EFFECT OF POST-EXTRACTION ALGAL RESIDUE SUPPLEMENTATION ON THE

THE THERUMEN MICROBIOME OF STEERS CONSUMING LOW-QUALITY FORAGE

A Thesis

by

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Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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August 2013

Major Subject: Animal Science

ABSTRACT

The rumen microbiome is a dynamic environment consisting of bacteria, protist, and fungi responsible for fiber degradation. Advances in molecular techniques have enabled description of bacterial microbiome via pyrosequencing. Cattle consuming low-quality forage are often supplemented with protein to increase forage intake and digestion, but effect on the rumen bacterial community is unknown. Thus, increasing post-extraction algal residue (**PEAR**) and cottonseed meal (**CSM**) supplementation was provided to steers consuming oat straw to observe the rumen microbiome within the liquid and solid fraction. Weighted UniFrac analysis indicated different fraction-associated communities with greater similarity across treatments in the solid fraction. *Bacteroidetes* was the predominant phyla detected in all samples (>65%). Within *Bacteroidetes*, *Prevotella* was the most abundant genus. In the liquid fraction, *Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiaceae* increased with PEAR provision ($P < 0.05$). Similar proportions of bacteria between unsupplemented control and CSM supplemented steers indicate factors other than N supply may impact ruminal bacteria populations. A second experiment evaluated the effect of supplemental CSM or dried distillers' grain (**DDG**) for Brahman steers consuming rice straw. Total digestible OM intake and total tract OM digestion increased linearly with additional CSM and DDG provision ($P < 0.01$). Provision of CSM increased ruminal ammonia linearly ($P < 0.01$), but DDG supplementation resulted in a quadratic response ($P = 0.02$). Overall, protein supplementation may increase LQF utilization via changes in the rumen microbiome.

DEDICATION

I would like to dedicate my thesis to my family: my Heavenly Father, Dad, Mom, Zach and Jenn.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Molecular analysis of 16S rRNA sequence data

Although the premise of host-microbe interrelationship had long been established (Krause et al., 2013), the function of the rumen was unknown for many years. Once rumen bacteria were cultured under anaerobic, *in vitro* conditions, substantial gains were made in describing microbial populations by using roll-tube techniques (Hungate, 1969) or most probable number estimates (Dehority et al., 1989). Many bacterial strains were successfully isolated, phenotypically and physiologically characterized based on substrate affinity and fermentative abilities. However, recent improvements in molecular techniques have unlocked a different perspective on microbial communities. Molecular techniques, unbiased by the culturing aptitude of an individual species, indicate over 90% of bacterial species in the rumen were not previously known to exist (Whitford et al., 1998; Tajima et al., 2000). This new field, studying many genomes simultaneously, has been termed metagenomics (Handelsman, 2004). The most common technique to study and compare bacterial communities uses the 16S ribosomal RNA (**rRNA**) gene to describe microbiome richness, diversity, and composition. While metagenomic principles are undoubtedly a powerful tool to investigate unexplored aspects of the rumen microbiome, a thorough understanding of the many caveats in analysis is required to make strategic improvements that impact fermentative efficiency and overall production.

Extraction of nucleic acid from environmental samples is necessary for all downstream molecular methods. Although there are many extraction protocols optimized for different samples types, all are built on similar principles. Initially, mechanical and/or chemical cell lysis ensures cell particles are sufficiently disturbed using garnet or glass beads and a lysis buffer. Traditional extraction methods mix phenol chloroform with an aqueous sample to form a lower, organic layer containing protein and an upper, aqueous layer with genetic material during centrifugation. Extracted DNA can be recovered using a spin column or ethanol precipitation. The vast majority of extractions use commercially available kits with slight alterations to optimize quantity and quality of extracted DNA.

The small subunit (**SSU**) of the 16S rRNA gene is present in all bacteria and contains about 1,500 nucleotides composed of highly conserved and hypervariable regions. Conserved regions serve as primer sites for *in vitro* amplification by PCR (Ludwig et al., 1994) or for universal probes to amplify large phylogenetic domains such as bacteria or archaea. Resulting amplified sequences are referred to as amplicons. Hypervariable regions are unique to individual genera, species, or even strains and can be used to discern specific population differences within a microbiome (Amann et al., 1990; Raskin et al., 1997). Appropriate primer selection is critical to ensure the hypervariable region amplified provides the greatest amount of information about the microbiome of interest without a bias against an important population of interest. Different universal primers are more effective than others and particular hypervariable regions of the 16S rRNA gene are more taxonomically informative (Liu et al., 2007;

Soergel et al., 2012). Analysis of the nine main hypervariable regions indicated the V1-V4 region estimated specie richness with greater accuracy, but amplification of any region was unable to faithfully predict the richness described by full-length16S sequences (Kim et al., 2011). However, partial 16S sequences from all evaluated hypervariable regions described the microbial community structure similar ($P > 0.25$) to the full-length 16S sequences using UniFrac analysis (Kim et al., 2011). The 16S rRNA gene does not evolve uniformly across its length and hypervariable regions vary in nucleotide length (Schloss, 2010). Therefore, choosing different hypervariable regions can impact downstream analysis of microbial diversity. Longer hypervariable regions are able to account for a greater percent of the phylogenetic variation as compared to the entire 16S rRNA genes (Schloss, 2010). The wealth of 16S sequence data makes it the most common choice for a marker gene, but diversity results can be biased by multiple copies (up to 15) of the 16S gene in a single bacterial genome (Klappenbach et al., 2001).

Amplification of specie-specific regions has increased sensitivity enabling detection of microbial populations present below detection levels of universal primers in pyrosequencing, such as *Megasphaera elsdenii* (Klieve et al., 2003). However, the incredible diversity and range of population densities found in most microbiomes prevents accurate quantification of all bacteria species thereby limiting description of ecologically "rare" taxa. Universal oligonucleotide probes amplify broad domains of microbes providing a sufficient quantity of nucleic acid to evaluate diversity of the entire microbiome. Community fingerprint analysis was the first technique to create a broad

snapshot of a microbial environment and describe ecological changes (Muyzer et al., 1993). Denaturing gradient gel electrophoresis (**DGGE**), terminal restriction fragment length polymorphism (**TRFLP**), and automated ribosomal intergenic spacer analysis (**ARISA**) are the main fingerprint methods used to describe microbial communities. While DGGE is based on the sequence-specific melting behavior of amplicons, TFRLP uses polymorphic terminal fragments after restriction digestion at specific target sites (Marsh, 1999; Zoetendal et al., 2004). Observations indicate DGGE can detect differences in taxa representing only 1% of the total population (Muyzer et al., 1993). However, it can be challenging to separate DNA fragments with a limited amount of sequence variation and bands must be cut out of the gel to be sequenced for taxonomic identification (Muyzer and Smalla, 1998). Alternatively, ARISA determines the length between the 16S and 23S genes in bacteria (Fisher and Triplett, 1999). This intergenic region can be up to 1,400 bp long and unique for most bacterial species. Oligonucleotide primers are utilized in PCR to amplify the intergenic regions, and then capillary electrophoresis separates the amplicons. Peaks in the electrophoretogram correspond to the minimum number of bacterial species present in a sample (Fisher and Triplett, 1999). Although community fingerprint analysis is useful for showing shifts in microbial ecology, the technique has a limited ability to describe the present taxa. Moreover, DGGE, TFRLP, and ARISA underestimate species richness due to the long-tail distribution of diverse microbiomes and have been mostly replaced by next-generation sequencing approaches (Bent et al., 2007).

Recent advances in DNA sequencing technology have dramatically changed the potential to investigate microbial ecology using high throughput molecular techniques. Sanger sequencing was the original sequencing technology pioneered by Frederick Sanger (Sanger et al., 1977). Although the technology produced extremely long sequence reads greater than 1,000 nucleotides, the necessary time and expense limited the access and incorporation of the technique to a few fields of science. Developments in sequencing-by-synthesis technology have changed sequencing availability as the paralleled nature of the technology has significantly reduced cost and time required. Although the output reads are shorter in length, the thousands or millions of additional reads generated result in a similar level of detection compared to full-length 16S rRNA gene (Youssef et al., 2009).

The Illumina HiSeq 2000 and Roche 454 FLX Titanium are currently the most commonly utilized sequencing platforms and are considered second generation technology. Although the HiSeq 2000 generates more reads per run at a lower cost per bp, the 454 FLX Titanium reads are much longer in length (400-500 vs. 100 bp). The initial step in sequencing fragments strands of DNA and ligates the strands to adapters. For the 454 platform, each ligated strand is attached to an individual bead to facilitate emulsion PCR amplification of DNA. Beads with single-stranded DNA templates are loaded into a picotiter plate for pyrophosphate sequencing (Rothberg and Leamon, 2008). A DNA polymerase adds individual nucleotides to the single-stranded DNA molecule releasing a pyrophosphate-initiated enzyme cascade including luciferase resulting in light emission that is correlated to the base pair (Wooley et al., 2010).

Alternatively, Illumina's platform utilizes dual adapters followed by bridge amplification. Once the bridges are broken, sequencing-by-synthesis occurs and a laser detects the color of light from the fluorescent label attached to each added base pair (Rothberg and Leamon, 2008). Thousands of reactions occur simultaneously, thus leading to the term highly paralleled, high throughput sequencing. Oxford Nanopore's GridION, Life Technology's ion torrent, and Pacific Bioscience's PacBio RS represent next-generation sequencing technology that seeks to improve the speed, read output, and sequence length while decreasing the cost and error rate in sequencing. These platforms have not been widely used in metagenomics and will not be discussed further.

Although DNA sequencing platforms were designed with whole genome sequencing in mind, adding a unique barcode sequence to each sample's amplicons can multiplex many samples or treatments into a single pyrosequencing run. Multiplexing is also termed pyrotagging or barcoding, and it refers to the process of adding a unique identifier sequence to all amplicons in a sample. Multiplexing has made sequencing and analysis of many microbial communities dramatically more efficient and cost-effective (Foster et al., 2012). As sequencing platforms continue to generate more reads per run, barcoding will continue to increase the efficiency of pyrosequencing multiple microbiomes simultaneously (Liu et al., 2007).

Once the reads have been sequenced, generating meaningful quantitative analysis remains the largest challenge in metagenomics, often referred to as the "bioinformatics bottleneck" (Teeling and Glöckner, 2012). Thus, the areas of computational biology and bioinformatics have become increasingly more important to microbial ecologist. There

are multiple challenges in analysis of 16S sequence data that need to be addressed: quality filtering, clustering of operational taxonomic units (**OTUs**), taxonomic assignments, and estimates of community diversity.

Quality filtering of sequence reads is critical to accurate analysis. Any artifacts from upstream processing in the sequence data must be corrected or removed prior to analysis. Most notable filtering steps include trimming, homopolymer removal, chimera removal, and denoising. During 454 sequencing, the quality of the base calls decrease at the end of a sequence read (Balzer et al., 2010). A read needs to be trimmed when the quality score of the base pair call is below 25 within a sliding window of 50 bp. A quality score of 25 is generally considered the threshold of acceptability. Quality-filtering by setting proper thresholds for minimum number of high-quality calls for a read, maximum number of consecutive low-quality calls, and eliminating reads with ambiguous bases, improved the accuracy of within-sample diversity (alpha diversity) estimates from Illumina amplicon sequencing (Bokulich et al., 2013).

When sequencing an entire genome, single base pair call errors are overlapped and masked by sufficient coverage depth. Therefore, quality filtering is not as stringent. However, in 16S downstream analysis, single bp errors appear as novel bacteria (Schloss et al., 2011). Additionally, the 454 platform has issues with homopolymers, a sequence of identical bases, because repetitive bases are recorded as a higher peak in the flowgram representing multiple base pairs. Longer homopolymers are more difficult to sequence, and the read quality is dependent on the precise calibration of base calls (Quince et al., 2009).

Chimeric sequences are artifacts created during PCR when incomplete extension occurs in a cycle. In the next cycle, a DNA strand may attach where the original parent left off if the initial region is similar. The amplicon is completed by the second parent resulting in a hybrid amplicon for amplification in subsequent cycles. Without chimera detection, the hybrid amplicon sequence would appear as previously unclassified and inflate species richness. There are multiple tools for chimera detection including ChimeraSlayer (Haas et al., 2011), UCHIME (Edgar et al., 2011), Perseus (Quince et al., 2011), and DECIPHER (Wright et al., 2012). ChimeraSlayer and UCHIME implement reference-based and de novo filtering, while Perseus only uses de novo filtering. DECIPHER executes a search-based approach to find short fragments uncommon in the phylogenetic group where a query sequence is classified and compares them to another phylogenetic group (Wright et al., 2012). De novo filtering of chimeras assumes more abundant sequences are less likely to be chimeric and uses them as the reference set (Quince et al., 2009). Chimeras are thought to represent from 5 to 45% of reads from pyrosequencing (Haas et al., 2011), and chimera removal should be considered a prerequisite step in pyrosequencing data analysis.

Noise is variation introduced through experimental procedures that distorts true diversity. In 454 sequence amplicons, noise can artificially inflate the number of OTUs detected (Quince et al., 2009). Therefore, denoising is an essential step to preserve alpha diversity and the correct number of species. Pyrosequencing and PCR steps are the main contributors of noise. PyroNoise is an algorithm created to cluster the flowgram output directly from the 454 sequencer instead of sequences to model 454 sequencing errors

(Quince et al., 2009), but computational cost hindered its utility. A new implementation of PyroNoise was combined with SeqNoise to form AmpliconNoise (Quince et al., 2011). SeqNoise algorithm performs alignments and uses sequence frequency to determine different rates of nucleotide errors in PCR. A comparison of AmpliconNoise to Denoiser (Reeder and Knight, 2010) and Single-linkage preclustering (Huse et al., 2010) indicated AmpliconNoise was more successful at removing noise from an artificial community of a known composition, albeit at a greater computational cost. Deficiencies identified in AmpliconNoise include a limited ability to differentiate fine-scale diversity as read abundances are not fully used when calculating distance between sequences and clusters; also an experiment-specific training data set is required to set custom parameters and determine how conservative the algorithm infers real diversity. As a result a novel algorithm, DADA, was created. DADA incorporates a model of substitution errors into a clustering algorithm and groups error-containing reads to clusters from a single sample genotype (Rosen et al., 2012). Computing performance of DADA is an order of magnitude faster than AmpliconNoise with an improvement in detecting fewer false positives. However, DADA has yet to be used in a published experiment.

An OTU is a group of organisms with 16S rRNA gene sequences with a defined level of similarity. Commonly, OTU is used in describing bacterial community composition as a proxy for species when 16S rRNA sequences are 97% similar (Weinstock, 2012). Inflation of OTU estimates leads to increased alpha and beta diversity, thus the correct determination of OTU or taxa is critical. The initial step is

picking OTUs from quality-trimmed and chimera-checked sequences based on the definition of OTU. De novo or open clustering formerly used complete linkage and average linkage based algorithms, but now greedy clustering algorithms have increased the efficiency of this process (Sun et al., 2012) However, without quality filtering and use of a reference set, OTU numbers can be overestimated. Reference or closed clustering also uses greedy algorithms to sequentially cluster OTUs starting with the most abundant sequence followed by direct comparisons at the specified similarity level to previously formed clusters. Once OTU clusters have been formed, a representative sequence is chosen for each OTU for subsequent downstream analysis. Typically, the most abundant sequence is chosen, but other options include random selection or the longest read within an OTU cluster. There are many clustering methods including: CD-HIT (Li and Godzik, 2006) basic local alignment search tool (**BLAST**; (Altschul et al., 1990) , UCLUST (Edgar, 2010), SEED (Bao et al., 2011) and several options within mothur (Schloss et al., 2009). CD-HIT uses a greedy incremental algorithm that orders sequences by decreasing length and selects the longest read as the seed of the first cluster. Other sequences are compared with existing seeds and if defined similarity is not met, it becomes the seed of a new cluster (Li and Godzik, 2006). A recent addition to the CD-HIT package, CD-HIT-OTU, implements multi-cluster steps to remove noise resulting in similar performance to AmpliconNoise (Li et al., 2012). BLAST compares sample sequences against a reference database of sequences for clustering by locating short matches between the two sequences (Altschul et al., 1990). UCLUST uses a similar greedy incremental approach, but includes heuristics, USEARCH algorithm, to

improve speed of sequence comparison (Li et al., 2012). Compared to the original CD-HIT, UCLUST was able to compute clusters of greater similarity with less computational resources (Edgar, 2010). SEED was created to work with high volumes of short reads from the Illumina platform, and it is mainly utilized for identification, enumeration, and removal of redundant sequences. To cluster sequences users can define parameters with up to three mismatches and three base pair overhanding ends (Bao et al., 2011). Mothur has options to use the nearest neighbor, average neighbor, or furthest neighbor algorithms to cluster sequences at the specified similarity (Schloss et al., 2009).

Reference alignments are constructed from large deposits of 16S sequence data and include template alignments of the 16S rRNA gene of many organisms. The most common databases of 16S sequence data include Greengenes (McDonald et al., 2012), SILVA (Quast et al., 2013), and the Ribosomal Database Project (**RDP**; Cole et al., 2009). Construction of each database affects the quality of the template alignments. For example, the RDP database does not align the hypervariable regions in contrast to SILVA and Greengenes (Schloss, 2013). Multiple sequence aligners (**MSA**) are tools that facilitate matching sequence reads to template sequence alignments in a database. The Nearest Alignment Space Termination (**NAST**) algorithm is commonly used for this purpose and implemented as PyNAST (Caporaso et al., 2010a) in QIIME (qualitative insights into microbial ecology; Caporaso et al., 2010b). INFERNAL is another MSA that constructs consensus RNA secondary structure profiles, covariance models, and uses them to search a reference set for homologous sequences or create new multiple

sequence alignments (Nawrocki et al., 2009). Other MSAs, including MUSCLE (Edgar, 2004) and ESPIRIT (Sun et al., 2009), work independent of reference databases. One disadvantage of these de novo MSAs is the inability to utilize the secondary structure of the 16S rRNA molecule to bolster alignment confidence.

Assignment of taxonomic identifications requires a large, high-quality database of known 16S sequences and an efficient, appropriate method to compare one's sequence reads to the database. Methods of identification include global alignment of sequence taxonomy (**GAST**), BLAST, RDP classifier, and C16S. GAST uses the sequence alignment to compare against the full-length 16S rRNA genes reference data sets and their pre-computed phylogeny (Huse et al., 2008). The GAST distance indicates how close the alignment is to the nearest sequence and is easily visualized in VAMPS (http://vamps.mbl.edu/). BLAST can also be used for taxonomic assignments by searching a database of 16S sequences from known genomes. Every sequence is assigned to an organism whose 16S sequence represented the best hit, but the process is not very effective for metagenomic samples with many uncharacterized species (Ghosh et al., 2012). In contrast, the RDP Classifier is a naïve Bayesian algorithm that uses "words" (short strings of 8 base pairs) matching to find the nearest genus based on defined confidence level (Wang et al., 2007). The sample sequence is compared to the reference sequence and the number of matching words between the two sequences is used to assign most likely taxa at the lowest possible taxonomic level. One-hundred iterations of taxonomic identification are computed and correct identification is based on whether the specified bootstrap confidence score (typically 0.5-0.8) is met. The RDP

Classifier can effectively classify unknown bacteria at higher levels of taxonomic identification (phyla, class, order, family, and genus). A new method called C16S has been developed for assigning taxonomy in metagenomic samples using information from the 16S gene. C16S implements a hidden Markov model-based algorithm with two phases that results in assignments similar to the RDP Classifier with less computational costs (Ghosh et al., 2012). The first phase assigns the 16S sequence to a precomputed, genus-specific hidden Markov model, while the second phase adjusts the taxonomic level of identification based on the quality of the initial assignment. Methods of taxonomic assignment will continue to improve in robustness and facilitate greater microbiome description.

