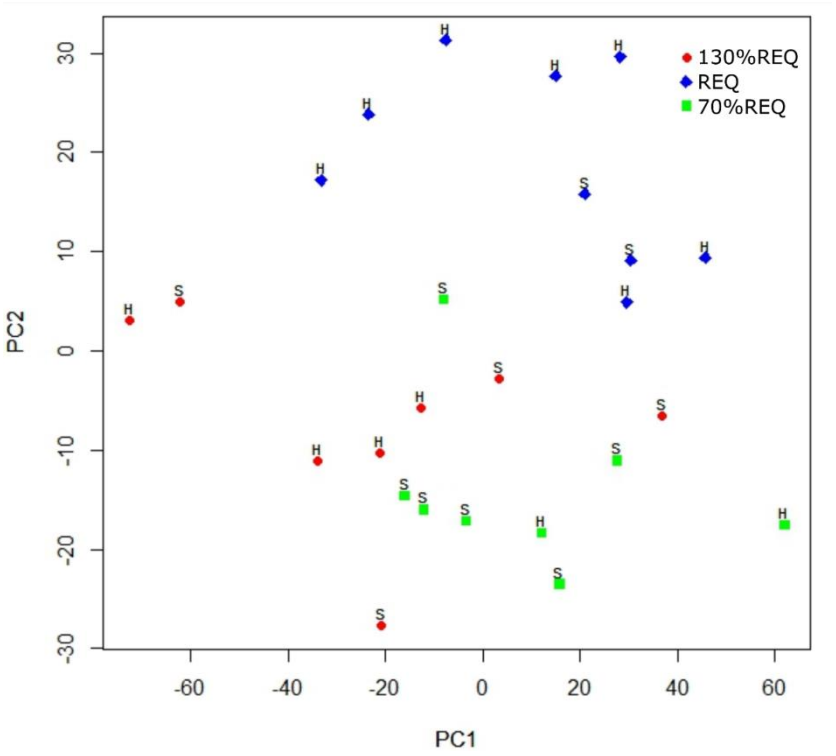


Figure 4.3. Gene dendrogram based on topological overlap measure (TOM) dissimilarity showing the co-expression modules labeled by colors determined by weighted gene co-expression network analysis (WGCNA).

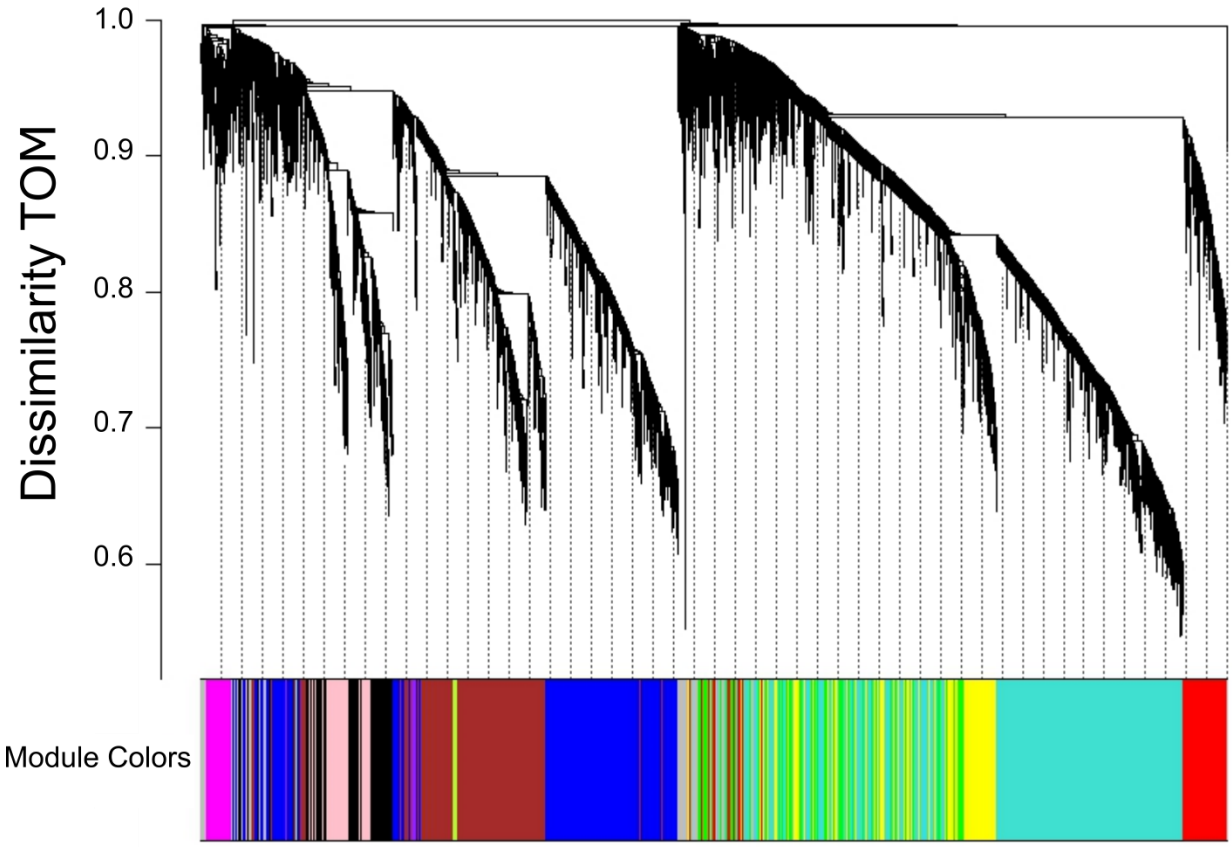


Figure 4.4. Effect of maternal plane of nutrition during mid-gestation on weighted gene co-expression network analysis modules 1- 6 of progeny longissimus muscle transcriptome at 392 d of age. Subscripts denote statistical significance at false detection rate corrected *P*-values ≤ 0.01.

