METAGENOMICS OF THE BOVINE RUMEN WITH DISTILLER'S GRAINS

A Dissertation

by

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ABSTRACT

The collective prokaryotic diversity in the rumen was examined by performing a meta-analysis of the 16s rRNA gene sequences available. This included 34 studies as of December 2016, with 22,003 OTUs, representing 68 phyla and 788 genera. A strong clustering of the rumen microbiota by study suggesting that technical differences between laboratories cause significant differences in the observed diversity. However, some factors produced sufficient changes in the gut microbiota to influence clustering patterns including genus of animal, fraction of the rumen, sample collection method, primer type, and diet. Regardless of experimental factor, the samples representing the solid or fiber fraction of the rumen separated from the liquid fraction.

The diversity of the rumen microbiome allows producers to feed many agricultural co-products that otherwise have limited value and would be discarded. One such product is distillers' grains, which have been utilized by the U.S. beef cattle industry for decades. However, few studies have examined the effect of these co-products and dietary changes on the rumen microbiome. Our first microbiome study with co-products included a comparison of *Bos indicus* and *Bos taurus* fed distillers' grains. Characterization of the rumen bacterial populations and their function has provided insights into these differences and the response to distillers' grains supplementation. Differences in relative abundance in key bacterial taxa were observed across species. Our second metagenomic study is focused on elucidating the effect of corn-based, sorghum-based, and treated sorghum-based distillers' grain diets in finishing feedlot

diets on the rumen microbial ecology and function. Both studies found limited dietary influence on the rumen microbiome as based on beta diversity metrics; however, diversity was greatest in a lower quality diet than the high-quality finishing diet. The effect of fraction was greatest in the microbial populations within the confines of a low-quality diet and lessened with a high-quality diet. Individual animal or host effect was a large influence on differences across samples in both studies.

DEDICATION

To Sybil Anne Sheets for helping me walk more slowly

Science, as you know, my little one, is the study
of the nature and behaviour of the universe.
It's based on observation, on experiment, and measurement,
and the formulation of laws to describe the facts revealed.

In the old times, they say, the men came already fitted with brains
designed to follow flesh-beasts at a run,
to hurdle blindly into the unknown,
and then to find their way back home when lost
with a slain antelope to carry between them.
Or, on bad hunting days, nothing.

The women, who did not need to run down prey,
had brains that spotted landmarks and made paths between them
left at the thorn bush and across the scree
and look down in the bole of the half-fallen tree,
because sometimes there are mushrooms.

Before the flint club, or flint butcher's tools,
The first tool of all was a sling for the baby
to keep our hands free
and something to put the berries and the mushrooms in,
the roots and the good leaves, the seeds and the crawlers.
Then a flint pestle to smash, to crush, to grind or break.

And sometimes men chased the beasts
into the deep woods,
and never came back.

Some mushrooms will kill you,
while some will show you gods
and some will feed the hunger in our bellies. Identify.
Others will kill us if we eat them raw,
and kill us again if we cook them once,
but if we boil them up in spring water, and pour the water away,
and then boil them once more, and pour the water away,
only then can we eat them safely. Observe.

Observe childbirth, measure the swell of bellies and the shape of breasts,
and through experience discover how to bring babies
safely into the world.

Observe everything.

And the mushroom hunters walk the ways they walk
and watch the world, and see what they observe.
And some of them would thrive and lick their lips,
While others clutched their stomachs and expired.
So laws are made and handed down on what is safe.
Formulate.

The tools we make to build our lives:
our clothes, our food, our path home…
all these things we base on observation,
on experiment, on measurement, on truth.

And science, you remember, is the study
of the nature and behaviour of the universe,
based on observation, experiment, and measurement,
and the formulation of laws to describe these facts.

The race continues. An early scientist
drew beasts upon the walls of caves
to show her children, now all fat on mushrooms
and on berries, what would be safe to hunt.

The men go running on after beasts.