Measures of diversity are foundational principals in microbial ecology that allow changes in community composition to be detected. Regardless of approach, biodiversity estimates seek to describe the richness and evenness of an environment from a species estimate or phylogenetic perspective. Richness refers to a basic count of OTUs where more OTUs indicate a richer environment, and evenness describes the similarity of species' population sizes in community. The combination of richness and evenness represent the diversity of the microbiome. Alpha diversity represents the diversity present within a single microbiome, while beta diversity is the difference in diversity between two microbial communities (Whittaker, 1972).

Depending on how well-defined the microbiome of interest may be, diversity can be studied from an OTU or species approach; these terms are interchangeable for the discussion of diversity estimates. Parametric and nonparametric estimation as well as

community phylogenetic methods are utilized to describe alpha diversity from environmental samples. Nonparametric approaches are most common for estimates of alpha diversity and OTU richness in small samples, and they are based on mark-release-recapture statistics without an assumed species abundance model. Methods pioneered by Anne Chao and colleagues (Chao and Bunge, 2002; Chao et al., 2006) are the most common and widely implemented in available software such as mothur and QIIME. Often used richness estimators including Good-Turing, Chao1, Abundance Base Coverage Estimator (**ACE**), and Chao-Bunge, are mathematically simple and straightforward (Bunge, 2011). Specifically, Chao1 uses the frequency of OTU singletons and doubletons to estimate the minimum richness of an environment (Bohannan and Hughes, 2003). Because Chao1 is only based on observed OTUs, it does not account for unobserved OTUs and therefore can underestimate diversity. It is also subject to bias from varying abundance of PCR-amplified genes (multiple copies of 16S rRNA gene) and the inclusion or exclusion of outlier species that are very abundant, but a similar bias across multiple communities would still facilitate adequate comparisons. Furthermore, singletons from pyrosequencing have been shown to be artifacts and subsequent removal eliminates use of Chao1 (Kunin et al., 2010; Tedersoo et al., 2010; Gihring et al., 2012).

The Shannon index is the most common nonparametric estimator of alpha diversity originally described in Shannon and Weaver (1949). By estimating both OTU richness and evenness, changes in the index can be difficult to interpret (Magurran, 2004). The index does assume all species from a given community are represented in

the sample. Large variation in sequencing depth affects estimated diversity and thus sample size (number of sequences per sample) should be standardized prior to estimation using the Shannon index. Improvements to the Shannon index were made by (Buzas and Hayek, 1996); splitting the index value into a richness and evenness value enhanced understanding of changes in diversity. Simpson's index (*D*) describes the probability of randomly selecting two individuals from a large community belonging to the same OTU (Simpson, 1949). The index seeks to capture the variance of the species abundance distribution and is weighted by relevant abundances of predominant species in a sample (Magurran, 2004). Often the reciprocal is used ($1/D$) such that an increased value indicates more even abundances of species within a community.

Alternatively, parametric methods can estimate the number of unobserved OTUs by fitting sample data to an expected model of relative OTU abundance. With a relatively small sample from a diverse environment, diversity can be estimated using Poisson, lognormal, or other models. However, drawbacks include an unclear choice of the appropriate abundance model and necessary assumptions of the estimated total specie number and most abundant OTU (Bohannan and Hughes, 2003). Recent advancements in this area of statistical research have led to development of parametric mixture models (Bunge, 2011). Although more computatively rigorous due to maximum likelihood estimation of parameters, these models are accurate in high-diversity populations. They mix multiple parametric models such that rare OTUs and abundant ones are estimated with different models. CatchAll represents the first program to implement parametric species richness modeling in an accessible form while still

computing coverage-based nonparametric estimates (Bunge, 2011). Diversity of the cattle fecal microbiome was successfully estimated with CatchAll demonstrating the ability to discern richness within a community (Shanks et al., 2011).

Diversity of a microbiome can also be evaluated from a phylogenetic standpoint. Comparing the shape of assembled phylogenetic trees from multiple samples can indicated changes in diversity. Specifically, lineage/time plots use differences between the constant predicted rate and the observed rate to represent variation between communities. One disadvantage is the assumption that diversity in the sample characterizes the diversity in the environment (Bohannan and Hughes, 2003). Phylogenetics has also been used to predict the number of copies of the 16S gene in a bacterial genome to more accurately describe the relative abundances in a microbiome (Kembel et al., 2012).

Similarity indices are used to estimate beta diversity by comparing bacterial communities from different environments. The classic comparison methods are strictly based on the incidence of shared or unique species between two samples. Jaccard (Jaccard, 1902), Sørenson (Chao et al., 2006), and an updated version of the Simpson index (Lennon et al., 2001) are the most commonly used, but all fail to account for abundance and tend to bias similarity downward. Specifically, the Jaccard index is a simple ratio of shared species compared to total species in a pair of samples (Foster et al., 2012). Another set of indices including Morisita-Horn and Bray-Curtis, utilize species abundance within a population. Morisita-Horn is unique in that it is nearly independent of sample size. However, this is because it is very sensitive to the most

abundant species, and thus insensitive to changes due to rare taxa (Magurran, 2004). Moreover, it has been shown to underestimate similarity systematically (Chao et al., 2006). Alternatively, the Bray-Curtis index only applies when sequence reads across samples are equal, but it accounts for both presence and absence of OTUs. Although the sample size can be standardized to an equal sampling depth, this is only an acceptable assumption when the two communities have the same total of number of individuals susceptible to sampling (Chao et al., 2006).

Another common beta diversity statistic is UniFrac because it allows simultaneous evaluation of many microbiomes. UniFrac uses the phylogenetic distance and accounts for different degrees of similarity between different sequences (Lozupone and Knight, 2005). Output from UniFrac can easily be implemented in standard multivariate statistics using Principle Coordinate Analysis (**PCoA**). Additionally, a weighted UniFrac option incorporates species abundance into the diversity measure.

There are a multitude of steps and tools required for complete analysis of 16S sequence data. Fortunately, several groups have developed programs to host much of the available software and facilitate a complete pipeline analysis with one interface. These tools enhance the ability to participate in metagenomics research without an extensive background in bioinformatics or programming. The two most commonly used open-source pipelines are QIIME (Caporaso et al., 2010b) and mothur (Schloss et al., 2009). While mothur is written in C++ language, QIIME was built using the PyCogent toolkit and both can be implemented in a variety of computing environments. Other pipeline analysis tools exist that may or may not allow for complete sequence data

process include: MG-RAST (Meyer et al., 2008), RDP (Cole et al., 2009), SnoWMAn (Stocker et al., 2011), VAMPS (http://vamps.mbl.edu), and the CD-HIT package (Wu et al., 2011; Li et al., 2012). With continued pipeline development, metagenomics research will continue to expand to a broader cross-section of the scientific community.

Understanding the challenges associated with using 16S rRNA sequence data to describe microbiomes is critical for proper experimental design, analysis pipeline construction, and meaningful interpretation to strategically manipulate microbial ecology for benefits in production.

Current perspective of bacteria in the rumen microbiome

Ruminants are important contributors of meat, milk, fiber, and draft worldwide. Considering cellulose is the most abundant organic molecule on earth, the ability to effectively ferment cellulolytic materials uniquely positions ruminants to utilize vast resources globally without directly competing with humans. Continuous fermentation in the rumen is driven by a diverse and competitive microbiome consisting of bacteria, archaea, protozoa, and fungi. Regardless of the substrates provided by the diet, volatile fatty acids (**VFA**) and microbial crude protein (**MCP**), the principle products of fermentation, are responsible for addressing a large portion of host energy and protein requirements. While VFAs can diffuse directly across rumen epithelium, MCP is absorbed in the small intestine as amino acids, di-, and tripeptides. Ammonia is also released by protein degradation for microbial growth in the rumen, absorbed through the ruminal wall to be detoxified in the liver to urea and subsequently recycled throughout the body, or excreted in urine as urea. Fermentation end products released through

eructation, including CO_2 and methane, represent a loss of energy and contribute to greenhouse gases. Hydrogen was considered the main energy source for methane released by *Archaea*, but recent findings suggest that a new class of methanogens can metabolize methylamines (Poulsen et al., 2013). The adaptive nature of the rumen microbiome allows ruminants to convert a wide array of low- and high-quality feedstuffs into high-quality microbial crude protein via fermentation. However, countless nutritional strategies potentially create equally as many unique microbiomes which when described will enable a greater understanding of the host-microbe relationship and its impact on animal performance.

Historical understanding of rumen microbiology was founded on culture based techniques pioneered by Robert Hungate (Hungate, 1966). Successful simulation of anaerobic conditions *in vitro* facilitated significant discoveries expanding the knowledge of the rumen microbiome through improved descriptions of bacterial species such as *Streptococcus bovis* (Russell and Hino, 1985) and *Megasphaera elsdenii* (Counotte et al., 1981). In isolated cultures, substrates and products of many bacterial strains were described in detail, providing the foundational understanding of the function for classes of bacteria. Advent of nucleic acid-based, molecular technology has ushered in a new culture independent perspective of microbial ecology unbiased by the culturing aptitude of microbial species. Bacterial species long accepted as having prominent roles in rumen function were detected at relatively low levels with molecular techniques. For example, the well-described cellulolytic genera, *Ruminococcus*, has not been found in quantities > 2% from fiber-adherent rumen fractions (Krause et al., 1999; Stevenson and

Weimer, 2007; Koike and Kobayashi, 2009) suggesting a lesser role in fiber degradation than originally believed. Although cultured species may not be the most prevalent, they may indicate the function of associated uncultured species. The most comprehensive studies will use a combination of techniques to enable comparisons to decades of culture based knowledge.

Ruminant nutrition has traditionally focused on measuring performance, voluntary intake, fermentation parameters, rate of passage, diet digestibility, and nitrogen metabolism in order to describe a diet's ability to meet animal requirements. Each of these measures is inseparably linked with the rumen microbial community. Feeding strategies, additives, and supplements are used to optimize animal performance, yet the mode of action is often unknown. Rumen microbiology research historically fails to correlate differences in bacterial populations, function, or phylogeny to meaningful responses in the animal. Utilization of new, high-throughput techniques allow microbial communities to be described in greater resolution than ever before. With nucleic acid-based, high-throughput approaches, a microbial understanding of proven nutritional strategies can set the foundation for new advancements in animal production.

Molecular approaches have advanced rapidly during the last 20 years and can be applied to the rumen microbiome. However, the multitude of steps required for analysis and the various methods to accomplish each step makes comparisons between studies challenging. Each rumen functions as its own system and thus, no two systems are the same. Moreover, the substrates provided (ration) directly influence the microbiome

structure. Due to these factors, review of literature will include pertinent details of methodology, analysis, and design.

The rumen is divided into interconnected sacs by pillars that along with the reticulum make up one large fermentation chamber. Feed particle size is reduced over time through rumination and fermentation. Small dense particles settle in the ventral sacs while fibrous materials float toward the dorsal sacs. Variation in particle composition and pH within the rumen has correlated to changes in the bacterial community. Microbial populations in various parts of the reticulorumen were estimated using TRFLP community fingerprinting technique (Fernando, 2008). Reticulum and rumen contents from three steers fed a similar feedlot ration demonstrated *Actinobacteria* and *Bacteroidetes* phyla were more prevalent in the reticulum relative to any part of the rumen (12 vs. 7% and 19 vs. 12%, respectively). Similar work comparing five locations within the rumen of three dairy cows observed consistent communities in the rumen using fingerprint analysis (Li et al., 2009). Understanding variation inherent in the rumen underscores the need for consistent rumen sampling methods to increase the ability to detect microbiome variation between animals or treatments. Although rumen fistulas allow convenient sample collection, surgery cost may limit the number of animals sampled. An alternative route of rumen sample collection uses an oral stomach tube to obtain rumen fluid. Lodge-Ivey et al. (2009) observed no significant differences in bacterial communities from rumen fluid taken by stomach tube compared to rumen fistula based on DGGE community fingerprinting. However, evaluating fiber-adherent bacterial populations may not be feasible using a

stomach tube nor correspond to other areas of the rumen. Microbial populations may be subject to diurnal variation, but no differences were observed for three dairy cows on TMR diet -3, +3, and +9 h after feeding (Li et al., 2009).

Different bacterial communities present in liquid and solid fractions were first observed using DGGE and ARISA by Larue et al. (2005). Identifying specific taxa more prevalent within the fibrous fraction in the rumen provides insight into the functional role of a particular species. While many have noted rumen microbial populations are animal specific, Weimer et al. (2010) demonstrated the host effect on community composition. Ruminal contents from two cows consuming a similar diet with divergent fermentation profiles (ruminal pH = 6.9 vs. 6.1; total VFA concentration = 57 vs. 77 m*M*) were completely exchanged. Samples taken prior to and after the exchange indicated the pre-exchange rumen pH and VFA levels returned to before transfer levels within 24 h after the exchange. Moreover, the bacterial community returned to prior structure in 14 d for one cow and 61 d for the other based on ARISA fingerprinting technique. Mechanisms to describe the relationship between the host and microbiome are not well defined. Diet preferences as well as feeding and drinking behavior are likely to be critical in regulation of the microbial community composition.

In addition to bacteria, the rumen microbiome consists of protozoa, archaea, and fungi. While most research focuses on either bacterial or archaea species, Kittelmann et al. (2013) characterized the entire microbiome populations of cattle on various diets. A mixture of barcoded amplicons were used from all three domains for simultaneous pyrosequencing. While 1,000 reads per sample were sufficient to describe protist and

fungal diversity, 5,000 reads were not sufficient to describe the bacterial community using OTU defined at 97% similarity. DGGE was used on archaea amplicons and compared to pyrosequencing results. While pyrosequencing provided greater resolution at 20,000 reads per sample, sequences not captured by DGGE represented only 0.63% of the total sequences. Although DGGE effectively showed shifts in the community, pyrosequencing has the ability to evaluate phylogenetic relationships in the community without creation of a clone library. Pairwise comparisons across all kingdoms using Spearman's rank correlation indicated a positive association of *Methanobrevibacter ruminatium* clade with the *Fibrobacteraceae* family suggesting a functional relationship. Simultaneous pyrosequencing and complete taxonomic analysis of the rumen microbiome may provide a broader perspective of symbiotic and antagonistic microbial relationships in the rumen. Describing complex interactions within the rumen can contribute to understanding observed physiological performance differences in response to nutrition or management. With the dearth of ruminal protist and fungal pyrosequencing efforts, additional research is needed to ensure database sequences reflect all species found in the rumen.

Understanding sequencing depth and diversity present in a microbial community is required to adequately describe a microbiome of interest. Variation in ruminal ecology of 16 dairy cows on a common diet was evaluated using pyrosequencing (Jami and Mizrahi, 2012). At a depth of 9,500 reads per sample, almost 5,000 total OTUs were identified. Each sample had an average of 1,800 OTUs; however, only 157 OTUs were identified in every rumen sample which aligned to 32 genera. Real-time PCR

estimates, a more sensitive quantification technique, were compared to the relative abundance observed in pyrosequencing. Across all samples, greater than 0.87 Pearson correlation was observed between pyrosequencing and real-time PCR estimated abundances for the four evaluated species. Under these rumen conditions and the sequencing depth, pyrosequencing was accurate for quantitative abundance of *Fibrobacter succinogenes* S85, *Megasphaera elsdenii* T81, and *Ruminobacter amylophilus* H18, but *Succinivibrio dextrinosolvens* 22b was underrepresented by pyrosequencing. Pairwise Bray-Curtis index showed a similarity of only 51% between samples, but the weighted Unifrac metric estimated average phylogenetic similarity among samples at 82%. This suggests although OTUs vary between animals, communities share OTUs phylogenetically related below the OTU defined similarity level of 97% (genus, order, etc.). These results underscore taxonomic variation present between animals consuming a 30% roughage, 70% concentrate diet. The relatively small, core microbiome (32 genera) could indicate additional taxa may not have significant functional contributions or that the functional similarity between animals is greater than observed taxonomic similarity.

There are two main nutrition strategies in the dairy industry: seasonal, forage-based diet and concentrate-based, total mixed ration (**TMR**). Maintaining a healthy and effective rumen microbiome is a key component to optimize feedstuff utilization and increase milk production. De Menezes et al. (2011) determined the effect of grazing or a TMR on the liquid and solid rumen bacterial community structure using pyrosequencing. Four dairy cows were used in a crossover design with two weeks for diet adaptation.

Bacteroidetes and *Firmicutes* represented over 80% of the total sequences for all samples at the phyla level. There was 10.5% dissimilarity between bacterial populations on pasture and TMR mostly attributed to differences in *Bacteroidetes* and *Firmicutes*; the relative abundance of *Firmicutes* was greatest in the TMR liquid fraction and the lowest in the TMR solid fraction suggesting a more defined niche compared to the forage diet. However, the 14.9% dissimilarity between liquid and solid fractions was due to differences in *Fibrobacteres* and *Actinobacteria*; the solid fraction favored *Fibrobacteres,* but *Actinobacteria* was more predominant in the liquid fraction. *Prevotellaceae*, *Lachnospiraceae*, *Ruminococceae* were the most abundant families. *Prevotellaceae* was more prevalent on pasture diet regardless of fraction. *Prevotella* strains have been shown to produce propionate (Strobel, 1992), thus increased *Prevotella* abundance could be a factor in limiting methane production as shown in a side-by-side trial (O'Neill et al., 2011). *Fibrobacteraceae* and *Spirochaetaceae* families did not contribute 10% of sequences combined, but were associated with the solid fraction regardless of diet suggesting a role in fiber degradation.

Similar work by Kim and Yu (2012) evaluated the rumen bacterial community using real-time PCR to quantify cultured and uncultured species in liquid and solid fractions of Jersey cows on a forage diet and Holstein cows on a TMR. Universal 16S primers were used to measure total bacteria and specie-specific primers amplified informative regions of the 16S gene. Jersey cows had 1.7×10^8 (16S rRNA copies/µg DNA) total bacteria while Holstein cows had 5.1×10^8, but there was no observed difference between liquid and solid fractions. Although the genus *Prevotella* accounted

for over 25% of absolute abundance in all samples, the well-described, *Prevotella ruminicola* represented only about 1% of absolute abundance. However, the genus *Prevotella* primers may have caused an overestimation because the forward and reverse primers matched non-*Prevotella* sequences in public databases. Although there was not an observed effect of diet or sample fraction on the relative abundance of *Prevotella*, absolute abundance increased in the liquid fraction of concentrate-fed Holsteins. The experimental design caused diet effects to be confounded with breed.

Increased ethanol production in the United States has resulted in increased utilization of the subsequent coproduct, distillers' grains, in beef cattle rations. Callaway et al. (2010) evaluated bacterial diversity in the rumen of cattle on feedlot diets with dried distillers' grains (DDG) included at 0, 25, and 50% replacing a commercial, concentrate feed. Pooled samples were pyrosequenced from two steers on each diet to describe the ruminal bacteria populations. Analysis of samples taken prior to the experiment indicated 74 genera with *Prevotella* as the most abundant. Averages across three feedlot diets resulted in detection of 54 species demonstrating a decrease in species richness with addition of concentrate to the diet. For diets containing 50% DDG, *Prevotella* and *Bacteroides* increased 152 and 276% in relative abundance while *Succinivibrio* populations decreased 406% compared to the 0% DDG diet. Moreover, *Firmicutes*:*Bacteroidetes* declined with increasing levels of DDG inclusion. The proteolytic ability of *Prevotella* and *Bacteroides* has been previously observed (Attwood and Reilly, 1995; Reilly et al., 2002); the response to DDG may be explained by the increase in dietary crude protein 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 feedlot diets and the unknown composition of the commercial feed prevents accurate diet comparisons.