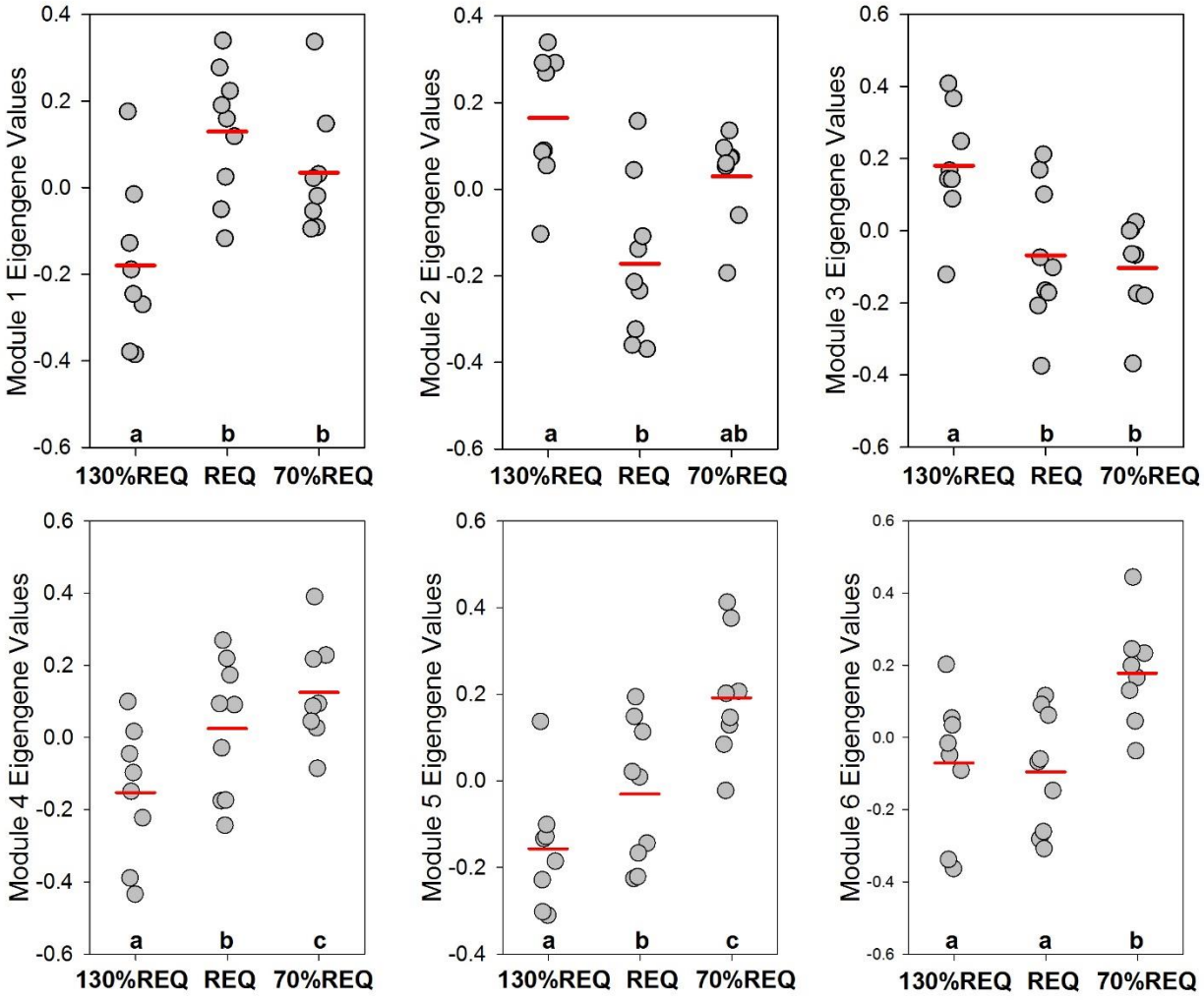


Figure 4.5. Effect of maternal plane of nutrition during mid-gestation on weighted gene co-expression network analysis modules 7- 11 of progeny longissimus muscle transcriptome at 392 d of age. Subscripts denote statistical significance at false detection rate corrected *P*-values ≤ 0.01.