The scientists walk more slowly, over to the brow of the hill
and down to the water's edge and past the place where the red clay runs.
They are carrying their babies in the slings they made,
freeing their hands to pick the mushrooms.

By Neil Gaiman

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CHAPTER I

INTRODUCTION

The symbiotic microbiota of the ruminant affects the animal's health and performance including, but not limited to pathogen load, immune function, toxin degradation, and greenhouse gas production. Despite, its importance the scientific community is still debating what controls the diversity and richness of the rumen microbial ecosystem. To explore the multitude of controls, 16s rRNA gene microbiome datasets were compiled into a meta-analysis along with the incorporation of the metadata for each experiment and test how the following factors may influence the overall microbial diversity and community structure of the rumen: 16s RNA gene target region (primer design), collection methodology, industry, DNA extraction technique, sequencing technology, diet, ruminant species, and fraction.

It is hypothesized that diet is the major driving force of the microbial ecosystem diversity, relative abundance, and function in the rumen, however other have theorized that individual animal is the greater determinant. This is explored via two microbiome datasets both of which incorporate distiller's grains into the diet of beef steers. Distiller's grains are a coproduct feed from ethanol production that are used as cost-effective feed supplements for primarily cattle. Research on the animal level has demonstrated that distillers' grains are an effective feedstuff or supplement leading to increases in average daily gains and other performance metrics. However, despite the importance of the

rumen microbiome and distillers' grains to the beef cattle industry limited research has been done demonstrating the effect of one on the other.

Bos indicus (Bi) typically perform better than *Bos taurus* (Bt) when consuming a low-quality diet; however, the response to supplementation is generally greater in Bos taurus. The underlying mechanisms supporting these responses have not been elucidated. Characterization of differences in rumen bacterial populations and their role in the two subspecies may provide functional and metabolic insights. Advent of next-generation DNA sequencing technologies has opened the door to explore the heretofore-unknown complexity and diversity of the rumen gastrointestinal tract. Prior to 2005, rumen microbiome research depended on culture based or first-generation low throughput technologies. As a result, there have been a limited number of publications on the rumen metagenome of Bt and none for Bi. Obviously, there have been no comparative rumen metagenome studies among these breeds. Chapter II provides metagenomic characterization and quantification of rumen bacterial ecosystems between Bt and Bi fed a low-quality forage diet with divergent levels and type of supplemental protein and degradability. Our approach will be to use the 16S rDNA model employing the Roche 454 pyrosequencing platform.

Relatively few evaluations have focused on distillers' grains products from sorghum feedstocks. The few that exist have generally shown that distillers grains from grain sorghum feedstocks have a lower feeding value (based on growth performance) than distillers grains corn-based feedstocks (Owens, 2008; MacDonald,

2011). Chemical treatment of roughages (alkaline base such as CaO, CaOH, NaOH) improves digestibility and consumption by beef cattle (Klopfenstein, 1978; Sundstol, 1988). However, it is unknown if and what the subsequent response to the rumen microbiome will be. Chapter III will examine the diversity, microbial diversity, and function of the rumen microbes under corn- and sorghum-based and treated sorghum distillers' diets using Whole Genome Shotgun (WGS) sequencing.

Therefore, the purpose of these studies aims towards elucidating the effect of distillers' grains, breed, time, fraction, pre-treatment, and protein type on the bacterial population metabolic potential and diversity. This study will generate high quality rumen metagenomic datasets centered on hypothesis driven studies. For the past ten years, many researchers have working on analyzing sequencing data from rumen samples; however perhaps because of a need for better sequencing depth, computation, or databases, they have for the most part been unable to integrate this data into the biology of the rumen or the production capacity of the ruminant. To successfully move forward in this field, we need to connect the rumen microbiome to the nutrition and metabolism of the animal as a whole in order to improve real world impact.

This work will help improve the foundation on which other researchers can utilize. Inclusion of the *Bos indicus* bacterial population data into the databases will help expand our understanding of the core microbiome of the ruminant likewise for the meta-analysis of the rumen microbiome presented in Chapter II. Along the same lines, there is

a lack of datasets of the rumen under low-quality forages, it is important to know how the rumen is impacted under substandard conditions as compared to high-quality diets.