The rumen has a dynamic microbial composition that is directly impacted by transitions during the animal's life. The segmented nature of the beef industry and distinct phases of dairy management represent necessary time points of rumen microbiome adaptation. Ensuring proper development and adaptation of the rumen benefits animal productivity and well-being. Young dairy calves are started on milk replacer, but transition to starter rations decreases labor and can improve rumen development. Inclusion of chopped forage (bromegrass hay) in starter diets increased average daily gain and feed efficiency compared to similar diets without forage (Coverdale et al., 2004). Observed performance increases could be linked with forage effects on promotion of muscular rumen development (Tamate et al., 1962), stimulation of rumination and saliva production (Hodgson, 1971), and subsequent regulation of ruminal pH.

Pyrosequencing techniques can be implemented to understand the rumen microbiome changes associated with management during rumen development. Jami et al. (2013) evaluated the rumen bacteria in dairy calves at 1-3 d, 2 months, 6 months, and 2 years of age. Rumen fluid samples were collected via stomach tube from five calves in each age group. Defining OTU at 97% similarity and including singletons in analysis, rarefaction curves indicated 11,000 reads per sample were sufficient to describe rumen bacteria for calves at 1-3 d and 2 months of age, but insufficient for cattle at 6 months

and 2 years of age. As expected, greater bacteria populations were present in older calves and cows compared to newborn and 2 month old calves. In 1-3 d old calves, *Firmicutes* was the dominant phylum. Specifically, *Streptococcus* appeared more abundant at d 1 and then rapidly declined to less than 0.1% at 2 months of age. Relative abundance of phylum *Bacteroidetes* increased with age; within the phylum, genus *Bacteroides* was the most abundant at 1-3 d but decreased with age as relative abundance of *Prevotella* increased. Because the rumen bacterial community in newborn calves is not similar to mature cattle, a dramatic change from primary colonization to mature animal must occur. From day 1 to 3 there was a distinct decrease in aerobic and facultative anaerobes coupled with an increase in bacteria associated with obligatory anaerobic function. *Ruminococcus flavefaciens*, a cellulolytic species, was detectable in the rumen at 1 day of age, suggesting possible alternate functions, dependence on other bacteria to meet nutritional requirements, or potential environmental contamination. Some ruminococci can grow on glucose, albeit at very slow rates, and prefer cellobiose (Russell, 2002). Diversity indices, OTU numbers, and similarity within group increased with age. Although only 5 different animals were used for each age group, the mature rumen environment was a more homogeneous, restricted niche relative to the rumen of a newborn calf. Greater dependence on products of microbial fermentation by mature cows to address nutritional demands may dictate a narrower range of functionality within the microbiome compared to newborn calves. Furthermore, newborn calves have an esophageal groove that shunts milk from the esophagus to the omasum limiting ruminal digestion. Understanding the natural progression of the rumen microbiome and

function is necessary to evaluate changes in the microbial environment caused by diet or management.

When cattle transfer production phases in the beef industry, there is typically an accompanying dietary change requiring rumen microbes to adjust. After weaning calves are classically stocked on available forage which is often winter wheat in the southern US. Pitta et al. (2010) evaluated the transition from Bermudagrass hay (11% CP) to wheat pasture (20% CP) in fourteen yearling cattle using pyrosequencing. Across solid, liquid, and whole rumen digesta samples, an average sequencing depth of about 2500 reads per sample was obtained. The liquid fraction of the hay diet contained the greatest number of bacterial genera identified with 149, while the whole digesta fraction from the wheat diet only had 118 genera that aligned at greater than 95% similarity. Rarefaction curve, ACE, and Chao1 estimated greater diversity in all three sample factions of the hay diet compared to wheat. Shannon index analysis indicated the average bacterial alpha diversity was higher for animals on the hay diet regardless of fraction (6.3 vs. 5.5, at 97% similarity level) were not similar. *Prevotella* was the most abundance genera observed and was greater in liquid fraction for both diets. *Rikenella* was found in greater abundance in the hay diet relative to wheat and was the second most abundant genera. Considering the hay diet contained greater NDF (68 vs. 44%) and was less digestible (57 vs. 80%, *in vitro* DM digestibility), greater species richness may be required to degrade additional cellulose in harvested C4 grasses compared to grazed C3 grasses.

Fernando et al. (2010) used a multifaceted approach including TRFLP, quantitative real-time PCR, and 16S library sequencing, to evaluate changes in the

rumen microbiome of four steers during adaptation from a high forage diet to a high concentrate. Step-up diets consisted hay-to-grain ratios of 80:20, 60:40, 40:60, and 20:80. An additional four steers remained on prairie hay for the duration of the trial. Results of TRFLP indicate the first major change in the rumen environment occurred after the shift from diet 60:40 to diet 40:60, and the change in bacterial community was even more pronounced on diet 20:80. At the time, only 30-50% of the terminal restriction fragments could be annotated to phylogenetic assignments, and only 115 bacterial genera were identified. 16S libraries were constructed from two steers on 20:80 and two steers on prairie hay. The library from steers on prairie hay had 398 different OTUs, while the library from steers on the concentrate diet (20:80) had 315 OTUs. However, only 24 OTUs were shared between libraries. Steers on the prairie hay diet had a significantly greater number of bacteria from the *Fibrobacteres* phylum, but fewer bacteria from the *Firmicutes* and *Bacteroidetes* phyla. However, there was a greater proportion of unclassified bacteria from the sequence library of steers on prairie hay compared to the high-concentrate diet (33 vs. 10%) suggesting previous sequences deposited in the database could have biased the taxonomic assignments (Fernando, 2008). Database bias declines with additional sequence deposits and unclassified bacteria would likely be reduced upon reanalysis of the sequence data. Additionally, *Firmicutes* was well represented under both dietary conditions suggesting the phylum may be a core bacterial component of the rumen.

Results from PCR analysis indicated an increase in *Megasphaera elsdenii*, *Streptococcus bovis*, *Selemonas ruminatium*, and *Prevotella bryantii* during adaptation

to high-concentrate diet (Fernando et al., 2010). Culture-based research has demonstrated *Megasphera 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). *Streptococcus bovis* is an amylolytic, facultative anaerobe known to increase with addition of starch to the diet and decreased ruminal pH (Slyter, 1976; Owens et al., 1998). Although *Streptococcus bovis* increased 2-fold on the first step-up diet, populations decreased on the remaining three diets suggesting the step-up diets were effective in adaptation to a high concentrate diet. However, *Butyrivibrio fibrosolvens* and *Fibrobacter succinogenes* populations decreased with addition of concentrate to the diet as expected. Although both have fibrolytic capabilities, *Fibrobacter succinogenes* decreased more rapidly during adaptation compared to *Butyrivibrio fibrosolvens*. The 40-fold decrease of *Fibrobacter succinogenes* is similar to observations of Tajima et al. (2001). *Butyrivibrio fibrosolvens* can also utilize maltose and sucrose (Russell and Baldwin, 1978) and significant decreases were not evidenced until a 20-fold reduction occurred on diet 4. Although TRFLP only provides a broad perspective of a microbial community, using sequencing of 16S libraries and quantitative real-time PCR in tandem with TRFLP provides a well-rounded approach to detect adaptation to concentrate-based diets.

Zened et al. (2013) evaluated changes in the rumen microbiome of dairy cows on four diets using a Latin square design. Diets consisted of either high or low levels of starch (34 and 20% starch, respectively) and inclusion of sunflower oil (5 and 0%, respectively). At an average depth of 7,000 sequences per sample, the average read

length was 393 nucleotides. With OTUs defined at 95% similarity, the number of OTUs was greatest in the low starch diet (2939) and was the lowest on the high starch diet with sunflower oil (1729). Shannon alpha diversity index supported greater diversity in the low starch diet compared to the high starch (7.2 vs. 6.3) and is a similar response observed by Pitta et al. (2010) and Fernando et al. (2010) with addition of starch to the diet. Richness estimated by Chao1 and ACE was much higher than OTUs, but was significantly decreased by inclusion of starch in the diet. Based on PCoA of the bacterial communities, there was more dissimilarity observed for cows on high starch diet, whereas cows on low starch diets were more similar. Across all diets, over 80% of the bacteria were represented by the phyla *Bacteroidetes* and *Firmicutes*. Although phyla *Fibrobacteraceae* and *Spirochaetes* were quantitatively minor in relative abundance, both decreased significantly with inclusion of high starch similar to previous work. While the decrease in *Spirochaetes* was similar to response observed by de Menezes et al. (2011), *Fibrobacteraceae* increased on TMR (higher starch diet). Additionally, *Rikenella* was observed at greater relative abundances on low starch diets similar to Pitta et al. (2010) potentially due to limited affinity for amylose and sunflower oil.

Beyond descriptions of species richness and abundance, the function and range of metabolic substrates vary between bacterial species. Greater understanding of species' functional roles will facilitate more meaningful interpretation of pyrosequencing-based results. Substantial variation between rumen microbiomes of steers fed a common diet was described by Brulc et al. (2009). Their broader interests

sought to ascertain differences in metabolic potential to degrade cellulose in addition to defining microbial community structure. Within three steers on an identical diet, the number of OTUs (97% similarity) per sample ranged from 161 to 259 based on full-length 16S libraries with a total of 510 unique OTUs. Furthermore, ACE and Chao1 curves at 97% similarity indicated approximately 2,000 full-length 16S sequences were sufficient to describe OTUs present. Nonmetric multidimensional scaling indicated the phylogenetic makeup of the full-length 16S libraries differed from the pyrosequenced 16S sequences. Pyrosequencing was also utilized to evaluate genes present in the rumen metagenome related to fiber degradation. A wide diversity of glucoside hydrolases belonging to 35 families was present. Alternatively, only three families of carbohydrate-binding modules and three dockerin modules were detected. This finding contrasts previous belief that fibrous plant material degradation is linked specifically to hydrolysis of the main chains of cellulose and hemicellulose; rather, the wealth of enzymes that breakdown the side chains (galactans and arabinans; Minic and Jouanin, 2006) of these polymers appear to be important for initial colonization of fiber.

In many studies, *Prevotella* has been observed as the dominant bacterial species in the rumen in various conditions (Whitford et al., 1998; Stevenson and Weimer, 2007; Pitta et al., 2010). Moreover, three ruminal species of the *Prevotella* genus can account for as much as 70% of the rumen bacterial population (Stevenson and Weimer, 2007; Jami and Mizrahi, 2012). The *Prevotella* group was originally classified as succinate producing bacteria by (Bryant et al., 1958), and the required nutrient, hemin, sorted it into two subspecies. The four characterized rumen *Prevotella* species include *Prevotella*

ruminicola, *Prevotealla bryantii*, *Prevotella albensis*, and *Prevotella brevis* (Avgustin et al., 1997; Bekele et al., 2010). Cultivated *Prevotella* strains display highly varied genetic divergence (Ramšak et al., 2000) suggesting uncultured *Prevotella* also have diverse functions. While different degrees of polysaccharide-degrading ability have been described in four species (Matsui et al., 2000), large clusters of *Prevotella*-related sequences have been associated with the fiber fraction of ruminal digesta (Koike and Kobayashi, 2009) and also the liquid fractions (Pitta et al., 2010). Even though *Prevotella* can be the most abundant species observed, cultured species only accounted for a small fraction of the total *Prevotella* population (Stevenson and Weimer, 2007; Bekele et al., 2010). The whole genome of a *Prevotella ruminicola* and *Prevotella bryantii* strain were sequenced to evaluate their genetic similarity (Purushe et al., 2010). The genome size and number of genes were similar between the strains, but the G + C content varied from 48 to 39%. Moreover, of the approximately 40 local syntenic blocks (groups of four or more genes), only 14 syntenic blocks were shared between the species. Most of the syntenic blocks were associated with polysaccharide metabolism and transport enzymes (Purushe et al., 2010). Sequenced ribosomal intergenic spacer clone libraries revealed *Prevotella* was more abundant in animals fed forage only diet compared to animal on forage and concentrate diet (Larue et al., 2005).

Beef cattle production is tied to the animal's ability to efficiently convert feedstuffs to product. Residual feed intake (**RFI**) is a tool used to select animals that perform similarly to their counterparts with less feed intake. Although efficiency increases can be quantified with RFI, the causal relationship has not been well defined.

Several groups have evaluated the rumen microbiome as a potential explanation for performance differences. Rius et al. (2012) fed 16 dairy cows selected for low or high RFI a similar diet of fresh ryegrass and evaluated intake, digestion, N retention, and the microbial environment. Although no differences were observed for intake, pH, volatile fatty acid profile, or urinary N output, low RFI cows had greater digestion of N and OM in addition to higher ruminal ammonia levels. Pyrosequencing results did not detect significant differences in the bacterial communities detected by PCoA analysis. However, the relative abundance of *Lachnospiraceae* was higher in low RFI cows (28 vs. 24%) while *Fibrobacteraceae* was higher in high RFI cows (2.6 vs. 3.3%). Overall, there was no observed difference detected in the microbial community as it relates to changes in ruminal fermentation. Similar work by Wiley et al. (2011) evaluated the rumen microbiome of high and low RFI Brahman bulls at high and low stocking rates on Bermudagrass (15% CP). After pyrosequencing and taxonomic assignment, main genera were grouped by affinity for different substrates as determined in culture-based experiments. There were limited differences detected in the substrate affinity of bacterial populations between high and low RFI bulls. The practice of assigning bacteria to broad substrate affinity groups to monitor changes in rumen ecology is uncommon and does not account for unknown functions of many uncultured bacterial species. Hernandez-Sanabria et al. (2010) also found inconclusive differences between high and low RFI groups using community fingerprinting techniques (DGGE), suggesting greater sequencing depth may be required to discern subtle differences in the rumen

microbiome. Additionally, pyrosequencing can help determine the effectiveness of an efficient microbiome (low RFI) on multiple diets.

Although high-concentrate, feedlot and TMR diets have been more extensively studied using microbiological techniques, cattle are often maintained on more economical, lower quality forage diets in beef operations. Chemical treatment of low-quality straws disrupt the cellulolytic structure within the cell wall and promotes greater forage utilization, but understanding of the microbial response to forage treatment is limited. Nguyen et al. (2012) evaluated the effect of urea and lime treated rice straw on intake, digestion, fermentation, and the rumen microbial environment of swamp buffalo in a 4 × 4 Latin square design. A 2 × 2 treatment arrangement consisted of ad libitum access to rice straw or urea and lime treated rice straw with additional provision of a 15% CP supplement containing 0 or 4% urea. Rumen fluid was collected to estimate cellulolytic, amylolytic, and proteolytic bacteria using the roll-tube technique as well as to measure methanogen and cellulolytic populations with real-time PCR. Forage intake of urea and lime treated rice straw was increased compared to untreated rice straw, but additional urea N in the supplement did not affect forage intake. Organic matter digestion increased from 59 to 66% by addition of urea to supplement of untreated rice straw treatments indicating added supplemental N increased fermentation. The highest OM digestibility observed was 74% for the 4% urea supplement of urea and lime treated rice straw. Microscopic counts of protozoa decreased with increased level of N provision via treated forage or urea inclusion in the supplement, but a contrasting response was detected for fungal zoospores. Increased passage rate with greater N

provision could have decreased protozoa counts. No response was observed to treatment for amylolytic or proteolytic bacteria. However, an increase of cellulolytic bacteria was observed with increased provision of degradable intake protein. Total bacteria populations were estimated by real-time PCR and ranged from 3.3 to 4.9×10^{12} copies per ml of rumen contents. Of three cellulolytic species evaluated, only increases in *Ruminococcus albus* corresponded to increased ruminal ammonia and OM digestion; the smallest population was observed in untreated rice straw without urea and the greatest population for the treated straw with urea included in the supplement (3.8×10^9 and 3.2×10^{10}, respectively). Additional research is needed to determine consistent responses of cellulolytic species to supplemental N and importance of cellulolytic species to overall diet digestion.

The dynamic and adaptive qualities of the rumen microbiome allow ruminants to utilize many industrial co-products including DDG, corn gluten, cottonseed meal and hulls. Similar to any fermentation process, a change in substrates alters resulting end products. Within the cow-calf phase of beef production, spring calving herds often have access to low-quality forage (less than 7% crude protein; **LQF**) during mid to late gestation when energy requirements increase. Insufficient N does not maintain efficient fermentation and ultimately leads to performance losses. Small amounts of high protein co-products are often used to increase intake and utilization of LQF via improvements in forage digestibility (Köster et al., 1996; Mathis et al., 1999; Winterholler et al., 2012). Moreover, less frequent supplementation (2 d/week) also increases forage utilization via nitrogen recycling, but may favor hyper ammonia producing bacteria in the rumen

(Bohnert et al., 2002; Farmer et al., 2004). While the physiological response to protein supplementation is well documented with increases in ruminal ammonia, passage rate, and nitrogen retention, there is a scarcity of literature related to the rumen microbiome on a LQF diet. Next generation pyrosequencing has been used to describe rumen ecology on forage grazing, feedlot concentrate, and dairy TMR type rations, but the effect of an exclusive LQF diet on the microbiome has yet to be evaluated with pyrosequencing.

Recent advances in algal biofuel production could create a market for the co-product, post-extraction algal residue (**PEAR**), to be utilized as a protein supplement in cow-calf operations (Drewery, 2012). The high protein level (18% crude protein) of PEAR is similar to other supplements; however, the high ash content (44%) is unique. Drewery (2012) observed similar improvements in forage intake and digestion in response to isonitrogenous levels of PEAR and CSM. Accordingly, our objective is to evaluate the effect of PEAR and CSM supplementation on the rumen microbiome of steers consuming LQF. To meet the objective, the rumen bacterial community will be surveyed by pyrosequencing of 16S rRNA gene sequences to estimate species abundance, diversity, and phylogenetic relationship within the microbiome and relate them to changes in digestion, intake, and rumen fermentation. Research results will provide the first perspective of the rumen metagenome on a LQF diet and relate fermentation improvements to changes in the rumen bacterial community. We hypothesize there will be different bacterial taxa associated within liquid and solid fractions as well as a response in the microbiome to increasing PEAR supplementation.

CHAPTER II

EFFECT OF POST-EXTRACTION ALGAL RESIDUE SUPPLEMENTATION ON THE RUMEN MICROBIOME OF STEERS CONSUMING LOW-QUALITY FORAGE

Overview

Cattle consuming low-quality forages (**LQF**) require protein supplementation to increase forage utilization and ruminal fermentation. Production of algal biomass for biofuel would result in large quantities of post-extraction algal residue (**PEAR**) that has the potential to elicit similar LQF utilization responses to cottonseed meal (**CSM**); however, its effect on bacterial communities is unknown. Five ruminally and duodenally cannulated Angus steers in a 5 × 5 Latin square had ad libitum access to oat straw. Treatments were infused ruminally and consisted of an unsupplemented control (**CON**), PEAR at 50, 100, and 150 mg N/kg BW, and CSM at 100 mg N/kg BW. Rumen samples were collected 4 h after supplementation on d 14 of each period. Amplification of the V4-V6 region of the 16S rRNA gene and 454 pyrosequencing was performed on liquid and solid rumen samples. After denoising, chimera checking, and quality trimming, 8364 ± 2745 sequences were generated per sample. Weighted UniFrac analysis and Morisita-Horn index demonstrated different community composition between liquid and solid fractions. Greater homogeneity was observed within solid samples. At the phyla level, *Bacteroidetes* characterized more than 75% of sequences in the solid fraction, while relative abundance of *Firmicutes* in the liquid fraction increased linearly with PEAR supplementation ($P = 0.02$). *Prevotella*

represented over 25% of sequences in all treatments and decreased in the solid fraction with increasing PEAR provision (linear, $P = 0.01$). *Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiaceae* increased in the liquid fraction with greater PEAR supplementation (linear, $P \leq 0.03$). *Fibrobacter* and *Treponema* decreased in liquid fraction with increasing PEAR (linear, $P < 0.1$). Results suggest increased forage utilization may be linked to changes within the liquid fraction of the rumen microbiome.