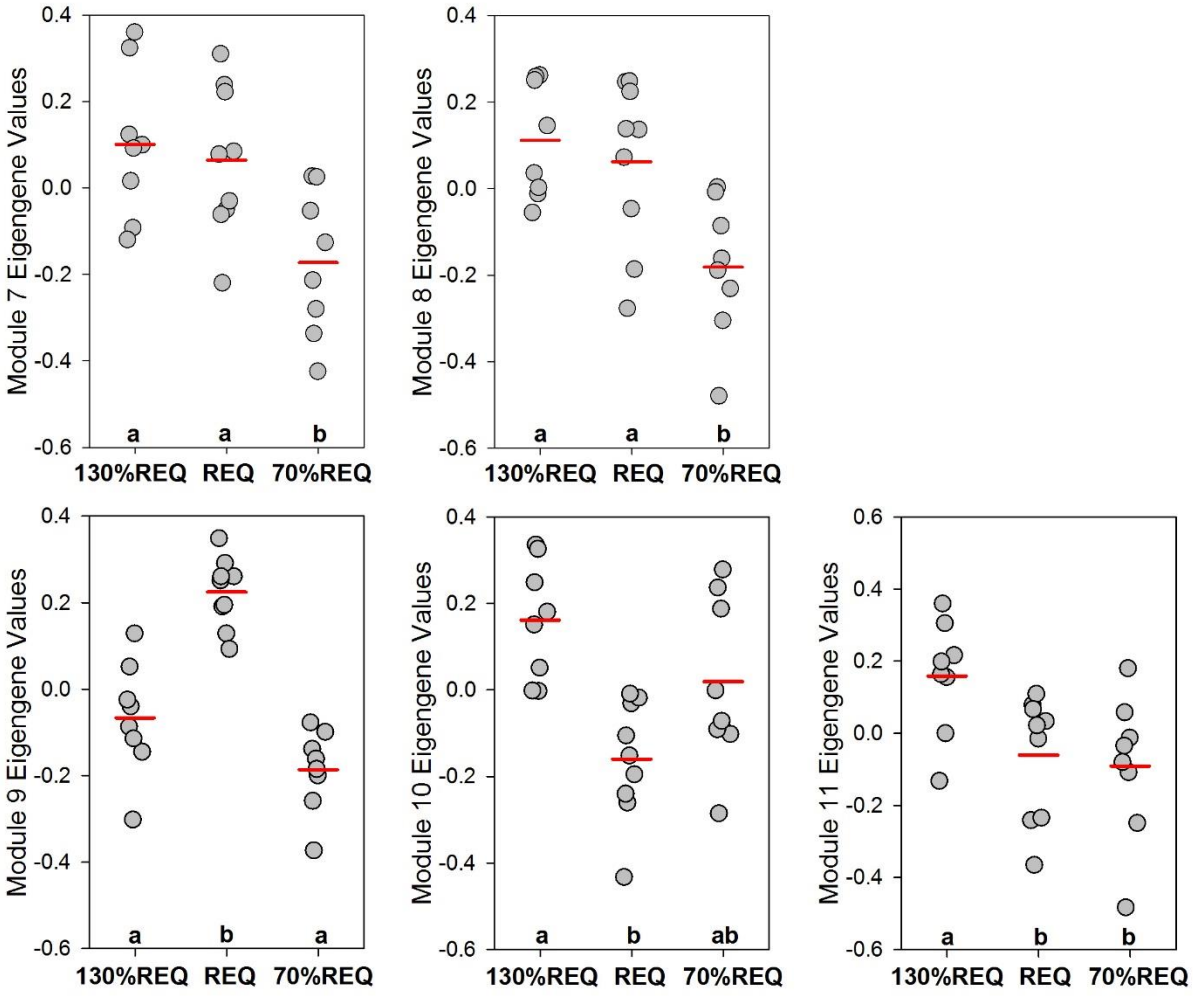


Figure 4.6. Correlation heat map between module eigengenes (ME) and phenotypic measures using Pearson correlations. All correlation coefficients are listed (P < 0.01). The scale bar colors denote the correlation coefficients with 1 indicating a perfect positive correlation (orange) and -1 indicating a perfect negative correlation (blue). Phenotypic traits include: finishing dry matter intake (**DMI**), hot carcass weight (**HCW**), backfat (**BF**), yield grade, intramuscular fat (**IMF**), marbling score (400 - 499 = small), Warner-Bratzler shear force (**WBSF**), cook yield percentage, longissimus muscle (**LM**) area, as well as insulin and glucose area under the curve (**AUC**) values determined during a glucose tolerance test (Wilson, 2015).