CHAPTER II

META-ANALYSIS OF THE RUMEN MICROBIOME OF RUMINANTS USING 16S rRNA GENE BASED HIGH-THROUGHPUT SEQUENCING

Introduction

Until the 1980s, rumen microbiology was based on culture-dependent techniques. The advent of 16s rRNA gene-targeted analysis allowed the detection and enumeration of microorganisms without the bias of culturing (Stahl *et al*., 1988). This high-throughput sequencing enabled a larger breadth of understanding and insight into the microbial ecology of the rumen by capturing the rumen microorganisms that had previously eluded cultivation.

However, this new technology is not without its limitations. The wide variety of steps and methods used to generate and analyze the microbial population introduces a high level of bias and makes comparisons across studies problematic. For example, previous studies have found that the choice of primer location within the 16s rRNA gene had a greater influence on prokaryote community composition than the treatment or fraction (Pitta *et al*., 2014 a; Pitta *et al.,* 2014 b). Similarly, DNA extraction and sampling method of rumen samples were also found to alter overall microbial community structure (Henderson *et al*., 2013). Keeping all methods the same, diet has been found to be the single greatest influence over rumen microbial diversity, even more so than animal species (Henderson *et al*., 2015). Under this meta-analysis's search

criteria, as of December 2016, 72 studies on the rumen microbiome using the 16s rRNA genes have been reported. Therefore, this field of study may have reached a point where comparison across studies is feasible; thereby enabling insights into whether the 16s RNA gene target region, DNA extraction technique, or other user-based criteria obscures or outweighs biologically meaningful differences.

In addition, individual studies tend to have a narrow scope in terms of animals sampled, diets, species, and time points; but collectively, studies have a large range of experimental conditions tested. This, combined with an often-limited depth of coverage, has perhaps led to an incomplete view of the rumen microbiome within individual studies. Therefore, the true prokaryotic diversity of the rumen might better be viewed through the usage of meta-analysis or pooled data. A similar meta-analysis currently exists of the rumen microbiome (Kim *et al*., 2011). This analysis was performed on the curated 16s rRNA gene sequences; however, it was limited to the Ribosomal Database Project (RDP) database and did not include metadata. Likewise, Edwards *et al*. (2004) pooled and analyzed the three published bacterial 16s rRNA gene rumen libraries available at the time (Whitford *et al*. 1998; Tajima *et al*. 1999, 2000), however this was based on clone libraries, rather than pyrosequencing data. Due to the limited number and size of meta-analyses to date, this study represents an important step in the integration of new studies and analysis of improved databases.

With this study, we have compiled 16s rRNA gene based sequences of the rumen with the goal of providing a census of the total ruminal prokaryotic population or pan-

microbiome. In addition, we wish to incorporate the metadata of each experiment and test how the following factors may influence the overall microbial diversity and community structure of the rumen: 16s RNA gene target region (primer design), collection methodology, industry, DNA extraction technique, sequencing technology, diet, ruminant species, and fraction.

Methods

As of December 2016, the following databases were used to collect the studies that included an *in vivo* analysis of the rumen microbiome, utilizing the 16s rRNA gene from ruminant species: CAB abstracts, AGRICOLA, Proquest Dissertations and Theses, Proquest Biological Sciences, SCOPUS, and PubAg were searched using the inclusion string: microb* or metagen* and rumen* and 16s or 454. This resulted in 34 datasets included in the downstream analysis after three exclusion criteria and the availability of data was considered (Figure A1).