Introduction

The dynamic and adaptive qualities of the rumen microbiome allow ruminants to utilize many industrial co-products. Within the cow-calf phase of beef production, spring calving herds often have access to low-quality forage (less than 7% CP; **LQF**) during mid to late gestation when energy requirements increase. Small amounts of high protein co-products are often used to increase intake and utilization of LQF via improvements in forage digestion (Köster et al., 1996; Mathis et al., 1999; Winterholler et al., 2012). While the physiological response to protein supplementation is well documented with increases in ruminal ammonia and passage rate (Bandyk et al., 2001) , there is a scarcity of literature related to the rumen microbiome on a LQF diet. Next-generation pyrosequencing has been used to describe rumen ecology with grazing, feedlot, and dairy diets, but the effect of protein supplementation to a basal diet of LQF on the microbiome has yet to be evaluated with pyrosequencing.

Advances in algal biofuel production could create a large supply (1.03 Mt/bn L) of post-extraction algal residue (**PEAR**), to be utilized as a protein supplement in cow-calf operations (Drewery, 2012). The high protein level (18% CP; 32% CP on OM

basis) of PEAR is similar to other supplements. Similar improvements in forage intake and digestion were observed in response to PEAR and cottonseed meal (**CSM**; Drewery, 2012). Accordingly, our objective was to evaluate the effect of increasing PEAR supplementation on the rumen microbiome of steers consuming LQF and compare an isonitrogenous level of CSM supplementation. We hypothesize there will be different bacterial taxa associated within liquid and solid fractions as well as a response in the microbiome to increasing PEAR supplementation.

Materials and methods

Experimental design

The experimental protocol was approved by the Institutional Animal Care and Use Committee at Texas A&M University. The experiment was described in detail by Drewery (2012). Briefly, five ruminally and duodenally cannulated steers (198 ± 6 kg BW) were used in a 5 × 5 Latin square design to determine effects of provision and level of PEAR supplementation on low-quality forage utilization. Supplements consisted of three levels of PEAR: 50, 100, and 150 mg N/kg BW (**50P**, **100P**, and **150P**), an isonitrogenous level of CSM: 100 mg N/kg BW (**100C**), and an unsupplemented control (**CON**). Supplemental PEAR (55.5% OM, 17.9% CP, as a percent of DM) was obtained from algal biomass grown photosynthetically in an open pond, flocculated, dewatered, spray-dried, and extracted with a methyl pentane solvent. Steers were fed chopped oat straw (92.7% OM, 4.5% CP, and 80.3% NDF) at 0700 h and offered 130% of previous 5 d average to ensure ad libitum consumption. Supplemental PEAR and CSM (91.0% OM, 42.9% CP, and 24.2% NDF) were dosed ruminally daily by removing ruminal

contents, placing the supplements in the rumen, and returning the contents to the rumen. Steers were housed in an enclosed barn with ad libitum access to water and a trace mineral block. The five experimental periods consisted of 8 d for adaptation to treatment, 5 d for measurement of intake and digestion, and 1 d for determination of ruminal fermentation and microbiome sampling. Rumen contents (~ 1 kg) were collected 4 h after feeding from four locations within the dorsal and ventral sac of the rumen. Collected rumen contents were mixed and squeezed through four layers of cheesecloth for fluid sample collection, and the remaining solid material was mixed and sampled. All samples were snap frozen in liquid nitrogen and stored at -80°C until DNA extraction. Additional parameters measured including forage intake and digestion, ruminal volatile fatty acid concentration, ammonia, pH, as well as N retention, duodenal microbial flow and liquid passage rate were reported by Drewery (2012).

DNA extraction

DNA was extracted using the QIAamp DNA stool mini kit (Qiagen, Valencia, CA) protocol with modifications. Specifically, 3 ml of liquid or a similar volume of solid sample were mixed with 3 ml Buffer ASL and vortexed for 15 min. After centrifugation at 500 rcf for 1 min, 500 µl of supernatant was transferred to tubes with 0.15 mm garnet beads (Mo Bio Laboratories, Inc., Carlsbad, CA) and 1000 µl phenol chloroform. Vortexing for 5 min completed bacterial lysis and the suspension was incubated for 5 min at 95°C. Centrifugation pelleted stool particles and 400 µl of the supernatant was added to 1000 µl of Buffer ASL and 1 InhibitEX tablet to incubate for 1 min at room temperature. Samples were centrifuged for 7 min and 535 µl supernatant

was added to 25 µl of Proteinase K followed by 535 µl of Buffer AL. The samples were subsequently incubated at 70°C for 20 min. After addition of 535 µl of ethanol, the mixture was added to a DNA spin column and QIAamp DNA stool mini kit recovery protocols were followed to finish extraction. Extracted DNA was quantified and quality-checked using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and gel electrophoresis. Ethanol precipitation was performed on all samples possessing 260:280 ratio below 1.7. Extracted DNA was standardized to 20 ng/µl concentration for downstream amplification.

16S rRNA amplification and pyrosequencing

Amplification of the V4-V6 segment of 16S rRNA gene utilized barcoded primer tags and the universal eubacterial primers 530F (5′-GTGCCAGCMGCNGCGG-3′) and 1100R (5′-GGGTTNCGNTCGTTG-3′) as previously described (Dowd et al., 2008). A single-step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) were used under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds; 53°C for 40 seconds and 72°C for 1 minute after which a final elongation step at 72°C for 5 minutes was performed. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Pyrosequencing was performed with a Genome Sequencer FLX System (Roche, Branford, CT) using Titanium chemistry at the MR DNA Molecular Research Lab (Shallowater, TX).

Sequence read analysis

Sequences for each sample were processed using the QIIME pipeline v1.5.1 (Caporaso et al., 2010b). Initially, raw flowgrams for sequences were denoised, demultiplexed, and truncated at 415 bp using AmpliconNoise (Quince et al., 2011) with Perseus option for de novo chimera removal. Denoising accounted for error associated with pyrosequencing and PCR, while truncation was performed when the average base pair quality score < 25. 401,468 high quality sequences reads (8364 ± 2745 sequences per sample) were used for downstream analysis. Clustering of OTUs at 97% similarity with UCLUST (Edgar, 2010) generated 6,556 OTUs with 698 ± 148 OTUs per sample. Cluster representative sequences were aligned to the gg_12_10_otus database (secondgenome.com) with PyNAST (Caporaso et al., 2010a). The RDP Classifier (Wang et al., 2007) assigned taxonomic classification using a 0.8 bootstrap value based on near full-length sequences in the Greeengenes database (McDonald et al., 2012). Singleton OTUs were removed to prevent artificial diversity inflation (Kunin et al., 2010). After sample size standardization to smallest library size (2,600 sequences), OTU richness, alpha, and beta diversity were estimated. Metrics used include: observed OTUs, ACE (Chao and Lee, 1992), Shannon (Shannon and Weaver, 1949), Simpson's (Simpson, 1949), Bray-Curtis (Bray and Curtis, 1957), and Morisita-Horn indices (Magurran, 2004). Pairwise Bray-Curtis and Morisita-Horn similarity was used for cluster analysis by Unweighted Pair Group Method with Arithmetic (**UPGMA**). Radial trees provided visualization of treatment and fraction differences using FigTree v1.4 (http://tree.bio.ed.ac.uk/software/). Also, UniFrac distance measured phylogenetic

dissimilarity between samples by calculating the fraction of unshared branch length between pairs of communities (Lozupone and Knight, 2005; Hamady et al., 2009). Unweighted UniFrac distance matrices were used as inputs for clustering analysis to determine cause of variation within the taxonomic composition in the rumen. UniFrac-based Principle Coordinate Analysis (**PCoA**) provided visualization of grouping of similar microbiome environments.

Statistical analysis

Relative abundances of bacteria present > 1% at phyla and family taxonomic level or > 0.3% at the genus level were logit transformed [$z = \log(p/(1-p))$] to normally distribute residuals, where *p* represents the relative abundance of a bacterial taxa. Transformed data was analyzed using the MIXED procedure of SAS 9.3 (SAS Inst. Inc., Cary, NC). Terms in the model included treatment, fraction, treatment × fraction, and period with treatment nested within steer included as a random effect. Treatment means were calculated using the LSMEANS option and back transformed [$p = 10^z / (1 + 10^z)$]. Orthogonal contrasts were implemented for means separation within liquid and solid fraction. Contrasts included linear and quadratic response to PEAR supplementation and direct comparison of 100P and 100C treatments.

Results

One animal observation (two samples) was removed for reasons unrelated to treatment. After pyrosequencing of 48 samples, 405,921 high-quality reads were used for downstream analysis. Average sequencing depth for each treatment ranged from 6,970 (50P) to 9,112 per sample (100C). Greater sequencing depth was obtained on

solid samples compared to liquid samples, 8,181 and 7,901 sequences per sample respectively. Clustering of sequencing at 97% similarity yielded a total of 6,556 OTUs with an average of 698 OTU's per sample. In the liquid fraction OTUs increased with additional PEAR (linear; $P = 0.04$). Over 99% of OTU sequences aligned to phyla in the Greengenes database, while 76 and 47% of sequences were assigned at the family and genus taxonomic level, respectively. Rarefaction analysis depicted an increase in observed OTUs with greater sequencing depth (Figure 1). After 10,000 sequences the rate of discovery of additional OTUs decreases. A quadratic line fits the scatter plot of OTUs observed for each sample and plateaus at 13,500 sequences per sample.

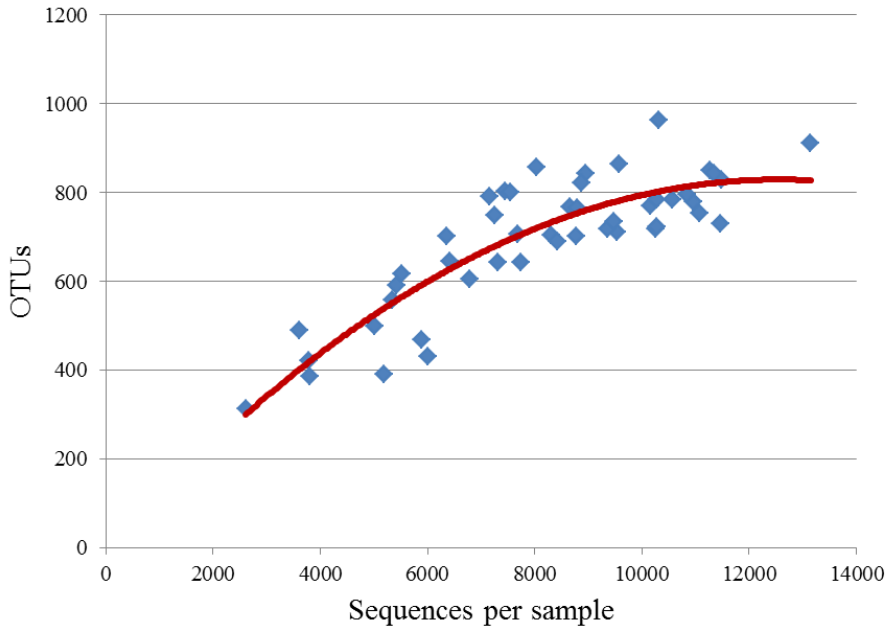

Figure 1. Effect of sequencing depth on detection of operational taxonomic units (OTUs) within 47 samples. One outlier was excluded due to greater than 17,000 sequences.

OTU richness, alpha and beta diversity

After rarefaction to 2,600 sequences per samples, richness estimates (ACE and observed OTUs) demonstrated decreased OTU richness in the liquid fraction with increasing PEAR supplementation (Table 1; $P < 0.1$). Within the solid fraction, a linear increase was observed in the Shannon index with increasing PEAR supplementation (Table 2; $P = 0.06$). The greatest observed OTUs in the solid fraction was detected for 150P (452 OTUs) and suggests the increase in the Shannon index may be due to increasing richness. However, no linear or quadratic trends in the solid fraction for OTU richness were detected in response to increasing PEAR ($P > 0.1$). Observed OTUs were greater in the liquid fraction than solid (452 vs. 428; $P = 0.03$). Additionally, an observed treatment × fraction interaction ($P = 0.04$) resulted from a decrease in the liquid fraction from 50P - 150P, while observed OTUs increased in the solid fraction with additional PEAR. Isonitrogenous 100P and 100C treatments resulted in similar alpha diversity and OTU richness among liquid and solid fractions ($P > 0.1$).

Table 1. Effect of increasing post-extraction algal residue supplementation on operational taxonomic unit richness at 97% similarity after rarefaction to 2,600 sequences per sample.

		Treatment[1]						Contrast *P*-value		
Item	Fraction	CON	50P	100P	150P	100C	SEM[5]	Linear	Quadratic	100P vs. 100C
No. observations		5	5	5	4	5				
ACE[2]	Liquid	805.3	838.4	762.6	658.2	838.9	67.9	0.05	0.28	0.38
	Solid	708.3	699.9	728.2	770.2	772.6	67.9	0.43	0.69	0.61
Observed OTUs[3,4]	Liquid	454.8	479.8	444.6	415.5	467.0	26.9	0.09	0.28	0.51
	Solid	419.0	401.4	432.6	452.0	435.0	26.9	0.16	0.46	0.94

[1]CON = unsupplemented control; 50P = 50 mg N/kg BW of PEAR; 100P = 100 mg N/kg BW of PEAR; 150P = 150 mg N/kg BW of PEAR; 100C = 100 mg N/kg BW of CSM.
[2]ACE = Abundance Coverage Estimator (Chao and Lee, 1992).
[3]Observed OTUs = number of unique OTUs detected in similarity after clustering at 97% similarity.
[4]Effect of sample fraction ($P = 0.03$); Treatment × fraction ($P = 0.04$)
[5]SEM = standard error of the mean

Table 2. Effect of increasing post-extraction algal residue supplementation on alpha diversity at 97% similarity after rarefaction to 2,600 sequences per sample.

		Treatment[1]						Contrast *P*-value		
Item	Fraction	CON	50P	100P	150P	100C	SEM[4]	Linear	Quadratic	100P vs. 100C
No. observations		5	5	5	4	5				
Shannon[2]	Liquid	6.55	6.92	6.97	6.84	6.87	0.25	0.85	0.29	0.75
	Solid	6.80	6.46	6.92	7.12	6.71	0.25	0.06	0.26	0.49
Simpson's[3]	Liquid	0.94	0.96	0.98	0.97	0.97	0.01	0.28	0.13	0.60
	Solid	0.97	0.96	0.98	0.98	0.97	0.01	0.20	0.35	0.56

[1]CON = unsupplemented control; 50P = 50 mg N/kg BW of PEAR; 100P = 100 mg N/kg BW of PEAR; 150P = 150 mg N/kg BW of PEAR; 100C = 100 mg N/kg BW of CSM.
[2]Shannon index as calculated by Shannon and Weaver (1949).
[3]Simpon's index as calculated by Simpson (1949).
[4]SEM = standard error of the mean

Weighted UniFrac principal coordinate analysis (**PCoA**) indicated a separation of liquid and solid fractions (Figure 2a). The first and second principal component accounted for 16.6 and 20.6% of the variation, respectively. Greater spatial heterogeneity was observed between liquid samples than solid samples. Moreover, weighted PCoA demonstrated solid bacterial communities were phylogenetically more

similar irrespective of treatment. Unweighted UniFrac PCoA also indicated distinct liquid and solid-associated communities, but the first and second coordinate only accounted for 12.2% of the variation combined (Figure 2b). Additional PCoA analysis of bacterial communities' association to ruminal ammonia, organic matter digestion, and NDF digestion reported by Drewery (2012) did not elucidate clear treatment effects.

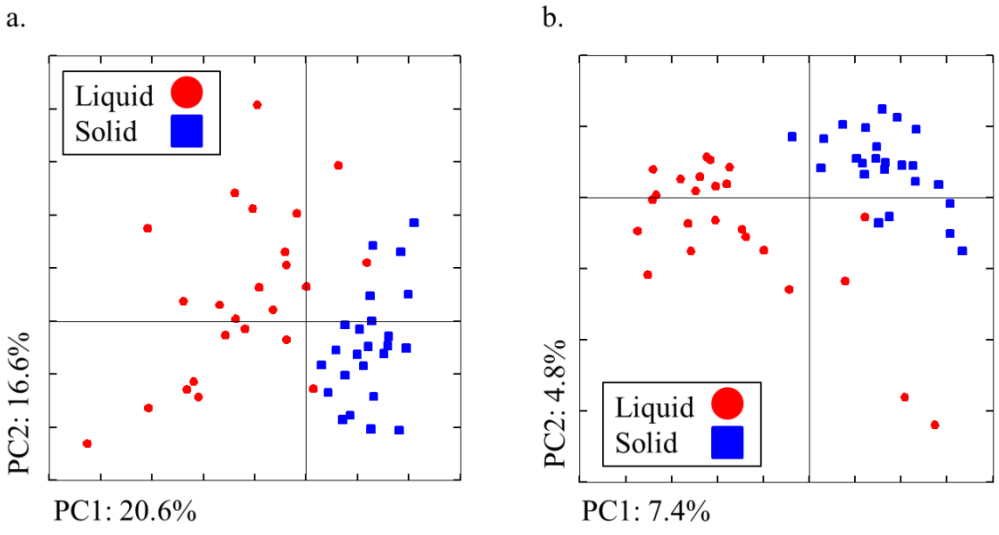

Figure 2. Effect of increasing post-extraction algal residue supplementation on beta diversity using principle coordinate analysis of weighted (a) and unweighted (b) UniFrac distance.

Two UPGMA-based cluster analyses were performed to visualize pairwise beta diversity metrics (Morisita-Horn and Bray-Curtis). Morisita-Horn pairwise similarity metric indicated greater homogeneity within the solid samples; only 4 samples did not cluster in the greater clade (Figure 3). In contrast, liquid samples clustered into 3 general clades with longer branch lengths signifying greater dissimilarity. Within both

fractions, treatment effects were not evident and CON samples were dispersed throughout. Bray-Curtis pairwise similarity metric clustered two-thirds of solid samples into a clade and over one-third of liquid samples (Figure 4). However, another one-third of samples from both fractions were paired for a given steer on one treatment.