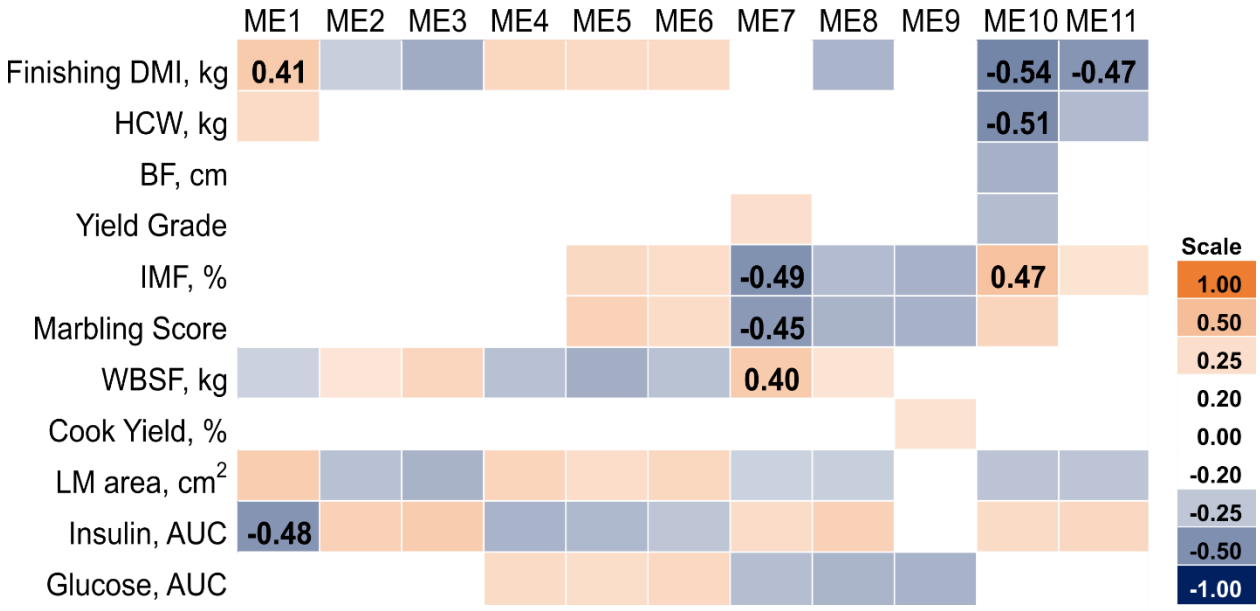


Figure 4.7. Effect of maternal plane of nutrition during mid-gestation on the most impacted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways determined by the Dynamic Impact Approach (DIA) analysis of longissimus muscle transcriptome at 392 d of age. Genes were assigned to module 1 and 7 by weighted gene co-expression network analysis (**WGCNA**). The parent KEGG sub-category for each pathway is provided in addition to the number of genes detected for a given pathway, the most activated gene of interest (**GOI**) determined by fold change, and the pathway specific impact as defined by the DIA.

Module 1 130%REQ vs. REQ

KEGG Sub-Category	KEGG Pathway	Number of Genes	GOI	Impact
Lipid Metabolism	Steroid biosynthesis	10	*SQLE*	
Energy Metabolism	Sulfur metabolism	5	*TST*	
Metabolism of Cofactors and Vitamins	Retinol metabolism	8	*CYP3A4*	
Lipid Metabolism	Steroid hormone biosynthesis	8	*CYP3A4*	
Lipid Metabolism	Synthesis and degradation of ketone bodies	5	*ACAT2*	
Xenobiotics Biodegradation and Metabolism	Drug metabolism - cytochrome P450	10	*MGST1*	
Amino Acid Metabolism	Glycine, serine and threonine metabolism	16	*TDH*	
Xenobiotics Biodegradation and Metabolism	Metabolism of xenobiotics by cytochrome P450	9	*MGST1*	
Lipid Metabolism	Biosynthesis of unsaturated fatty acids	8	*HACD2*	
Lipid Metabolism	Glycerolipid metabolism	20	*MOGAT3*	
Amino Acid Metabolism	Phenylalanine metabolism	8	*GLYAT*	
Metabolism of Terpenoids and Polyketides	Terpenoid backbone biosynthesis	10	*ACAT2*	
Digestive System	Bile secretion	13	*CYP3A4*	
Digestive System	Fat digestion and absorption	6	*MOGAT3*	
Endocrine System	PPAR signaling pathway	24	*PCK1*	

Module 7 130%REQ vs. 70%REQ

Carbohydrate Metabolism	Glycolysis / Gluconeogenesis	14	*PFKM*	
Carbohydrate Metabolism	Fructose and mannose metabolism	6	*PFKFB4*	
Carbohydrate Metabolism	Starch and sucrose metabolism	5	*UXS1*	
Carbohydrate Metabolism	Pentose phosphate pathway	5	*PFKM*	
Amino Acid Metabolism	Alanine, aspartate and glutamate metabolism	4	*ABAT*	
Nucleotide Metabolism	Purine metabolism	8	*PKM*	
Signal Transduction	Calcium signaling pathway	10	*MYLK2*	
Endocrine System	Insulin signaling pathway	8	*PHKA1*	

Figure 4.8. Effect of mid-gestation maternal nutrition on skeletal muscle mature microRNA relative expression in progeny over time. Significant effects are indicated by symbols: day effect (*; $P < 0.01$), treatment × day effect (§; $P < 0.05$), sex effect (‡, $P < 0.05$), and sire effect (¶; $P < 0.05$).