The raw files were obtained and metadata files were generated either from the body of the text or from the authors. The data was then processed using the default settings in the Quantitative Insights Into Microbial Ecology (QIIME) analysis pipeline (1.8.0). Quality filtering consisted of rejecting reads <200 nucleotide (nt) and >1000 nt, excluding homopolymer runs >6 nt, accepting 0 barcode corrections and 0 primer mismatched. Because the regions of the 16s rRNA gene differed between studies, a closed or reference-based approach was utilized, enabling us to avoid denoising the data. Operational taxonomic units (OTUs) were selected at 97% pairwise identity using the

latest release (13_8) of the GreenGenes taxonomy reference. Data that did not have ≥97% identity to any of the reference sequences in the GreenGenes database were not assigned to OTUs, and thus not further considered in these analyses. GreenGenes was also used to calculate weighted (abundance based) and unweighted (presence/absence based) UniFrac distances between communities. Principal Coordinates Analysis (PCoA) was applied to the distance matrices for visualization. Taxa summaries at the phyla and genus level were performed using the RDP classifier that were trained on the GreenGenes 97% reference data set using QIIME. The 16s rRNA data, and all of the metadata used to conduct this analysis, were from previously published studies and are available for download in the publicly accessible QIIME database (Qiita.ucsd.edu), and are summarized in Table 1.

Results and Discussion

A standardized bioinformatics pipeline was utilized (QIIME 2.0) as taxonomic results are strongly database dependent; for example the Firmicutes/Bacteroidetes ratio varied up to tenfold depending on the database utilized (Tanca *et al*., 2016; Manor and Borenstein, 2017; Siegwald *et al*., 2017). Since methodological steps pre- *in silico* varied greatly across different studies, an inherent difficulty in this study is determining authentic patterns in the rumen microbiome and experimental parameters. This paper collected the 16s rRNA gene datasets from a wide range of ruminants and pseudo-ruminants across diets, methodology, and life-stages in both production and wild environments, including 95 breeds of ruminants representing 25 genera and 36 species.

Their collective microbiome included 22,003 OTUs across 1,176 samples with an average of 3,087 sequences per sample. A total of 77 Phyla and 1,675 unique OTUs at the highest resolution were observed.

Firmicutes were the dominant phyla detected at 46.5% ± 23.5 followed by Bacteroidetes (33.1% ± 19.8) and Proteobacteria (11.6% ± 17.3), with an additional 74 prokaryotic phyla detected, representing 8.1% relative abundance (Figure A2). Diet may be a contributing factor for the large standard error, as Firmicutes are associated with forage, whereas Bacteroidetes are associated with grain (Fernando *et al*., 2010). Another source of variation is DNA extraction methodology, which has been documented many times to create biase for, or against, certain taxa with different methods sharing only 29.9 - 52.0% of the total OTUs recovered (Henderson *et al*., 2013; Fliegerova *et al.*, 2014; Brooks *et al*., 2015; Vebø *et al*., 2016; Keisam *et al*., 2016). Interestingly, there appeared to be just as much observed variation in relative abundance when looking at the genus host *Bos* alone; 30.7% Firmicutes ± 29.5, 24.1% Bacteroidetes ± 25.1, and 9.7% Proteobacteria ± 18.6. Therefore, animal taxa may not be substantially influential under this scenario. Three archaea phyla were detected: Euryarchaeota (1.26% ± 0.011), Crenarchaeotia (0.09% ± 0.006), and Parvarchaeota (0.05% ± 0.006). The archaea standard error was relatively smaller as compared to bacterial populations, indicating that these populations may not be as sensitive to diet, DNA extraction methodology or other experimental parameters.

Of the top 40 most abundant genera, as determined by GreenGenes (Table A1), 27 were found in 100% of the datasets examined, and 34 were found in 90%. Henderson *et al*. (2015), under the Global Rumen Census project, found *Prevotella*, *Butyrivibrio*, and *Ruminococcus,* as well as unclassified *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidales*, and *Clostridiales,* to be the seven most abundant genera (Henderson *et al*., 2015). Likewise, we found *Prevotella*, unclassified Clostridiales, *Ruminococcus*, unclassified Lachnospiraceae, unclassified Bacteroidales and *Butyrivibrio* to be in common with our findings, alternatively, the seventh most abundant microbe in our study was found to be *Bacillus*. Of the top 40 genera, 23 did not have representatives at the genus level within the culture database (% similarity to culture strain less than 95%). Most genera were not represented by cultures that belong to the same species, indicating that there is a need for more work to be done on the microbiological side to understand the rumen ecosystem. The Hungate 1000 project (www.hungate1000.org.nz) represents part of this effort.