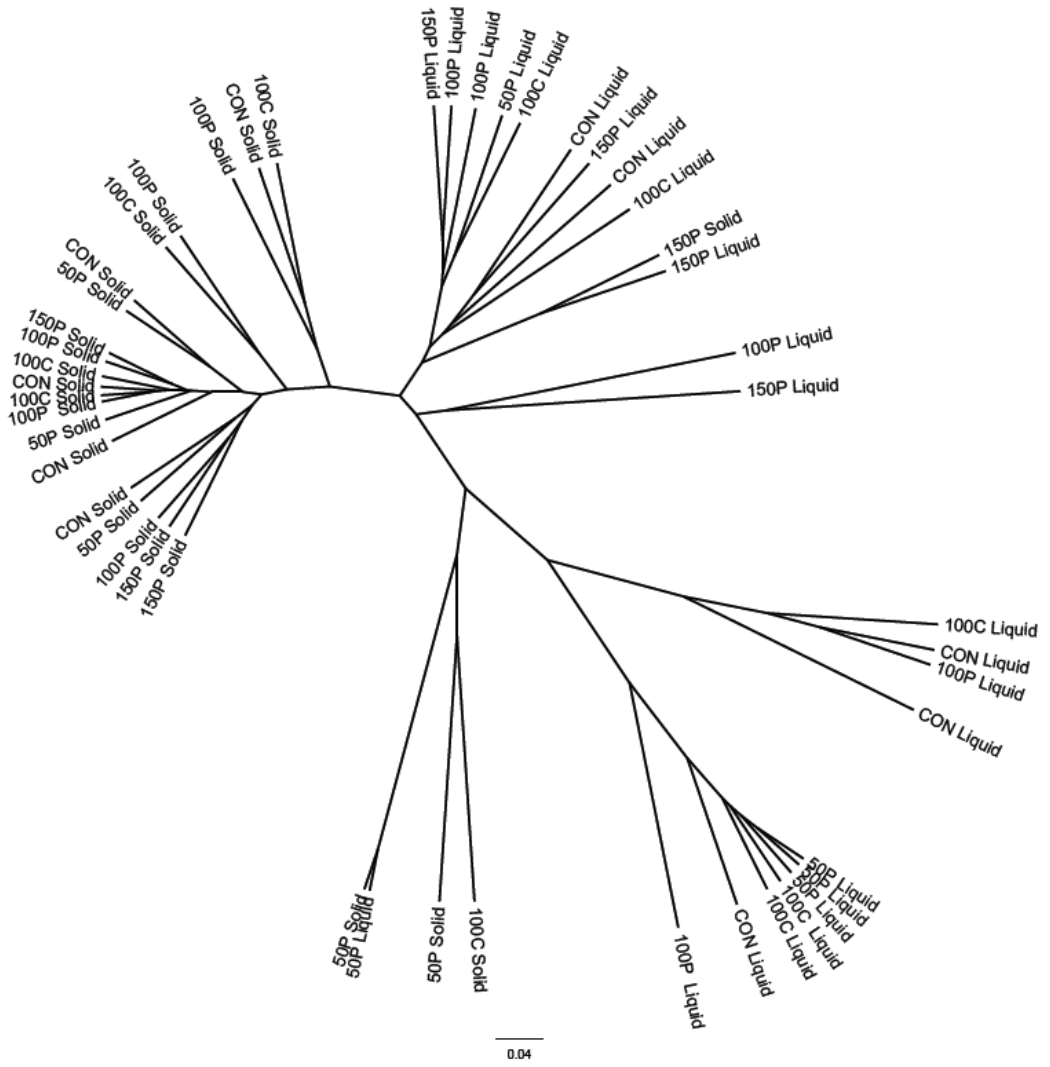

Figure 3. Effect of post-extraction algal residue supplementation on beta diversity using Morisita-Horn index with clustering analysis. CON = unsupplemented control; 50P = 50 mg N/kg BW of PEAR; 100P = 100 mg N/kg BW of PEAR; 150P = 150 mg N/kg BW of PEAR; 100C = 100 mg N/kg BW of CSM.

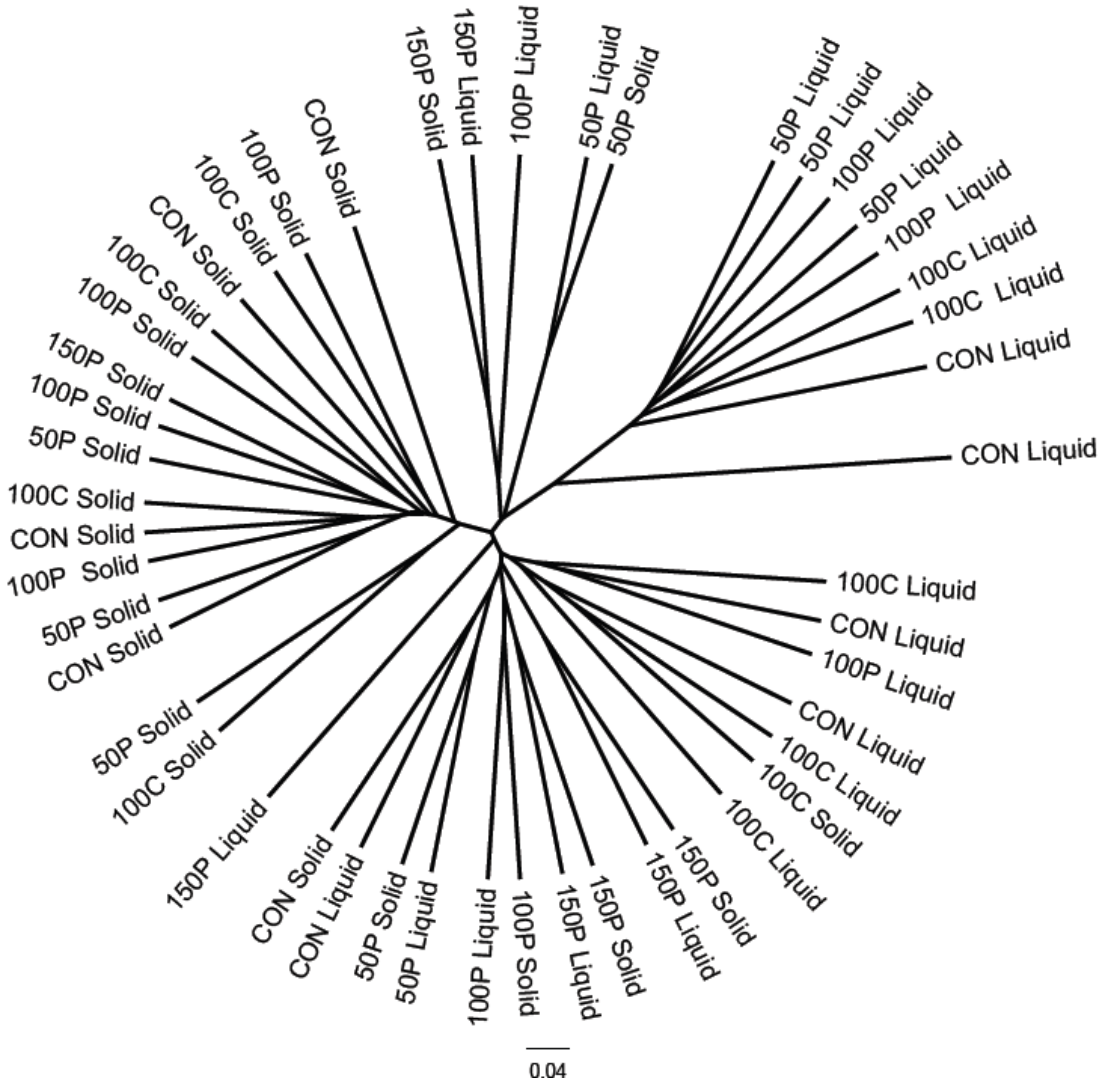

Figure 4. Effect of post-extraction algal residue (PEAR) supplementation on beta diversity using Bray-Curtis index with clustering analysis. CON = unsupplemented control; 50P = 50 mg N/kg BW of PEAR; 100P = 100 mg N/kg BW of PEAR; 150P = 150 mg N/kg BW of PEAR; 100C = 100 mg N/kg BW of CSM.

Effect on bacterial taxa

Across all samples *Bacteroidetes* and *Firmicutes* characterized 79.2 and 13.8% of all sequences (Figure 5). The average *Bacteroidetes*:*Firmicutes* (**B:F**) consisted was

5.7:1. Additional phyla detected including *Tenericutes*, *Fibrobacteres*, *Spirochaetes*, *Verrumicrobia*, and *Proteobacteria* were less than 5% of sequences in any sample.

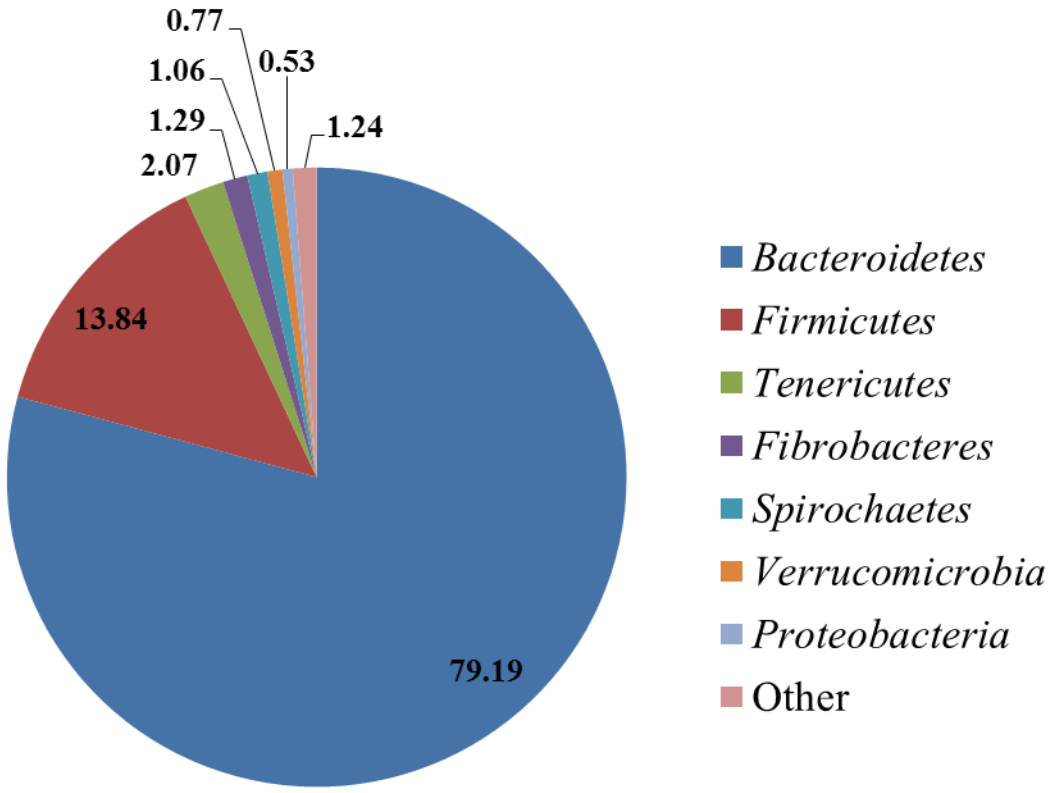

Figure 5. Relative abundance of ruminal bacteria phyla of steers consuming low-quality forage across all treatments.

Phylum *Bacteroidetes* represented the majority of the sequences in every treatment ranging from 66.4% (liquid 150P) to 82.9% (solid 100C; Table 3). Although no main fraction effect was observed ($P > 0.1$), contrasts indicated the treatment response varied between fractions. In the liquid fraction, relative abundance of *Bacteroidetes* decreased from 81.5% for CON to 66.4% for 150P (linear, $P = 0.08$).

Relative abundances were stable in the solid fraction and did not respond to treatment ($P > 0.1$). *Firmicutes* was the second most abundant phylum ranging from 8.7 (liquid 100C) to 20.8% (liquid 150P) in relative abundance. A treatment × fraction interaction was observed for *Firmicutes*; PEAR provision increased relative abundance in the liquid fraction (linear; $P = 0.02$), but no effects were observed within the solid fraction ($P > 0.1$). The relative abundance of *Firmicutes* was 179% greater in 100P than 100C ($P = 0.02$). *Tenericutes* were relatively more abundant in the liquid fraction (2.2 vs. 1.6%; $P < 0.01$). No significant treatment response was observed for *Tenericutes*; however, increasing PEAR numerically decreased the proportion in the liquid fraction. Sequences from phylum *Fibrobacteres* represented 1-2% overall and exhibited a treatment × fraction interaction ($P = 0.08$). Relative abundance of *Fibrobacteres* was similar between fractions for 50P, 100P, and 100C, but favored the solid fraction of CON and 150C. Increasing PEAR provision resulted in an inverse quadratic response within observed treatment levels in the solid fraction ($P = 0.01$), whereas *Fibrobacteres* decreased in the liquid fraction from 1.2 to 0.4% for CON and 150P, respectively (linear, $P = 0.09$). Moreover, a period effect was observed in *Fibrobacteres* relative abundance across all treatments ($P = 0.05$). A treatment × fraction interaction was observed for *Spirochaetes* ($P < 0.01$); this interaction was largely driven by a linear decrease in the liquid fraction ($P = 0.01$) and an inverse quadratic increase in the solid fraction ($P = 0.03$).

Table 3. Effect of increasing post-extraction algal residue supplementation on relative abundance of bacteria phyla[1]

		Treatment[2]						Contrast *P*-value		
Item	Fraction	CON	50P	100P	150P	100C	L vs. S[3]	Linear	Quadratic	100P vs. 100C
No. observations		5	5	5	4	5				
Bacteroidetes	Liquid	81.5	79.6	76.5	66.4	82.6	0.31	0.08	0.83	0.12
	Solid	76.9	82.9	80.9	80.1	81.8		0.69	0.23	0.80
Firmicutes[4]	Liquid	9.6	12.6	15.6	20.8	8.7	0.76	0.02	0.91	0.02
	Solid	14.9	12.0	14.1	12.6	13.0		0.90	0.76	0.72
Tenericutes	Liquid	2.6	2.4	2.3	1.6	2.1	<0.01	0.20	0.54	0.76
	Solid	2.3	1.5	1.3	1.4	1.4		0.41	0.26	0.73
Fibrobacteres[4,6]	Liquid	1.2	0.9	0.5	0.4	1.4	0.13	0.09	0.95	0.08
	Solid	1.8	0.6	0.7	2.0	1.0		0.11	0.01	0.59
Spirochaetes[5]	Liquid	1.3	1.2	0.7	0.5	1.1	0.94	0.01	0.67	0.18
	Solid	1.0	0.6	0.9	1.7	0.7		0.01	0.03	0.52

[1]Phyla listed were detected at greater than 1% relative abundance averaged across all samples.
[2]CON = unsupplemented control; 50P = 50 mg N/kg BW of PEAR; 100P = 100 mg N/kg BW of PEAR; 150P = 150 mg N/kg BW of PEAR; 100C = 100 mg N/kg BW of CSM.
[3]L vs. S = main effect of liquid vs. solid fraction.
[4]Treatment × fraction ($P < 0.1$)
[5]Treatment × fraction ($P < 0.01$)
[6]Period effect ($P = 0.05$)

Prevotellaceae was the most prevalent family observed across all treatments representing more than 25% of all sequences. Greater relative abundance of *Prevotellaceae* was observed in the solid fraction than the liquid (Table 4; 33.4 vs. 28.7%; $P < 0.01$). In the solid fraction, *Prevotellaceae* relative abundance decreased with additional PEAR (linear, $P = 0.01$), while proportions remained stable (27.4 - 29.8%) in the liquid fraction. Another predominant unclassified family found was a member of the order *Bacteroidales*. The unidentified *Bacteroidales* family favored the solid over the liquid fraction (20.0 vs. 13.6%; $P < 0.01$), but no treatment response was observed. Alternatively, family BS11 was relatively more abundant in the liquid ($P <$

0.01) compared to the solid fraction (19.3 and 12.6%, respectively). *Paraprevotellaceae* was more predominant in the liquid fraction ($P < 0.01$). Within the solid fraction, relative abundance of *Paraprevotellaceae* increased with additional PEAR (linear, $P = 0.05$). There was an observed treatment × fraction interaction for *Lachnospiraceae* ($P = 0.02$); although *Lachnospiraceae* was observed in greater proportions in the solid fraction ($P < 0.01$), populations within the liquid fraction increased with PEAR supplementation (linear, $P = 0.01$). Moreover, relative abundance of *Lachnospiraceae* in the liquid fraction of 100P was 195% greater than 100C ($P = 0.01$). *Ruminococcaceae* represented a greater proportion of the liquid fraction overall ($P < 0.02$) except for CON. Within the liquid portion, increasing PEAR supplementation supported a linear increase in relative abundance of *Ruminococcaceae* ($P = 0.02$). The treatment × fraction interaction for family S24-7 ($P = 0.07$) indicated the quadratic increase ($P \leq 0.05$) in relative abundance within the liquid and solid fraction changes in the magnitude of difference with PEAR. The response of S24-7 was the only treatment effect consistent in liquid and solid fractions. Overall, a greater proportion of sequences in solid samples were represented by S24-7 than liquid (3.8 vs. 1.5%; $P < 0.01$). *Clostridiaceae* within the liquid fraction increased in relative abundance with increasing PEAR (linear, $P = 0.01$); however, no observed treatment effects in the solid fraction caused a treatment × fraction interaction ($P = 0.7$). Additionally, a greater proportion of *Clostridiaceae* was represented in 100P compared to 100C (3.4 vs. 1.2%; $P < 0.01$). The unclassified family (Other) represented up to 3.5% of sequences from any treatment, but was more prevalent

Table 4. Effect of increasing post-extraction algal residue supplementation on relative abundance of bacteria families[1]

		Treatment[2]						Contrast *P*-value		
Item	Fraction	CON	50P	100P	150P	100C	L vs. S[3]	Linear	Quadratic	100P vs. 100C
No. observations		5	5	5	4	5				
Prevotellaceae	Liquid	29.8	27.4	28.2	29.2	29.0	<0.01	0.76	0.53	0.84
	Solid	35.8	38.1	30.4	27.5	36.0		0.01	0.35	0.16
Unidentified family 1[4]	Liquid	14.1	15.5	13.4	11.7	13.4	<0.01	0.19	0.43	0.99
	Solid	19.3	18.8	21.7	22.7	17.9		0.27	0.78	0.30
BS11	Liquid	21.6	19.7	15.9	16.8	23.4	<0.01	0.40	0.71	0.17
	Solid	10.2	11.4	12.2	16.6	13.5		0.19	0.64	0.73
Paraprevotellaceae	Liquid	5.8	5.2	2.9	4.5	5.4	<0.01	0.54	0.56	0.64
	Solid	3.6	3.7	4.3	5.3	3.3		0.05	0.47	0.16
Lachnospiraceae[5]	Liquid	1.9	3.1	3.9	5.8	2.0	<0.01	0.01	0.78	0.01
	Solid	4.6	3.7	4.2	4.3	4.5		0.72	0.53	0.82
Ruminococcaceae	Liquid	3.0	3.8	3.9	5.8	2.8	0.02	0.03	0.53	0.11
	Solid	3.5	2.8	2.9	3.1	2.6		0.83	0.35	0.63
S24-7[6]	Liquid	0.5	2.2	2.4	1.7	1.4	<0.01	0.61	<0.01	0.17
	Solid	2.5	4.7	4.6	2.9	4.5		0.47	0.05	0.94
Clostridiaceae[6]	Liquid	1.4	1.9	3.4	3.6	1.2	0.19	0.01	0.58	<0.01
	Solid	2.7	2.3	3.2	2.2	2.4		0.99	0.61	0.41
Other[4]	Liquid	1.6	2.1	2.0	1.4	1.6	<0.01	0.35	0.17	0.34
	Solid	2.3	2.4	3.5	2.1	2.1		0.99	0.20	0.07
Bacteroidaceae[5]	Liquid	2.2	2.5	2.5	1.0	1.8	<0.01	0.08	0.11	0.49
	Solid	1.2	1.3	1.5	1.6	1.0		0.57	0.94	0.31
Unidentified family 2[7]	Liquid	1.8	1.4	1.6	1.3	1.4	0.83	0.81	0.97	0.56
	Solid	2.1	1.4	1.2	1.3	1.4		0.38	0.32	0.69
Porphyromonadaceae[5]	Liquid	2.0	2.8	3.0	0.5	2.5	<0.01	0.02	0.02	0.74
	Solid	0.3	0.2	0.3	0.4	0.2		0.47	0.65	0.51
Fibrobacteraceae[6,8]	Liquid	1.2	0.9	0.5	0.4	1.3	0.14	0.09	0.98	0.08
	Solid	1.8	0.6	0.7	2.0	1.0		0.11	0.01	0.61
Veillonellaceae	Liquid	1.1	1.3	1.0	1.4	1.3	<0.01	0.91	0.76	0.45
	Solid	1.1	0.9	0.7	0.6	1.2		0.14	0.89	0.15

[1]Families listed were detected at greater than 1% relative abundance averaged across all samples.
[2]CON = unsupplemented control; 50P = 50 mg N/kg BW of PEAR; 100P = 100 mg N/kg BW of PEAR; 150P = 150 mg N/kg BW of PEAR; 100C = 100 mg N/kg BW of CSM.
[3]L vs. S = main effect of liquid vs. solid fraction.
[4]Order *Bacteroidales*
[5]Treatment × fraction ($P < 0.05$)
[6]Treatment × fraction ($P < 0.1$)
[7]Order RF39
[8]Period effect ($P = 0.05$)

in the solid fraction ($P < 0.01$). Family *Bacteroidaceae*, *Porphyromonadaceae*, and *Veillonellaceae* were relatively more abundant in the liquid fraction ($P < 0.01$).