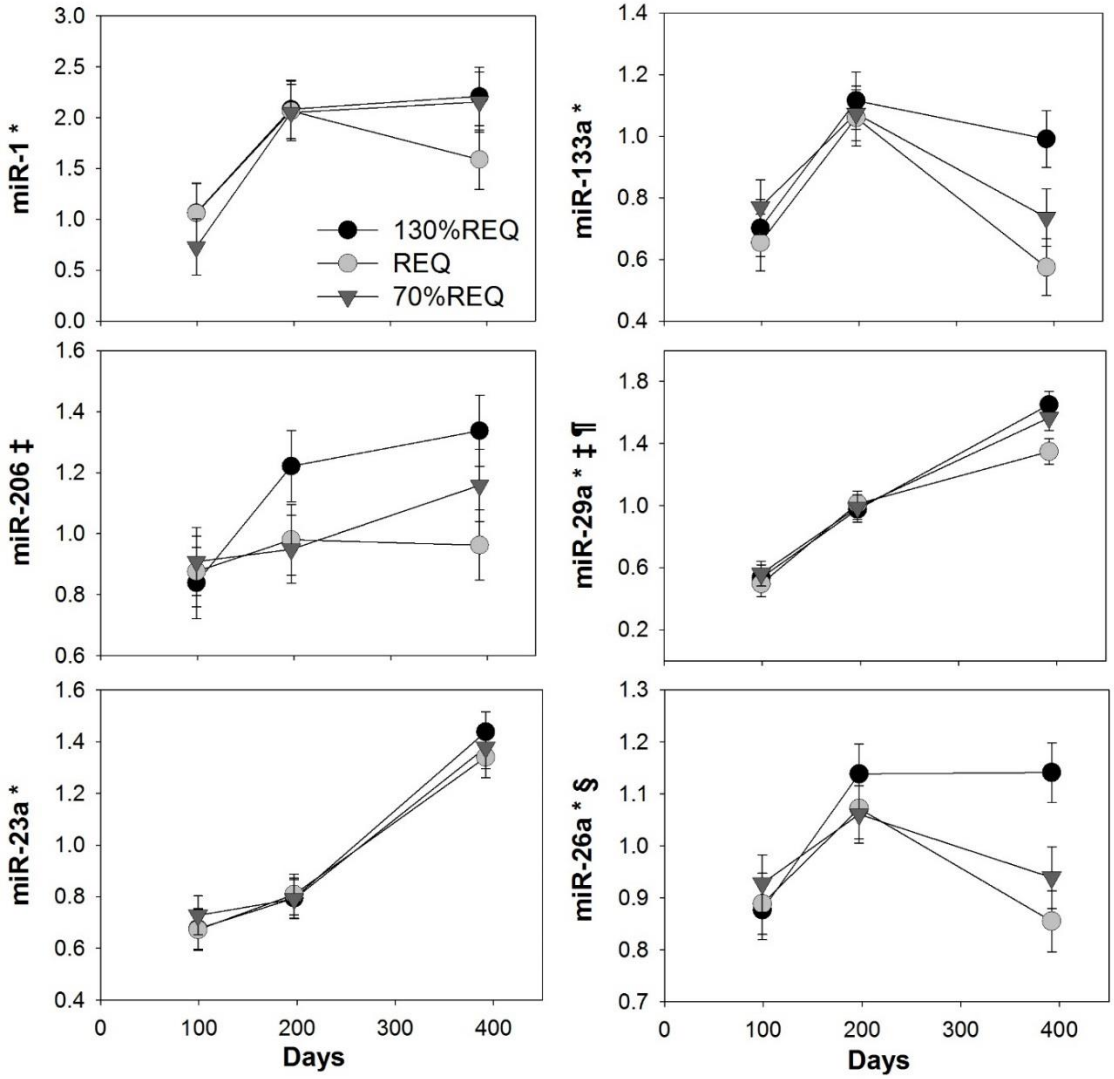
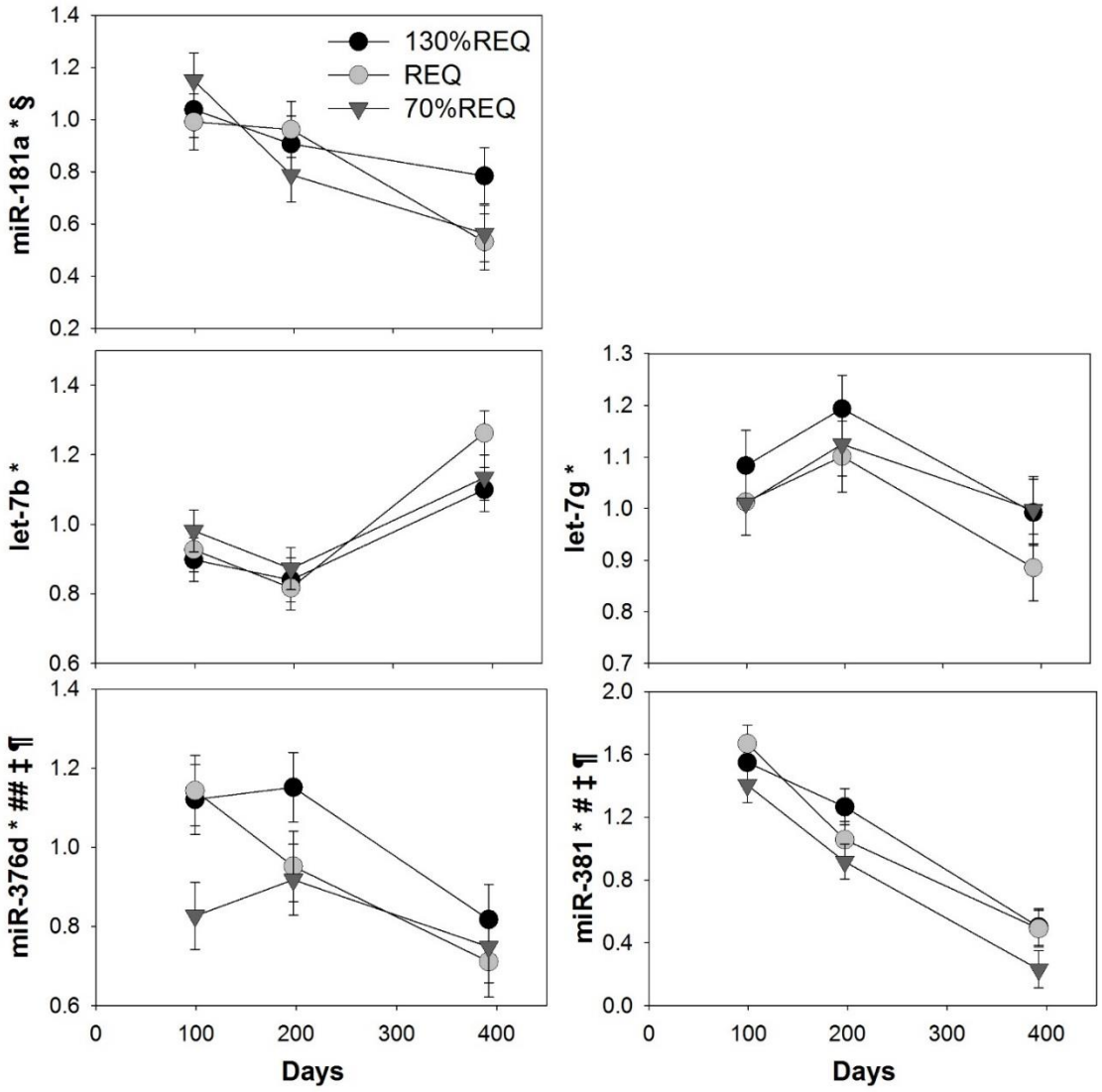