Taxonomic resolution at the genus level demonstrated all members of family *Prevotellaceae* belonged to genus *Prevotella* and were consistent with previously discussed effects (Table 5). *Clostridium,* the second greatest identified genus, ranged from 1.1 to 3.4%. An observed treatment × fraction interaction ($P = 0.06$) indicated there was a difference between fractions for CON, 100C, and 150P. The proportion of *Clostridium* increased in the liquid fraction with greater PEAR provision ($P = 0.02$). Genus CF231 was detected in greater relative abundance in the liquid fraction ($P = 0.02$) than the solid, with the exception of 150P. A quadratic response was observed in the liquid fraction ($P < 0.01$), while CF231 was greater in 100P in the solid fraction compared to 100C (2.4 vs. 1.5%; $P = 0.04$). Genus YRC22 was detected at consistent levels in both fractions except CON. Within the solid fraction, relative abundance of YRC22 increased with additional PEAR ($P = 0.08$) was highest at 2.9% on 150C. BF311, *Paludibacter*, *Succiniclastium* represented a greater proportion with the liquid fraction ($P \leq 0.01$). Specifically, the treatment response of *Paludibacter* differed by fraction ($P = 0.02$) with a nine-fold greater relative abundance in the liquid fraction, except for 150P. *Ruminococcus* also increased in the liquid fraction ($P = 0.01$) as observed for *Ruminococcaceae*, but less than one-quarter of the *Ruminococcaceae* sequences were assigned to known genera *Ruminococcus*. Over 60 and 82% of *Spirochaetes* sequences corresponded to genus *Treponema* in liquid and solid fractions, respectively. Although there was a similar response within fraction to phyla

Table 5. Effect of increasing post-extraction algal residue supplementation on relative abundance of bacteria genera[1]

		Treatment[2]						Contrast *P*-value		
Item	Fraction	CON	50P	100P	150P	100C	L vs. S[3]	Linear	Quadratic	100P vs. 100C
No. observations		5	5	5	4	5				
Prevotella	Liquid	29.8	27.5	28.4	29.4	28.9	0.01	0.71	0.52	0.89
	Solid	35.8	38.2	30.5	27.6	36.1		0.01	0.35	0.16
Clostridium[4]	Liquid	1.3	1.9	3.3	3.4	1.1	0.15	0.02	0.43	<0.01
	Solid	2.5	2.3	3.1	2.1	2.4		0.93	0.51	0.41
CF231	Liquid	1.9	2.7	3.0	1.5	2.8	0.02	0.11	<0.01	0.73
	Solid	1.7	1.7	2.4	2.0	1.5		0.28	0.59	0.04
YRC22	Liquid	2.1	1.3	1.4	2.6	1.7	0.48	0.15	0.03	0.55
	Solid	1.3	1.5	1.4	2.9	1.4		0.08	0.24	0.79
BF311[5]	Liquid	2.1	2.4	2.2	0.8	1.7	<0.01	0.07	0.13	0.60
	Solid	1.1	1.1	1.5	1.5	0.9		0.45	0.95	0.32
Fibrobacter[4,6]	Liquid	1.2	0.9	0.5	0.4	1.3	0.14	0.09	0.97	0.08
	Solid	1.8	0.6	0.8	2.0	1.0		0.11	0.01	0.61
Paludibacter[5]	Liquid	1.9	2.6	2.7	0.5	2.1	<0.01	0.02	0.02	0.66
	Solid	0.2	0.2	0.3	0.4	0.2		0.31	0.55	0.38
Succiniclastium	Liquid	1.0	1.3	1.0	1.4	1.1	0.01	0.90	0.77	0.75
	Solid	1.1	0.9	0.7	0.6	1.1		0.15	0.94	0.20
Ruminococcus	Liquid	0.6	0.7	0.9	1.2	0.6	0.46	0.01	0.49	0.21
	Solid	0.8	0.7	0.7	0.7	0.6		0.79	0.85	0.40
Treponema[5]	Liquid	0.7	0.7	0.4	0.4	0.7	0.03	0.05	0.81	0.15
	Solid	0.8	0.4	0.8	1.4	0.6		<0.01	0.03	0.52
Bacteroides	Liquid	0.2	0.3	0.3	0.2	0.2	0.31	0.59	0.15	0.60
	Solid	0.3	0.5	0.2	0.2	0.3		0.11	0.85	0.22
Butyrivibrio[4]	Liquid	0.2	0.3	0.3	0.2	0.2	0.78	0.14	0.81	0.10
	Solid	0.3	0.2	0.3	0.2	0.3		0.73	0.46	0.79

[1]Genera listed were detected at greater than 0.3% relative abundance averaged across all samples and assigned taxonomic identification.
[2]CON = unsupplemented control; 50P = 50 mg N/kg BW of PEAR; 100P = 100 mg N/kg BW of PEAR; 150P = 150 mg N/kg BW of PEAR; 100C = 100 mg N/kg BW of CSM.
[3]L vs. S = main effect of liquid vs. solid fraction.
[4]Treatment × fraction ($P < 0.1$)
[5]Treatment × fraction ($P < 0.05$)
[6]Period effect ($P = 0.05$)

Spirochaetes, relative abundance was greater in solid samples ($P = 0.03$) and increased with additional PEAR provision ($P < 0.01$). In contrast to family *Bacteroidaceae*, relative abundance of genus *Bacteroides* was not significantly impacted by treatment or solid fraction ($P > 0.1$). Less than 10% of sequences in family *Lachnospiraceae* were assigned to known genus *Butyrivibrio*, and thus identified OTUs did not correlate to treatment effects observed at the family level. Bacterial taxa associated more predominantly within the liquid or solid fraction are summarized in Table 6.

Table 6. Bacterial taxa associated more predominantly with liquid or solid fraction[1]

Liquid	Solid
Phyla	
Tenericutes	
Family	
BS11	*Prevotellaceae*
Paraprevotellaceae	Unidentified family 1
Ruminococcaceae	*Lachnospiraceae*
Bacteroidaceae	S24-7
Porphyromonadaceae	Other
Veillonellaceae	
Genus	
CF233	*Prevotella*
BF311	*Treponema*
Paludibacter	
Succiniclastium	

[1]Taxa listed were detected at greater than 1% relative abundance at the phyla and family level and greater than 0.3% at the genus level averaged across all samples.

Core microbiome

Bacterial taxa present in all samples within each fraction were considered part of the core microbiome. Similar to richness results, the core microbiome was larger for the solid fraction (42 OTUs) compared to the liquid fraction (30 OTUs). Within the solid fraction, genus *Prevotella* represented thirteen OTUs including *Prevotella ruminicola*, and order *Bacteroidales* accounted for ten OTUs (Table 7). Additionally, *Fibrobacter succinogenes*, two genus *Clostridium*, five family *Lachnospiraceae*, and two family *Ruminococcaceae* OTUs were ubiquitous in solid samples. At the phyla level, 67% of core OTUs were *Bacteroidetes* and 28% *Firmicutes*. In contrast, *Bacteroidetes* and *Firmicutes* were more similarly represented within the liquid samples at 57 and 43% respectively. Specifically, five order *Bacteroidales*, seven genus *Prevotella*, and four *Ruminococcaceae* OTUs were detected in all liquid samples (Table 8). Across both fractions, 20 OTUs were considered part of the core microbiome including *Prevotella ruminicola* and four other genus *Prevotella* and four order *Bacteroidales* OTUs (Table 9).

Table 7. Core microbiome OTUs detected in all solid fraction samples.[1,2]

Phyla	Class	Order	Family	Genus	Species
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	BF311	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	BS11		
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	BS11		
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	BS11		
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	YRC22	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	*ruminicola*
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Fibrobacteres	*Fibrobacteria*	*Fibrobacterales*	*Fibrobacteraceae*	*Fibrobacter*	*succinogenes*
Firmicutes	*Clostridia*	*Clostridiales*	*Christensenellaceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Succiniclasticum*	
Firmicutes	*Clostridia*	*Coriobacteriales*			
Tenericutes	*Mollicutes*	RF39			

[1]Taxonomic classification is provided at the lowest level based on RDP bootstrap score > 0.8.
[2]A total of 42 OTUs were detected in the solid fraction core microbiome.

Table 8. Core microbiome OTUs detected in all liquid fraction samples.[1,2]

Phyla	Class	Order	Family	Genus	Species
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	CF231	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	YRC22	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	BS11		
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	BS11		
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	BS11		
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	*ruminicola*
Firmicutes	*Clostridia*	*Clostridiales*	*Christensenellaceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Succiniclasticum*	
Firmicutes	*Mollicutes*	RF39			
Firmicutes	*Mollicutes*	RF39			

[1]Taxonomic classification is provided at the lowest level based on RDP bootstrap score > 0.8.
[2]A total of 30 OTUs were detected in the liquid fraction core microbiome.

Table 9. Core microbiome OTUs detected in all solid and liquid samples.[1,2]

Phyla	Class	Order	Family	Genus	Species
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	YRC22	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	BS11		
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	BS11		
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	*ruminicola*
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	
Firmicutes	*Clostridia*	*Clostridiales*	*Christensenellaceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*		
Firmicutes	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Succiniclasticum*	
Tenericutes	*Mollicutes*	RF39			

[1]Taxonomic classification is provided at the lowest level based on RDP bootstrap score > 0.8.
[2]A total of 20 OTUs were detected in the core microbiome.

Discussion

The objective of this study was to define the bacteria in the rumen microbiome of cattle consuming LQF and identify bacterial taxa responding to increasing PEAR supplementation. Furthermore, isonitrogenous CSM supplementation compared the effect of a traditional protein supplement to PEAR. Results detail the first in-depth evaluation of the rumen microbiome of cattle consuming LQF using pyrosequencing technology.

Regardless of treatment, community level differences between liquid and solid fractions were distinguished by UniFrac PCoA analysis and are supported by previous work (Larue et al., 2005; Brulc et al., 2009; Pitta et al., 2010). Bacteria more prevalent in the solid fraction likely have a role in the complex degradation of celluloytic compounds. While unweighted UniFrac PCoA analysis indicated divergence based on sample fraction, principle coordinates only explained 12% of the variation. However, weighted UniFrac PCoA demonstrated variation between samples was linked to relative abundance of detected bacteria rather than the phylogenetic relationship of bacteria by accounting for over 35% of the variation in the first two principle coordinates. While unweighted analysis has detected treatment differences with the rumen microbe previously (Lee et al., 2012), our result was not surprising due to the overall similarity of dietary treatments with the same basal forage representing a minimum of 85% of the total diet (Drewery, 2012). Additionally, unweighted PCoA analysis indicated similar distribution patterns of samples within the liquid and solid fraction suggesting the variation in phylogenetic relationship between detected species in each fraction was similar. Alternatively, weighted PCoA graphs indicated a smaller spread within the solid samples suggesting greater dissimilarity within liquid fraction could be linked to treatment effects. Labeling PCoA charts by treatments (not shown) did not elucidate any treatment effects suggesting overall between animal variation was greater than between treatments via whole community UniFrac phylogenetic beta diversity.

Clustering analysis using pairwise Morisita-Horn and Bray-Curtis similarity indices provided detailed visualization of treatment effects on bacterial communities.

Morisita-Horn cluster analysis was more effective in separating sample fractions, and shorter branch lengths suggest solid bacterial communities were more similar compared to liquid samples. While Bray-Curtis cluster analysis sorted 25 samples based on fraction type, 8 samples from both fractions were paired for a given steer on one treatment. Pairing by steer indicates that between animal variation was greater than treatment effect or sample fraction. Variation in the rumen microbiome between animals on common diets has been previously observed (Brulc et al., 2009; Rius et al., 2012); after switching rumen contents of two cows on a common diet, (Weimer et al., 2010) observed ruminal pH and VFA concentration reestablished within 24 h followed by the bacterial community. The ability of the Morisita-Horn index to differentiate between bacterial communities may be due to its emphasis on the most abundant species to determine similarity (Magurran, 2004). Accordingly, the observed differences between fractions are driven by the predominant taxa. Less similarity among liquid samples suggests taxa responding to protein supplementation are located in the liquid fraction.

Combining measures of richness and evenness, the Shannon index describes changes in alpha diversity. The linear increase in alpha diversity in the solid fraction with increasing PEAR could result from the numerical increase in richness detected by ACE and observed OTUs. Since neither richness estimate significantly increased in the solid fraction ($P > 0.1$), evenness may have also increased with PEAR provision. Shannon values (6.46 - 7.12) are slightly higher than observed by Pitta et al. (2010) and Lee et al. (2012) from cattle on Bermudagrass hay (6.28) and a finishing ration (6.05), respectively. Fundamental differences in the current study contributing to higher

diversity may include greater sequencing depth and greater cellulolytic content in the basal forage.

Supplemental PEAR decreased estimated of richness in the liquid fraction. While only 15% of the total intake of 150P was provided as supplement, decreased OTU richness has been documented as steers adapted from a hay diet to a feedlot ration (Fernando et al., 2010). Similarly, Pitta et al. (2010) observed greater richness for a hay diet compared to grazed wheat with greater differences in the liquid fraction.

Core microbiome can have various meanings (Shade and Handelsman, 2012); we defined it as OTUs present in 100% of samples or within a fraction. Differences in the core microbiome between fractions were similar to richness with greater core OTUs in the solid fraction. Across sixteen animals on a common dairy ration, 157 core OTUs were identified with greater than 50% identified as genus *Prevotella* (Jami and Mizrahi, 2012). Additional core OTUs between this experiment and Jami and Mizrahi (2012) include order *Bacteroidales*, order *Clostridiales*, family *Lachnospiraceae*, and family *Ruminococcaceae*.

Similar to prior pyrosequencing surveys of the rumen bacterial microbiome (de Menezes et al., 2011; Lee et al., 2012; Zened et al., 2013), *Bacteroidetes* and *Firmicutes* were the dominant phyla. However, B:F exceeded 9:1 in the liquid fraction of 100C, which is greater than previously pyrosequenced rumen environments. Interestingly, the most similar B:F ratio to 100C was CON (8.5:1). The most comparable previous B:F observations (3:1) occurred on a tropical forage-based diet providing supplemental protein at a level similar to 150P (Thoetkiattikul et al., 2013). In contrast, B:F ratio was

more consistent in the solid fractions (~6:1) suggesting a more similar function within the solid fraction microbiome.

Prevotella is well-documented as the main genus in rumen microbiome (Whitford et al., 1998; Stevenson and Weimer, 2007; Callaway et al., 2010). The variety of rations used in these experiments underscores the diverse metabolic potential of *Prevotella* species. *Prevotella* species have a documented role in metabolism of starch (Cotta, 1992), hemicellulose (Matsui et al., 2000), pectin (Dehority, 1991), and peptide or protein catabolism (Attwood and Reilly, 1995). Of the four culturable *Prevotella* species, *P. ruminicola* had the greatest ability to catabolize the xylan component of hemicellulose (Matsui et al., 2000). *In vitro* co-culture of *P. ruminicola* with cellulolytic *Fibrobacter succinogenes* or *Ruminococcus flavefaciens* increased cellulose degradation above the ability of either cellulolytic species alone (Fondevila and Dehority, 1996). These synergistic effects may be caused by presence of hemicelluloytic carbohydrate esterases within genome of *P. ruminicola* that degrade ferulic acid esters and acetyl-esters (Kabel et al., 2011). Isolation of *Prevotella* species from liquid and solid rumen factions indicated culturable species represented only 22% of the genus *Prevotella* (Bekele et al., 2010); divergent uncultured *Prevotella* phylotypes were observed in each fraction suggesting different niches for various *Prevotella* species. The *P. ruminicola* and *P. bryantii* genomes lack some xylanase enzymes possessed by members of *Bacteroidetes* suggesting an alternate method of xylan degradation (Dodd et al., 2011). Decreased *Prevotella* in the solid fraction with PEAR may have resulted from their lower proteolytic activity relative to other *Bacteroidetes* members in the solid fraction.

Overall, the relative abundance of *Prevotella* was similar to proportions observed for a hay diet (Pitta et al., 2010).

Firmicutes responded positively to PEAR supplementation in the liquid fraction, but 100C levels were similar to CON suggesting this response was driven by other nutrients besides N in the PEAR or more rapid N release from PEAR. Within phylum *Firmicutes*, a linear increase was observed for *Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiaceae* families in the liquid fraction with increasing PEAR in addition to greater relative abundance in 100P compared to 100C. Although no treatment effects were observed in the solid fraction, all three families were detected at 2-5% relative abundance suggesting some role in fiber degradation. Within each of these families, *Clostridium* was the only predominant genus identified. Although genus *Ruminococcus* and *Butyrivibrio* were detected, unassigned genera represented 75% of the sequences within *Ruminococcaceae* and *Lachnospiraceae*.

Within phylum *Firmicutes*, *Clostridia* was the predominant genus and increased with PEAR supplementation in the liquid fraction. Multiple *Clostridia* species including *Clostridium sticklandii* and *Clostridium aminophilum*, have demonstrated ammonia producing activity 20-fold greater than *Prevotella ruminicola* (Russell et al., 1988; Paster et al., 1993). These bacteria have been referred to as hyper ammonia producers or obligate amino acid fermenters and their excess release of ammonia reduces the benefit of protein to other bacteria and the host (Farmer et al., 2004). Monensin can reduce *C. sticklandii* populations, but *C. aminophilum* has shown to be largely resistant (Paster et al., 1993; Krause and Russell, 1996). Small populations of obligate amino acid

fermenters may be below typical detection levels for pyrosequencing. Drewery (2012) reported ruminal ammonia concentrations between 100P and 100C were not different 4 h after supplementation, but they suggest the ruminal ammonia apex for 100P was likely reached prior to sampling. The only genera identified at the species level were *C. stercorarium* and *C. hiranonis* as each represented a single OTU. *Clostridium stercorarium* has been associated with fiber degradation and contains known xylanases (Bérenger et al., 1985; Bronnenmeier et al., 1990), while *C. hiranonis* ferments saccharides (Kitahara et al., 2001). Neither species have been commonly observed in the rumen. Other cellulolytic *Clostridum* species isolated from the rumen include *C. chartatabidium* (Kelly et al., 1987), *C. cellobioparum* (Hungate, 1944), *C. lochheadii*, and *C. longisporum* (Hungate, 1957). The majority of novel *Clostridia* clones isolated from sheep on a hay diet were most similar to *C. thermosuccinogenes* which ferments glucose and fructose to succinate or acetate, or the fibrolytic *C. leptum* (Larue et al., 2005). Low abundance of ruminal *Clostridium* species or insufficient sequence length may have prevented detection and classification. Species-specific probes should be utilized to determine the taxa responding within the liquid fraction to supplemental PEAR.

Family *Ruminococcaceae* and genus *Ruminococcus* increased in the liquid fraction with increasing PEAR supplementation. Although the majority of the sequences were not identified at the genus level, fourteen *Ruminococcus* OTUs were identified including *R. bromii*, *R. callidus* (4), and *R. flavefaciens* (9). Thoetkiattikul et al. (2013) similarly observed few sequences within *Ruminococcaceae* identified with known genus

Ruminococcus. Probes designed for *R. flavefaciens* also amplified *R. callidus* underscoring their similarity (Stevenson and Weimer, 2007), and both have been associated with cellulose and hemicellulose hydrolysis (Dehority and Scott, 1967). Novel ruminococci with non-cellulolytic activities have been shown to be phylogenetically dissimilar from original cellulolytic ruminococci (Rainey and Janssen, 1995). Further phenotypic tests indicate this group of ruminococci can ferment glucose, maltose, and mannose (Liu et al., 2008). This group has been proposed to be renamed *Blautia*. With a more diverse range of function genus *Blautia* could be responsible for changes within *Ruminococcaceae* observed with supplementation in the liquid fraction. Although at family level, there is a difference between fractions, a fraction association was not observed within the identified genus *Ruminococcus*. Unidentified *Ruminococcaceae* members in this study are more prevalent in the liquid fraction even though identified *Ruminococcus* increased with PEAR supplementation.

Within family *Lachnospiraceae*, genera *Butyrivibrio* and *Psuedobutryivibrio* are thought to be the main butyrate producers in the rumen and are considered to be effective hemicellulose degraders (Diez-Gonzalez et al., 1999; Paillard et al., 2007). *Butyrivibrio* species have been mainly found in solid fraction (Whitford et al., 1998; Tajima et al., 1999; Koike et al., 2003), and some are noted for high proteolytic activity (Cotta and Hespell, 1986; Attwood and Reilly, 1995; Sales et al., 2000). Butyrivibrios are diverse metabolically; the 16S rRNA gene of *B. fibrisolvens* strains is not always similar and some have alternative methods of butyrate production (Russell, 2002). *Butyrivibrio fibrisolvens* is commonly detected by PCR probes (Forster et al., 1997) and

has been shown to decrease with adaptation to concentrate diets (Mrázek et al., 2006; Fernando et al., 2010). A newer isolate from the rumen, *B. proteoclasticus* (originally *Clostridium proteoclasticum*), can ferment xylan, peptides, amino acids, and hydrogenate unsaturated fatty acids (Kelly et al., 2010; Palevich, 2011). Although family *Lachnospiraceae* increased in relative abundance with PEAR in the liquid fraction, less than 8% of sequences classified to known genus *Butyrivibrio* and significant treatment effects were not detected. While PEAR and CSM are similar in ether extract (1.5 and 1.7%, respectively), the smaller particle size of PEAR would likely increase lipid content available for bacterial catabolism. Moreover, differences in glycolipid, phospholipid, triacylglycerol content could affect lipid metabolism. Therefore, unidentified members of *Lachnospiraceae* which responded to protein supplementation within the liquid fraction could be associated with increased availability of lipids.

Fibrobacteres at the phylum and genus level decreased with additional PEAR provision; a linear decrease in the liquid fraction and a quadratic decrease in the solid fraction were observed. Although traditionally accepted as a predominant cellulolytic species in the rumen (Stewart et al., 1997; Wanapat and Cherdthong, 2009), *Fibrobacter succinogenes* was only detected in half of the samples by pyrosequencing (Jami and Mizrahi, 2012). Our results indicated *Fibrobacter* represented < 2% in relative abundance, but detection of absolute abundance could demonstrate a different response to supplementation. De Menezes et al. (2011) observed *Fibrobacteraceae* levels > 5% in the solid fraction of a pasture diet supporting possibility of PCR bias in *Fibrobacter*

detection as discussed by Tajima et al. (2001). Others have demonstrated a decrease in relative abundance of *Fibrobacteraceae* with addition of dietary concentrate in the solid fraction (de Menezes et al., 2011) or overall (Thoetkiattikul et al., 2013). Nguyen et al. (2012) observed increased cellulolytic species by roll-tube technique with increasing urea content in isonitrogenous protein supplements (65 mg N/kg BW) fed to swamp buffalo consuming rice straw (2% CP). However, no change in absolute abundance of *F. succinogenes* was detected by real-time PCR with the increase in urea.

The response of genus *Treponema* to PEAR supplementation correlated to phyla *Spirochaetes* of which it represented greater than half of the sequences. The fraction × treatment interaction was caused by increasing dissimilarity between fractions with additional PEAR. A greater percentage of the phylum *Spirochaetes* in the solid fraction was identified *Treponema* compared to the liquid fraction (82 vs. 60%). *Treponema saccharophilum* can ferment pectin, starch, mannose, galactose, and arabinogalactan (Paster and Canale-Parola, 1982), while *T. bryantii* can metabolize xylose, mannose, galactose, and pectin (Paster and Canale-Parola, 1985). However, *T. bryantii* only makes up a small fraction of genus *Treponema* isolated from the rumen (Bekele et al., 2011). Although culturable Treponemes are noncellulolytic, they associated with the solid fraction and increased fiber degradation in co-culture with *Fibrobacter succinogenes* (Stanton and Canale-Parola, 1980; Kudo et al., 1987). Their association is supported by our results as they had similar responses to supplementation in the solid fraction. A positive correlation between *Fibrobacters* and *Spirochaetes* has also been observed by pyrosequencing and PCR (Tajima et al., 2001; de Menezes et al., 2011).

Recently, a draft genome of a novel *Treponema* isolated from the rumen suggests that it has a broader range of carbohydrate activity than *T. bryantii* with predicted cellulases, endohemicellulases, glycoside hydrolases, and carbohydrate esterase enzymes (Rosewarne et al., 2012).

In a similarly designed study to ours, *in vitro* rate of ammonia production increased after supplementation to steers consuming LQF with greater rates for higher levels of supplementation (Bell et al., 2013). However, most probable number estimates demonstrated a slight decrease in $\log_{10}$ cells/ml for the highest level of supplementation. This could indicate fewer bacteria are responsible for greater ammonia production via increased activity levels. Drewery (2012) observed supplemental PEAR stimulated an increase in forage OM intake and total digestible OM intake with the greatest level at 100P. Although OM and neutral detergent fiber digestion also increased quadratically, the peak at 50P attests to the competing dynamics of passage rate and digestion (Robinson et al., 1985). Although changes in bacterial relative abundance are linked to increasing PEAR provision, overall similarity between CON and 100C indicates changes in bacteria proportion may not explain improvements in intake and digestion associated with protein supplementation.

Conclusion

The primary objective of this experiment was to determine the effect of increasing PEAR supplementation on the rumen bacterial microbiome in steers consuming low-quality forage. Distinct bacterial communities between liquid and solid fractions were detected by weighted UniFrac analysis and indicated greater

heterogeneity within the liquid fraction. *Prevotella* was the predominant observed genus representing greater than 25% of sequences from each treatment. *Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiaceae* increased in relative abundance in the liquid fraction with greater PEAR provision. Our results suggest PEAR supplementation of LQF impacts the liquid fraction of the rumen microbiome; however, similarity of CSM supplemented and control treatment indicate additional factors affect improvements in forage utilization.

CHAPTER III

EFFECT OF COTTONSEED MEAL AND DRIED DISTILLERS' GRAINS SUPPLEMENTATION ON RICE STRAW UTILIZATION BY BRAHMAN STEERS

Overview

Seven ruminally cannulated Brahman steers were used in a 7 × 4 incomplete block design to determine the effects of cottonseed meal (CSM; 43.9% CP, 82.9% degradable protein) or dried distiller's grain (DDG; 27.5% CP, 43.6% degradable protein) supplementation on rice straw utilization. Treatments consisted of a control (no supplement) and three levels (60, 120, and 180 mg N/kg BW) of either CSM or DDG. Periods were 14 d with 9 d for adaptation and 5 d for data collection. Steers had ad libitum access to rice straw (4.7% CP, 68% NDF) and were fed supplements daily. Increased supplementation resulted in a linear increase ($P \leq 0.06$) in forage OM intake from 13.5 g/kg BW (control) to 16.1 and 15.5 g/kg BW for 180 mg N/ kg BW of CSM and DDG respectively, with no difference between sources ($P = 0.84$). Total digestible OM intake was increased by supplementation (linear, $P < 0.01$) from 8.0 g/kg BW (control) to 11.7 and 12.9 g/kg BW for 180 mg N/ kg BW of CSM and DDG respectively. A greater response was observed for DDG ($P = 0.05$) due to greater provision of supplement (g DM/d) to achieve isonitrogenous treatment levels. Total tract OM digestion increased (linear, $P < 0.01$) with CSM and DDG supplementation. Although CSM and DDG increased NDF digestibility (linear, $P \leq 0.06$) from 49.6% (control) to 53.7 and 54.9% at 180 mg N/kg BW respectively, estimated forage NDF

digestibility was not significantly increased ($P > 0.10$). Ruminal ammonia concentrations peaked 4 h after supplementation with the greatest concentration (4.0 mM) observed for 180 mg N/kg BW of CSM and the lowest concentration observed in the control (0.8 mM). Provision of CSM resulted in a linear increase ($P < 0.01$) in ruminal ammonia in contrast to the quadratic response ($P = 0.02$) observed with DDG supplementation. Total VFA production followed a similar trend with a linear increase for CSM and DDG supplementation, respectively ($P = 0.09$, $P = 0.01$). Protein supplements containing high and low levels of degradable intake protein were effective at improving intake and utilization of rice straw.

Introduction

Rice straw is a widely used forage resource for beef cattle in East Asia and is globally, the crop residue produced in the greatest amount (Lal, 2005). Although there are comparatively few acres of rice grown in the United States, over 2.5 million acres are grown in the Sacramento Valley of California, along the Mississippi River, and near the Texas gulf coast. Rice straw may offer an alternative to traditional roughage sources for beef cattle producers. Rice straw is considered a low-quality forage (< 7% CP) and may require supplemental protein to increase intake and digestion (Krishnamoorthy et al., 1982). Degradable intake protein (**DIP**) is the first limiting factor for utilization of most low-quality forages (Köster et al., 1996). Cottonseed meal (**CSM**) supplementation increased intake and ruminal ammonia concentrations of steers consuming prairie hay while also increasing *in vitro* dry matter disappearance after 24 h (McCollum and Galyean, 1985). Despite a lower DIP content, dried distillers' grains (**DDG**) have also

been shown to improve utilization of bermudagrass hay (Rambo, 2010). Provision of undegradable intake protein can increase ruminally available nitrogen supply via nitrogen recycling (Wickersham et al., 2008). Considering increased input costs in beef cattle operations, determining proper supplementation type and amount can optimize digestible OM intake and return on monetary expenditures. Therefore, the objective of this study was to determine the amount of CSM or DDG needed to optimize digestible OM intake and forage utilization in cattle consuming rice straw.

Materials and methods

All experimental procedures were approved by Institutional Animal Care and Use Committee at Texas A&M University. Seven ruminally and duodenally fistulated Brahman steers (average initial BW = 255 ±10 kg) were used in a 7-treatment, 4-period, incomplete block design. Steers were housed in individual pens (2.1 m x 1.5 m) in an enclosed, continuously lighted and climate controlled barn with ad libitum access to water and a trace mineralized salt block (≥96.0% NaCl, 1.00% S, 0.15% Fe, 0.25% Zn, 0.30% Mn, 0.009% I, 0.015% Cu, 0.0025% Co, and 0.001% Se; United Salt Corporation, Houston, TX). Rice straw (Table 10) was chopped through a screen (76 cm × 76 cm) and offered at 0630 h at 130% of average voluntary intake of the preceding 4 d to ensure access to forage did not limit intake. Supplement levels of DDG and CSM (60, 120, and 180 mg N/kg BW) were selected based on previous work using low-quality forage (Rambo, 2010) in addition to an unsupplemented control. Although CSM and DDG treatments were designed to be isonitrogenous, variability in CSM led to

overestimation of CP content. Supplements were offered daily at 0630 h, top dressed over the rice straw.

Table 10. Feedstuff composition.

Item, % of DM	Rice straw	CSM[1]	DDG[1]
OM	85.3	92.8	94.5
CP	4.7	43.9	27.5
Degradable intake protein[2], % of CP	61.3	82.9	43.6
NDF	68.1	25.5	37.0
ADF	50.3	16.6	13.3
Acid detergent insoluble ash	9.1	0.4	0.2

[1]CSM = cottonseed meal, DDG = dried distillers' grains
[2]Determined by *Streptomyces gresius* procedure (Mathis, 2001)

Experimental periods were 14 d long, with 8 d for adaptation to treatments and 6 d for collection. Calculations of intake and digestion were made from observations made on d 9 through d 13 (Cochran and Galyean, 1994). Straw and supplement samples were collected on d 9 through d 12 to correspond with ort samples collected on d 9 through d 12. Fecal grab samples were collected every 8 h on d 10 through d 13 advancing 2h each day and composited across days within steer. On d 14, rumen fluid samples were collected with a suction strainer (Raun and Burroughs, 1962), 19 mm diameter 1.5 mm mesh) prior to administration of treatments (0 h) and 4, 8, 12, 16, and 20 h after treatments were fed. Rumen fluid (8 ml) from each collection was combined with 2 mL of 25% metaphosphoric acid and frozen (-20° C) for subsequent determination of NH_3 and VFA concentration (Erwin et al., 1961). Rumen fluid pH was

measured immediately after collection with a portable pH meter with a combined electrode (Symphony pH meter, VWR International, Radnor, PA).

Laboratory analysis

Straw, supplement, orts, and fecal samples were dried in a forced-air oven for 96 h at 55°C, and then air equilibrated for 24 h and weighed to determine partial DM. Dried samples were ground in a Wiley Mill (No. 4, Thomas Scientific, Swedesboro, NJ) to pass through a 1mm screen. Straw and ort samples were composited across days. All samples were dried at 105°C in a forced-air oven for 24 h to determine DM, and then combusted at 450°C for 8 h in a muffle furnace to determine OM. Nitrogen content was determined on straw and supplement samples by total combustion (Rapid N Cube, Elementar, Mt. Laurel, NJ). Crude protein was calculated as N × 6.25. Straw and supplement CP degradability was estimated using a 48 h *Streptomyces griseus* protease procedure as described by Mathis et al. (2001). Straw, supplement, ort, and fecal samples NDF and ADF were analyzed using an Ankom Fiber Analyzer 200 (Ankom Technology, Macedon, NY) without sodium sulfite or correction for residual ash. Acid detergent insoluble ash (ADIA) was utilized as an internal marker and determined on supplement, straw, ort, and fecal samples by combusting ADF residues in Ankom bags for 8 h at 450°C in a muffle furnace. Fecal production was estimated using ADIA intake divided by fecal ADIA concentration, then total tract digestion coefficients were calculated for OM, NDF, ADF using procedures as described by Cochran and Galyean (1994). Forage NDF digestibility was calculated by accounting for predicted undigested supplement NDF using *in situ* NDF degradabilities observed by Winterholler et al.

(2012). Frozen rumen liquid samples were thawed and centrifuged; the supernatant were decanted and ruminal NH_3 and VFA concentrations were determined by a colorimetric procedure (Broderick and Kang, 1980) using a UV/VIS (Sigma Diagnostics, St. Louis MO) and gas chromatography (Vanzant and Cochran, 1994) respectively.

Statistical analysis

Intake and digestion were analyzed using the MIXED procedure of SAS 9.3 (SAS Inst. Inc., Cary, NC). Terms in the model included treatment and period with steer included as a random effect. Ruminal ammonia, pH, and VFA were analyzed using MIXED procedure. Terms in the models were treatment, period, hour, and hour × treatment with steer and treatment × period × steer included as a random effect. Treatment means were calculated using the LSMEANS option. Orthogonal contrasts were implemented for means separation. Contrasts included: 1) CSM vs. DDG, 2) CSM linear, 3) CSM quadratic, 4) CSM cubic, 5) DDG linear, 6) DDG quadratic, 7) DDG cubic, and 8) N source × N level. Coefficients required for orthogonal polynomial were obtained using IML procedure of SAS (SAS Inst. Inc., Cary, NC). When multiple contrasts were significant, data was visually interpreted to match the most appropriate correlation.

Results

One intake observation and a separate ruminal fermentation observation were removed for reasons unrelated to treatment. Supplemental CSM increased forage OM intake (**FOMI**) (linear, $P = 0.02$; Table 11) with a 119% increase above the unsupplemented control for CSM 180 mg N/kg BW (16.1 g/kg BW). Supplemental

DDG tended to increase FOMI (linear, $P = 0.06$); 180 mg N/kg BW treatment demonstrated a 115% increase compared to the control (15.5 g/kg BW). Source of supplemental N did not effect FOMI response ($P = 0.84$). Forage N intake responses followed a similar pattern to FOMI (linear, $P \leq 0.02$) while supplement N intake linearly increased ($P < 0.01$) with supplementation, as expected based on study design. However, a difference between CSM and DDG supplement N intake ($P < 0.01$) indicated treatments were not truly isonitrogenous. Supplemental CSM and DDG at the 120 mg N/kg BW treatment level did not increase FOMI above the response observed 60 mg N/kg BW treatment level. Additionally, supplemental CSM increased total tract OM digestion (linear, $P \leq 0.01$; Table 12), while similar increases were observed for DDG supplementation (linear, $P \leq 0.01$). Specifically, supplementation increased total tract OM digestion to 63.6 and 65.3% for CSM and DDG supplementation respectively at 180 mg N/kg BW compared to 58.7% for the unsupplemented control. Total tract NDF digestion increased linearly with DDG supplementation ($P = 0.01$) and tended to increase with CSM supplementation (linear, $P = 0.06$). However, NDF digestion for both supplements at the 120 mg N/kg BW treatment level was numerically lower than other supplemented treatments. Total digestible OM intake (**TDOMI**) was increased by CSM and DDG supplementation (Figure 7; linear, $P < 0.01$), although a greater was observed for DDG supplementation compared to CSM ($P = 0.05$). The greatest TDOMI was observed at 180 mg N/kg BW treatment level for CSM and DDG (11.7 and 12.9 g/kg BW) which was 146 and 161%, respectively of the unsupplemented control. Response of total OM intake was consistent with supplement effect on TDOMI.

Supplementation of CSM and DDG increased total NDF intake (linear, $P < 0.01$), but no difference was detected between CSM and DDG ($P = 0.13$). However, supplemental DDG tended to increase forage NDF intake (linear, $P = 0.06$), while supplemental CSM increased forage NDF intake (linear, $P < 0.01$). Supplement NDF intake was increased for DDG supplementation compared to CSM ($P < 0.01$) due to greater NDF composition and lower CP content. Forage NDF digestibility was not significantly affected by treatment ($P > 0.10$).

The treatment × hour interaction was significant for ruminal ammonia ($P < 0.01$; Table 13). Overall, concentrations peaked 4 h after feeding then returned to nadir before the next feeding event (Figure 6). Provision of supplemental N from CSM (linear, $P < 0.01$) and DDG (quadratic, $P = 0.03$) increased ruminal ammonia concentrations. Although observed ruminal ammonia concentrations did not differ between CSM and DDG ($P = 0.17$), an N source × N level interaction ($P = 0.03$) indicated ruminal ammonia response was not consistent between CSM and DDG at a given supplemental N level. Supplemental DDG increased total VFA concentration (quadratic, $P = 0.01$), and CSM tended to increase VFA concentrations linearly ($P = 0.09$). Supplemental DDG increased the molar percentage of propionate (linear, $P = 0.01$) and butyrate (quadratic, $P = 0.01$) which corresponded with a decrease in acetate:propionate (linear, $P < 0.01$). The main effect of hour was significantly related to acetate:propionate ($P < 0.01$) as it was the lowest 4 h after feeding before increasing until the next feeding event. Treatment did not affect ruminal pH ($P = 0.27$) with an observed range from 6.57 - 6.72.

Table 11. Effect of increasing amounts of cottonseed meal or dried distillers' grains supplementation on intake.