Figure 4.9. Effect of maternal mid-gestation weight gain on skeletal muscle microRNA expression in calves over time. Significant effects are indicated by symbols: day effect (*; $P < 0.01$), treatment effect (#; $P < 0.05$; ##; $P < 0.10$), treatment × day effect (§; $P < 0.1$), sex effect (‡, $P < 0.05$), and sire effect (¶; $P < 0.05$).)



APPENDIX

Table A.1. Ingredient composition of the basal lactation diet.

Ingredient, % DM	
Alfalfa hay	3.36
Grass hay	2.80
Corn silage	33.6
Alfalfa silage	9.32
Cottonseed	8.01
Soy hulls	4.66
Dry ground corn grain	21.0
Lactating supplement[12]	17.3

[1] Lactating supplement was formulated for 43.6% CP, 13.4% NDF, 7.1% ADF, 0.3% lignin, 5.47% crude fat, and 25.21 mEq/100g DCAD, and contained: 24.7% soybean meal, 26.16% bypass protein, 1.94% bypass fat, 7.53% blood meal, 4.3% sodium bicarbonate, 6.13% limestone, 2.26% dicalcium phosphate, 1.18% white salt, and <1% of each of the following: trace minerals, vitamin E.
[2] Additional wheat-barley pellet was topdressed on d 4 feeding.

Table A.2. Ruminal pH response parameters after SARA induction on d 5.[1,2]

	Non-SARA		SARA	
	Mean	SD	Mean	SD
No. of observations	7		5	
Average pH	6.50	0.18	6.13	0.06
pH nadir	5.90	0.21	5.38	0.09
AUC < 5.8	0.01	0.02	1.35	0.52
Time < 6.0, h	4.0	3.87	10.6	2.30
Time < 5.8, h	0.4	0.53	7.0	1.73
Time < 5.6, h	0.0	0.00	3.4	1.52

[1]Reinterpreted data from Luan et al (Luan et al., 2016).

[2]Non-SARA = cows (n = 7) in which ruminal pH was not < 5.6 for 3 h on d 5. SARA = cows (n = 5) in which ruminal pH was < 5.6 for 3 h on d 5.

Table A.3. Primers utilized for qPCR of ruminal bacteria.

Bacteria species		Primers (5` - 3`)	Source
Anaerovibrio lipolytica	F	GAAATGGATTCTAGTGGCAAACG	(Minuti et al., 2015)
	R	ACATCGGTCATGCGACCAA	
Butyrivibrio proteoclasticus	F	GGGCTTGCTTTGGAAACTGTT	(Minuti et al., 2015)
	R	CCCACCGATGTTCCTCCTAA	
Eubacterium ruminantium	F	CTCCCGAGACTGAGGAAGCTTG	(Stevenson and Weimer, 2007)
	R	GTCCATCTCACACCACCGGA	
Fibrobacter succinogenes	F	GCGGGTAGCAAACAGGATTAGA	(Stevenson and Weimer, 2007)
	R	CCCCCGGACACCCAGTAT	
Megaspheara elsdenii	F	AGATGGGGACAACAGCTGGA	(Stevenson and Weimer, 2007)
	R	CGAAAGCTCCGAAGAGCCT	
Prevotella bryantii	F	AGCGCAGGCCGTTTGG	(Stevenson and Weimer, 2007)
	R	GCTTCCTGTGCACTCAAGTCTGAC	
Selenomonas ruminantium	F	CAATAAGCATTCCGCCTGGG	(Stevenson and Weimer, 2007)
	R	TTCACTCAATGTCAAGCCCTGG	
Succinimonas amylolytica	F	CGTTGGGCGGTCATTTGAAAC	(Khafipour et al., 2009)
	R	CCTGAGCGTCAGTTACTATCCAGA	
Streptococcus bovis	F	TTCCTAGAGATAGGAAGTTTCTTCGG	(Stevenson and Weimer, 2007)
	R	ATGATGGCAACTAACAATAGGGGT	
Succinivibrio dextrinosolvens	F	TAGGAGCTTGTGCGATAGTATGG	(Khafipour et al., 2009)
	R	CTCACTATGTCAAGGTCAGGTAAGG	
Eubacterial primer 1	F	GGATTAGATACCCTGGTAGT	(Fliegerova et al., 2014)
	R	CACGACACGAGCTGACG	
Eubacterial primer 2	F	GTGSTGCAYGGYTGTCGTCA	(Maeda et al., 2003)
	R	ACGTCRTCCMCACCTTCCTC	
Eubacterial primer 3	F	CCTACGGGAGGCAGCAG	(Muyzer et al., 1993)
	R	ATTACCGCGGCTGCTGG	