	Treatment[1]								Contrasts[2,5]				
	Control	CSM			DDG					CSM		DDG	
Item	0	60	120	180	60	120	180	SEM[3]	CSM vs. DDG	L	Q	L	Q
No. of observations	4	4	3	4	4	4	4						
OMI, g/kg BW/d													
Forage	13.5	14.9	14.4	16.1	15.0	14.6	15.5	0.85	0.84	0.02	0.82	0.06	0.69
Supplement[4]	0.0	0.8	1.6	2.4	1.4	2.9	4.3	0.01	<0.01	<0.01	0.10	<0.01	0.76
Total	13.5	15.7	16.0	18.4	16.4	17.4	19.8	0.85	0.04	<0.01	0.83	<0.01	0.70
Digestible	8.0	9.4	9.8	11.7	10.1	10.6	12.9	0.65	0.05	<0.01	0.69	<0.01	0.81
NDF, g/kg BW/d													
Forage	10.7	11.8	11.4	12.8	11.9	11.6	12.3	0.68	0.93	0.02	0.73	0.06	0.57
Supplement[4]	0.0	0.2	0.4	0.7	0.6	1.1	1.7	0.04	<0.01	<0.01	0.63	<0.01	0.99
Total	10.7	12.0	11.8	13.5	12.5	12.8	14.0	0.67	0.13	<0.01	0.74	<0.01	0.57
Digestible	5.4	6.4	6.0	7.3	6.6	6.6	7.7	0.46	0.16	<0.01	0.74	<0.01	0.75
N intake, mg/kg BW/d													
Forage	102.1	113.8	111.3	122.5	113.0	113.0	120.5	6.33	0.93	0.01	0.96	0.02	0.71
Supplement[4]	0.0	54.5	108.8	163.0	59.3	118.4	178.0	0.87	<0.01	<0.01	0.35	<0.01	0.92
Total	101.8	168.4	220.3	285.3	172.4	231.4	298.6	6.24	0.24	<0.01	0.78	<0.01	0.71

[1]Control = 0 mg N/kg BW, CSM 60 = 54 mg N/kg of BW; CSM 120 = 108 mg N/kg of BW; CSM 180 = 162 mg N/kg of BW, DDG 60 = 59 mg N/kg of BW, DDG 120 = 119 mg N/kg of BW, DDG 180 = 178 mg N/kg of BW.

[2]L = linear, Q = quadratic, Overall N = effect of supplemental N from CSM and DDF regardless of source, CSM = effect of increasing level of CSM supplementation, DDG = effect of increasing level of DDG supplementation, CSM vs. DDG = effect of all levels of CSM compared to all levels of DDG.

[3]SEM = standard error of the mean

[4]N source × N level interaction ($P < 0.01$)

[5]All cubic contrasts ($P > 0.10$) except DDG supplement OM intake ($P > 0.03$).

Table 12. Effect of increasing amounts of cottonseed meal or dried distillers' grains supplementation on digestion.

	Treatment[1]								Contrasts[2,5,6]				
	Control	CSM			DDG					CSM		DDG	
Item	0	60	120	180	60	120	180	SEM[3]	CSM vs. DDG	L	Q	L	Q
Total Tract Digestion, %													
OM	58.7	60.2	61.4	63.6	61.5	61.0	65.3	1.48	0.36	<0.01	0.76	<0.01	0.47
NDF	49.6	53.0	51.2	53.7	53.2	51.7	54.9	1.62	0.50	0.06	0.70	0.02	0.87
Forage NDF[4]	49.7	53.0	51.0	53.4	52.5	50.4	53.2	1.74	0.67	0.12	0.72	0.15	0.99

[1]Control = 0 mg N/kg BW, CSM 60 = 54 mg N/kg of BW; CSM 120 = 108 mg N/kg of BW; CSM 180 = 162 mg N/kg of BW, DDG 60 = 59 mg N/kg of BW, DDG 120 = 119 mg N/kg of BW, DDG 180 = 178 mg N/kg of BW.

[2]L = linear, Q = quadratic, Overall N = effect of supplemental N from CSM and DDF regardless of source, CSM = effect of increasing level of CSM supplementation, DDG = effect of increasing level of DDG supplementation, CSM vs. DDG = effect of all levels of CSM compared to all levels of DDG.

[3]SEM = standard error of the mean

[4]Estimated based on in situ NDF digestibility of CSM and DDG (Winterholler et al., 2012)

[5]N source × N level interaction not significant for digestion ($P > 0.10$)

[6]All cubic contrast ($P > 0.05$)

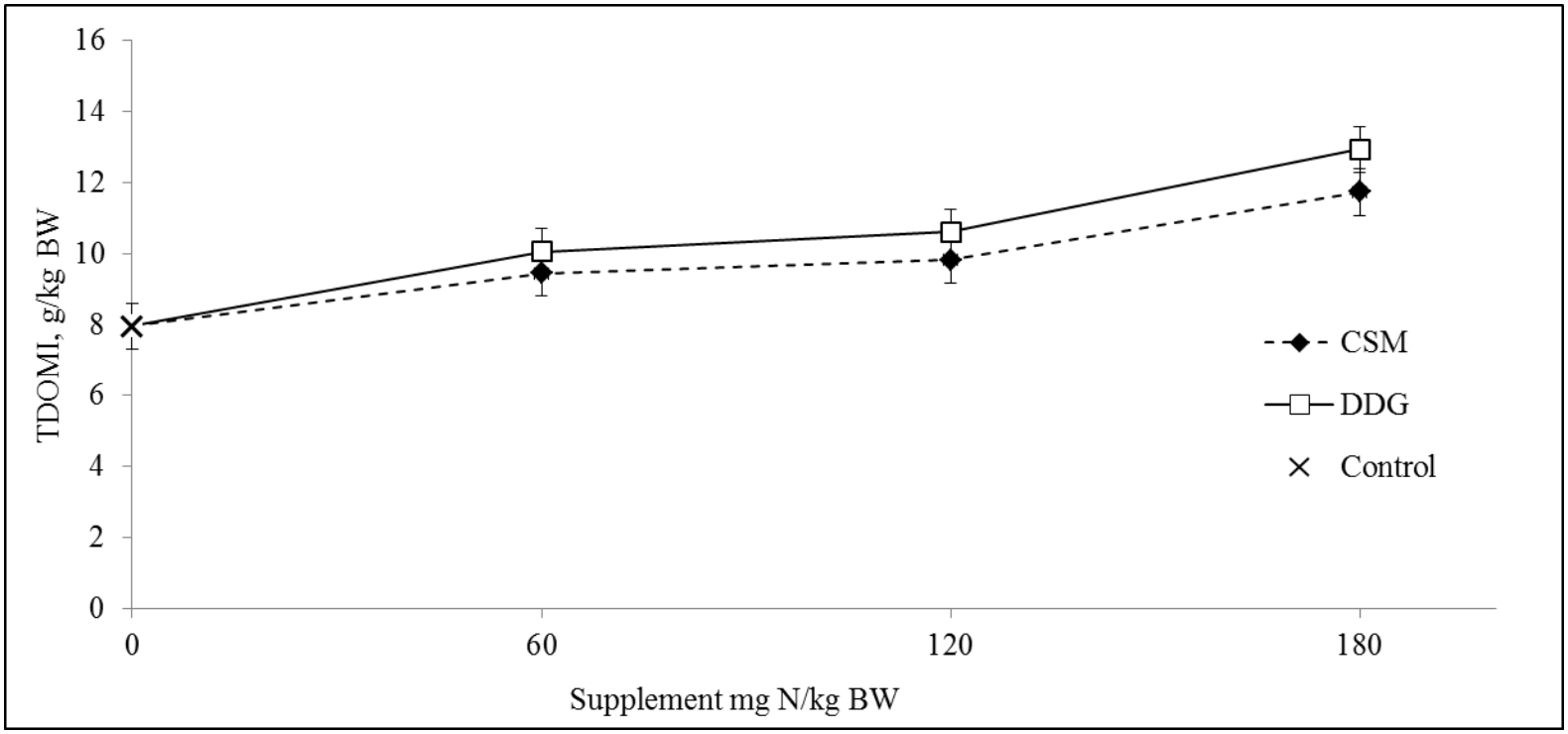

Figure 6. Effect of increasing amounts of cottonseed meal or dried distillers' grains on total digestible OM intake. CSM 60 = 54 mg N/kg of BW; CSM 120 = 108 mg N/kg of BW; CSM 180 = 162 mg N/kg of BW, DDG 60 = 59 mg N/kg of BW, DDG 120 = 119 mg N/kg of BW, DDG 180 = 178 mg N/kg of BW, Control = 0 mg N/kg of BW; CSM and DDG linear contrast ($P \leq 0.01$).

Table 13. Effect of increasing amounts of cottonseed meal or dried distillers' grains supplementation on ruminal ammonia, pH, and volatile fatty acid (VFA) production.

	Treatment[1]								Contrasts[2,6]				
	Control	CSM			DDG					CSM		DDG	
Item	0	60	120	180	60	120	180	SEM[3]	CSM vs. DDG	L	Q	L	Q
No. of observations	4	4	4	4	4	4	3						
Ammonia[4,5], m*M*	0.27	0.69	1.75	2.37	1.13	1.61	1.19	0.26	0.17	<0.01	0.70	0.02	0.03
pH[e]	6.69	6.72	6.66	6.64	6.64	6.57	6.67	0.05	0.17	0.23	0.50	0.50	0.10
Total VFA, m*M*	82.2	88.5	90.8	90.6	94.8	93.8	85.3	3.87	0.63	0.09	0.35	0.63	0.01
Acetate:propionate[4]	4.1	4.4	4.4	4.3	4.2	3.9	3.6	0.11	<0.01	0.09	0.05	<0.01	0.08
Molar Percentage													
Acetate[4,5]	73.9	74.5	73.8	73.4	72.9	71.1	70.4	0.39	<0.01	0.21	0.16	<0.01	0.72
Propionate[4,5]	18.2	17.2	16.9	17.2	17.5	18.4	19.6	0.45	<0.01	0.05	0.08	0.01	0.02
Butyrate	6.7	6.8	7.6	7.6	7.8	8.4	7.8	0.26	<0.01	<0.01	0.76	<0.01	<0.01
Isobutyrate	0.6	0.6	0.7	0.8	0.7	0.7	0.8	0.35	0.63	<0.01	0.91	<0.01	0.57
Isovalerate	0.4	0.5	0.6	0.6	0.6	0.8	0.8	0.06	0.01	<0.01	0.93	<0.01	0.20
Valerate[5]	0.3	0.4	0.4	0.4	0.4	0.5	0.6	0.29	<0.01	<0.01	0.62	<0.01	0.43

[1]Control = 0 mg N/kg BW, CSM 60 = 54 mg N/kg of BW; CSM 120 = 108 mg N/kg of BW; CSM 180 = 162 mg N/kg of BW, DDG 60 = 59 mg N/kg of BW, DDG 120 = 119 mg N/kg of BW, DDG 180 = 178 mg N/kg of BW.

[2]L = linear, Q = quadratic, Overall N = effect of supplemental N from CSM and DDF regardless of source, CSM = effect of increasing level of CSM supplementation, DDG = effect of increasing level of DDG supplementation, CSM vs. DDG = effect of all levels of CSM compared to all levels of DDG.

[3]SEM = standard of the mean

[4]N source × N level interaction ($P \leq 0.10$)

[5]Time × treatment interaction ($P \leq 0.05$)

[6]All cubic contrasts ($P > 0.10$)

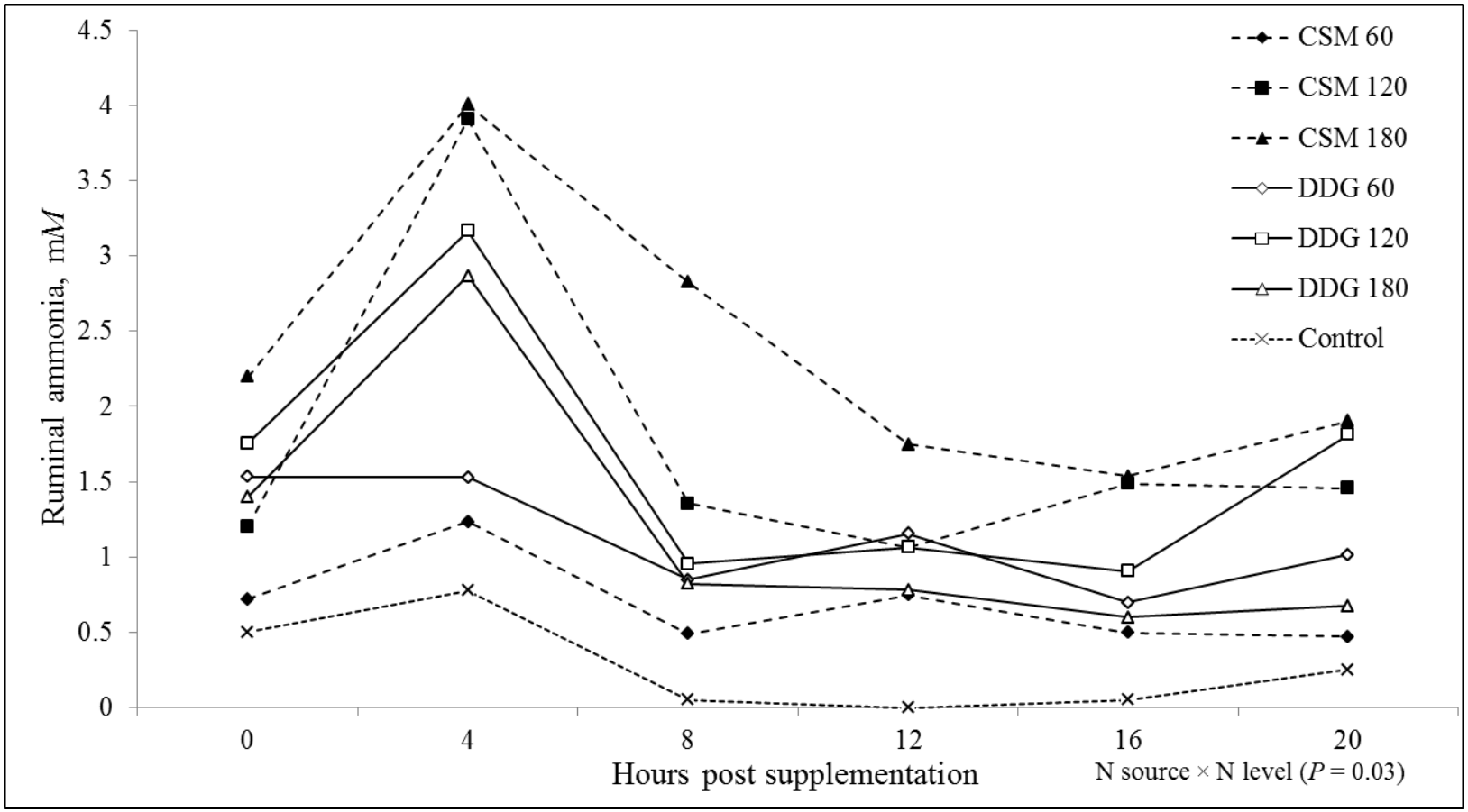

Figure 7. Effect of increasing amounts of cottonseed meal or dried distillers' grains on ruminal ammonia concentration. CSM 60 = 54 mg N/kg of BW; CSM 120 = 108 mg N/kg of BW; CSM 180 = 162 mg N/kg of BW, DDG 60 = 59 mg N/kg of BW, DDG 120 = 119 mg N/kg of BW, DDG 180 = 178 mg N/kg of BW, Control = 0 mg N/kg of BW; Treatment × hour ($P < 0.01$); SEM = ± 0.45.

Discussion

Protein supplementation increased TDOMI as expected and in agreement with previous work (Mathis et al., 1999; Rambo, 2010; Winterholler et al., 2012). Cann et al. (1991) observed a 108% TDOMI increase for steers consuming rice straw with a soybean meal-based supplement compared to an isonitrogenous urea-based supplement. Although urea supplementation did not increase TDOMI of sheep consuming rice straw, addition of molasses to urea supplement increased TDOMI by 117% compared to an unsupplemented control (Maeng and Chung, 1989). Although a linear increase in TDOMI was due to the linear increase in FOMI and OM digestion, protein supplementation levels were insufficient to elicit a quadratic intake response as reported by others (Köster et al., 1996; Mathis et al., 1999; Klevesahl et al., 2003). Steers receiving DDG had greater TDOMI responses because of the additional provision of supplement required to achieve congruent N levels and pre-experimental overestimation of CSM CP content. Supplemental DDG was provided at 175% OM of CSM and corresponded to greater total OM intake as well as OM digestibility for DDG treatments. Although response to DDG supplementation resulted in greater TDOMI, similarity in FOMI and forage NDF digestion suggests forage utilization was similar between CSM and DDG supplementation. In spite of their divergent DIP concentrations (82.9 vs. 43.6%), CSM and DDG supplementation increased FOMI suggesting ruminal available N was supplied via different mechanisms. Supplementation of CSM has been shown to increase intake, IVDMD, and solid passage rate of low-quality forage (McCollum and Galyean, 1985). Loy et al. (2007) and Rambo (2010) did not observe an increase in

FOMI in steers consuming forage supplemented with DDG, but the basal forage for each study was 7.4 and 8.2% CP respectively. Infusion of undegradable intake protein (**UIP**) has been demonstrated to increase FOMI quadratically in cattle consuming forage containing 4.7% CP (Wickersham et al., 2008). However, supplemental UIP is not as effective as DIP at increasing forage utilization (Bandyk et al., 2001; Wickersham et al., 2004) because the process is not 100% efficient and requires energy to form urea. *Bos indicus* cattle may have an increased ability to recycle N (Hunter and Siebert, 1985; Hennessy et al., 2000) and the similar FOMI response to CSM and DDG supplementation may support the possibility of increased N conservation by Brahman cattle.

Depressed forage intake on the 120 mg N/kg BW treatment relative to the 60 mg N/kg BW treatment appears to be an aberration within the data and a potential consequence of an incomplete treatment arrangement.

Protein supplementation increased diet digestibility similar to prior observations feeding prairie hay to beef cattle (Köster et al., 1996; Bandyk et al., 2001) and feeding rice straw to swamp buffalo (Wanapat and Pimpa, 1999; Nguyen et al., 2012). While equal levels of DIP and UIP increased OM digestion 116% above unsupplemented control (Bandyk et al., 2001), supplementation of 180 - 720 g DIP increased OM digestion from 44.6 to 53.4% averaged across supplemented treatments (Köster et al., 1996). Moreover, addition of urea to isonitrogenous protein supplements increased OM digestion of rice straw from 58.8 to 66.4% (Nguyen et al., 2012). Their results suggest rumen microbes responded to greater available N and increased forage utilization.

Although NDF digestibility increased with CSM and DDG supplementation from 49.6% (control) to a maximum of 54.9% (DDG 180 mg N/kg BW), estimated forage NDF digestibility was not affected by protein supplementation as it accounted for digestible NDF in CSM and DDG. Significant changes in forage NDF digestibility may have been not been observed due to reciprocal dynamics of passage rate and digestion (Robinson et al., 1985; Hoover, 1986).

Steers on the control diet had the lowest ruminal ammonia concentrations similar to other studies for unsupplemented cattle consuming low-quality forage (Hannah et al., 1991; Lintzenich et al., 1995; Bandyk et al., 2001). Ruminal ammonia concentrations were similar overall between CSM and DDG, but Figure 6 demonstrated the highest concentrations were for CSM 120 and 180 mg N/kg BW. Considering concentrations at 4 h was similar for 120 mg N/kg BW and 180 mg N/kg BW for CSM and DDG, peak concentrations may have been occurred prior to sampling. Average ruminal ammonia concentrations were much lower than reported optimal level for ruminal fermentation of 3.6 m*M* as classically observed by Satter and Slyter (1974).

Ruminal total VFA concentrations were unaffected by CSM and DDG supplementation with an average of 89 m*M* which is similar to other published values (McCollum and Galyean, 1985; Nguyen et al., 2012). Steers receiving increasing levels of DDG supplementation had decreased acetate:propionate expressed as molar proportions.

CHAPTER IV

CONCLUSION

The primary purpose of this experiment was to determine the effects of increasing CSM and DDG supplementation on utilization of rice straw by Brahman cattle. Intake, digestion, and ruminal ammonia responded linearly to CSM and DDG supplementary N and were maximized at180 mg N/kg BW treatment level. Total digestible OM intake was greater for DDG supplementation due to greater provision to achieve isonitrogenous treatment levels. Overall, results suggest protein supplements with divergent levels of degradable intake protein are effective in increasing utilization of rice straw. Brahman cattle can effectively use rice straw as a low-cost roughage with protein supplementation.

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