Table A.4. Primers utilized for qRT-PCR of rumen epithelium tissue.

Gene	Accession #	Primers[1]	Primers (5'-3')	bp[2]	Source
CXADR	NM_174298.4	F.644	TCCGACTCACAGAAACTGCC	106	(Walker et al., 2014)
CXADR		R.749	CCGTACAGGTGTATGTCCCG		
CLDN1	NM_001001854	F.480	GGCATCCTGCTGGGACTAATAG	100	(Minuti et al., 2015)
CLDN1		R.579	CAGCCATCCGCATCTTCTGT		
CLDN4	NM_001014391	F.695	CCCCAGCCAGCAACTACGT	103	(Minuti et al., 2015)
CLDN4		R.797	TCACAGATTGCAGTGAGCTCAGT		
JAM2	NM_001083736.1	F.592	CCCCATCGGAACAAGGTCAA	129	(Walker et al., 2014)
JAM2		R.720	GACATCGCAGCTCTACCACA		
OCLN	NM_001082433.2	F.466	GCCATTTTCGCCTGTGTTG	101	(Minuti et al., 2015)
OCLN		R.566	CCAAAGGCACTTCCTGCATAA		
TJP1	XM_582218.8	F.2965	GCACATAGGATCCCTGAACCA	107	(Minuti et al., 2015)
TJP1		R.3071	TGCTTCCGGTAGTACTCCTCATC		
TLR2	NM_174197.2	F.2238	CTGGCAAGTGGATTATCGACAA	102	(Jacometo et al., 2015)
TLR2		R.2340	TACTTGCACCACTCGCTCTTCA		
TLR4	NM_174198.6	F.555	TGCGTACAGGTTGTTCCTAACATT	109	(Jacometo et al., 2015)
TLR4		R.664	TAGTTAAAGCTCAGGTCCAGCATCT		
IGFBP3	NM_174556.1	F.542	GCGCCCTTACCTGCTACC	86	(Grala et al., 2014)
IGFBP3		R.627	CAGCCTGGTTCTCTGTGCT		
IGFBP5	NM_001105327.2	F.513	GTCCAAGTTCGTGGGAGGAG	89	this study
IGFBP5		R.601	AGGGCCCCTGCTCAGATTTC		
DSG1	NM_174045.1	F.775	AGACAGAGAGCAATATGGCCAGT	88	(Steele et al., 2012)
DSG1		R.862	TTCACACTCTGCTGACATACCATCT		
CMTM6	NM_001035066.1	F.419	TTCACTTTGACACATGACAATACCA	103	(Minuti et al., 2015)
CMTM6		R.521	CACGGAGCATAAAGGAGAACTCA		
ERC1	NM_001205419.1	F.2981	CCTCCCATTCCGGTCAAAG	105	(Naeem et al., 2012)
ERC1		R.3085	GTCTGATGTACAACTTGAGCTTGCTT		
MRPL39	NM_001080730.2	F.602	AGGTTCTCTTTTGTTGGCATCC	101	(Bionaz and Loor, 2007)
MRPL39		R.502	TTGGTCAGAGCCCCAGAAGT		

[1]Primer direction (F = forward; R = reverse) and hybridization position on the sequence.

[2]Amplicon size in base pair (bp).

Table A.5. Effect of SARA induction on relative abundances of bacterial families in the solid fraction using 16S rRNA sequencing.[1]

	Non-SARA		SARA		*P*-value[2]		
	d 1	d 6	d 1	d 6	SG	Day	SG × Day
Firmicutes							
Lachnospiraceae	33.27	24.34	27.75	27.96	0.87	0.14	0.12
Ruminococcaceae	15.05	15.40	17.29	19.54	0.44	0.54	0.65
Clostridiales[4]	16.54	14.44	15.46	13.05	0.76	0.14	0.92
Lactobacillales[4]	1.57	7.83	4.57	0.02	0.71	0.79	0.11
Mogibacteriaceae	3.94	3.64	3.41	2.61	0.42	0.26	0.61
Veillonallaceae[3]	1.84	1.67	0.59	1.41	0.38	0.24	0.14
Christensenellaceae	1.18	0.79	0.93	0.89	0.78	0.11	0.21
Clostridiaceae	1.90	1.82	1.82	1.69	0.86	0.75	0.98
Erysipelotrichaceae	0.21	0.22	0.23	0.17	0.78	0.18	0.11
Actinobacteria							
Coriobacteriaceae	6.37	5.85	7.14	6.32	0.74	0.49	0.88

[1]Non-SARA = cows (n = 7) in which ruminal pH was not < 5.6 for 3 h on d 5. SARA = cows (n = 5) in which ruminal pH was < 5.6 for 3 h on d 5.

[2]SG = SARA grouping of cows based on ruminal pH as Non-SARA or SARA.

[3]Data were logit transformed to ensure normality of residuals.

[4]Listed at the lowest level of taxonomic assignment (order).

Table A.6. Effect of SARA induction on relative abundances of bacterial families in the liquid fraction using 16S rRNA sequencing.[1]

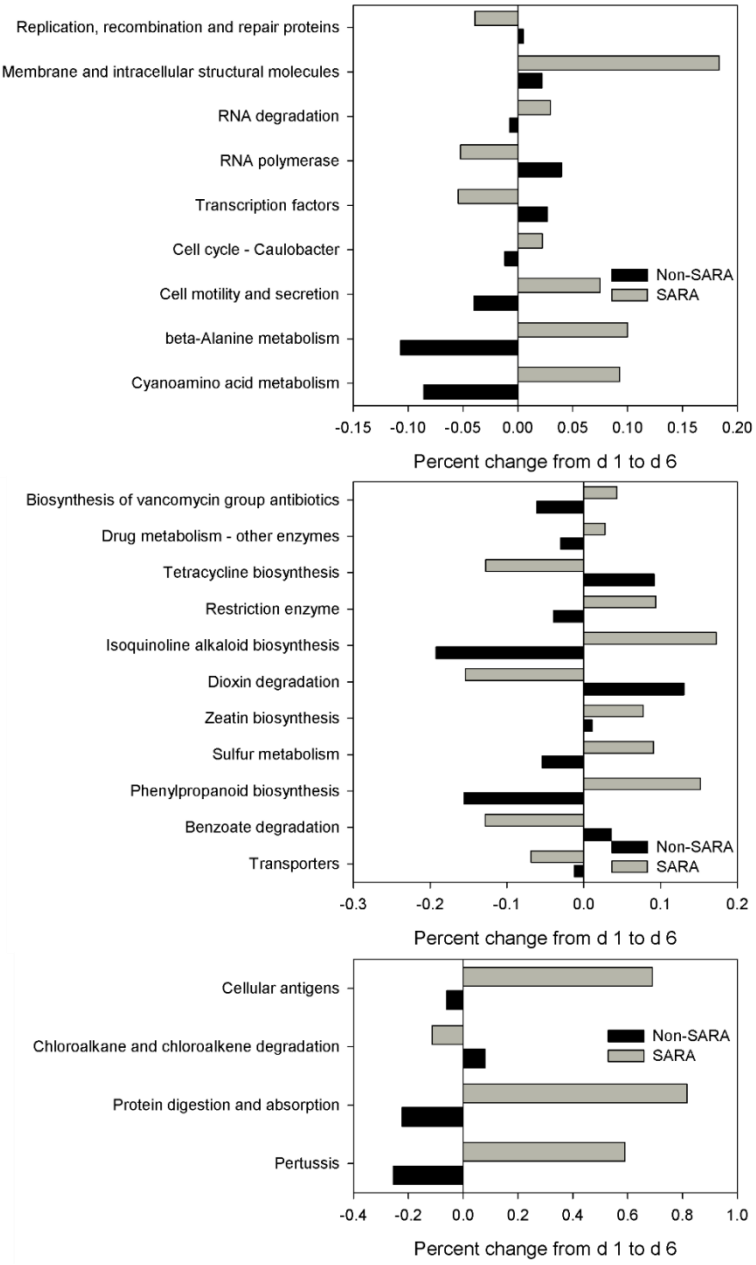
	Non-SARA		SARA		*P*-value[2]		
	d 1	d 6	d 1	d 6	SG	Day	SG × Day
Bacteroidetes							
Paraprevotellaceae	4.92	4.96	5.45	6.41	0.39	0.39	0.43
Firmicutes							
Ruminococcaceae	13.67	12.97	12.87	14.38	0.67	0.21	0.39
Clostridiaceae[3]	0.25	0.21	0.20	0.24	0.91	0.93	0.46
Mogibacteriaceae	0.48	0.39	0.41	0.41	0.78	0.44	0.41
Carnobacteriaceae	0.20	0.10	0.20	0.27	0.73	0.86	0.45
Veillonallaceae	0.16	0.22	0.24	0.34	0.49	0.11	0.86

[1]Non-SARA = cows (n = 7) in which ruminal pH was not < 5.6 for 3 h on d 5. SARA = cows (n = 5) in which ruminal pH was < 5.6 for 3 h on d 5.

[2]SG = SARA grouping of cows based on ruminal pH as Non-SARA or SARA.

[3]Data were logit transformed to ensure normality of residuals.

Figure A.1. Effect of SARA induction on the predicted metagenome pathways in the solid fraction. Values represent the percentage change in expression of a given pathway from d 1 to d 6. Positive values indicate an increased representation on d 6 compared with d 1 of a given pathway in the predicted metagenome, while negative values describe a percent decrease on d 6 of a predicted pathway.



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