Study parameters as a whole were the primary determinants of the rumen microbiota. Collection methodology and industry (grouped by dairy, milk, fiber, dual usage, and wild animals) appear to have some influence on beta diversity (Figure A3b and c). However, previous studies have found sampling methodology (rumen cannula vs. esophageal tubing) to not significantly influence the rumen microbiome (Lodge-Ivey *et al*., 2009; Paz *et al*., 2016). The influence of industry was surprising given the wide range of ruminants included in each category. For example, dairy included species of

goat, camels, water buffalo, cows, and sheep. This may be a reflection of diet as animals consuming TMR (Total Mixed Ration) were observed to have 88 unique OTUs, second only to pasture based diets with 418 (Figure A4b). On a principal coordinate analysis (PCoA) level, there was no spatial separation by primer or DNA extraction. It is well established that primer choice does bias for, or against, certain taxa (Pitta *et al*., 2014a; Myer *et al*., 2016; Fischer *et al*., 2016). The lack of differences in UniFrac distance measurements may be due to confounding variables surrounding study methodological choices such as DNA extraction, collection methodology, sample handling, or sequencing technology. It is worthwhile to note that V1-V3 region was the most popular primer region constituting 50% of all studies and QIAGEN kit for DNA extraction (35%). Future studies may want to consider utilizing these techniques in an effort to make rumen 16s rRNA gene experiments more comparable. Fraction, as expected, had a noticeable effect on spatial heterogeneity of the prokaryotic rumen microbiome (Figure A3d). As a whole, it is unclear what is causing the long tail on PC axis 1; the sample points do not have any experimental design facets in common, except for being wild animals, but not of the same breed or genera.

Across all datasets, there are 142 OTUs common with the five predominant ruminant genera studied (Figure 4a), which could be considered the core bacterial microbiome. Despite their ubiquity, 42% of these taxa do not have representatives in culture collections at the genus level or lower. *Bos* and *Ovis* have the highest number of unique OTUs, which is likely a result of more rigorous sequencing. There were only 9

taxa shared across all ruminant and pseudo-ruminant genera including *Prevotella*, S24-7 (family), Clostridiales (order), Lachnospiraceae (family), *Butyrivibrio*, *Coprococcus*, Ruminococcaceae (family) and *Ruminococcus.* Notable absences include *Fibrobacter* in Odocoileus (a deer genus), *Succiniclasticum* in Rupicapra (a goat-antelope genus), and *Clostridium* in Dama (a deer genus).

One aspect not reported on in this study is the preservation method utilized after sample collection. Many studies do not report sample treatment and handling post-collection such as rumen fluid handing (cheesecloth squeezed, nylon bag, centrifuged, or filtered), time at room temperature, potential oxygen exposure, storage temperature and cryoprotectants. Even low levels of oxygen can change the rumen microbial ecosystem, metabolite formation, taxa composition, and metabolism (Hobson and Stewart, 2012) Therefore, it is reasonable to conjecture that superfluous headspace or exposure to oxygen could also alter the results of 16s rRNA gene sequencing. Other studies have documented bias that has been introduced via rumen fluid treatment, as well as DNA preservation method and time spent in the freezer (Mitchell and Takacs-Vesbach, 2008; Contreras *et al*., 2010; Fliegerova *et al.*, 2014; Granja-Salcedo *et al*., 2017). This bias presents an extra difficulty in making studies comparable, in addition to other experimental parameters such as primer design, DNA extraction, collection methodology, and sequencing.

Annotation of sequencing data depends on databases that are known to be weak in rumen microbial entries (Wallace *et al*., 2017). Projects such as this, along with the

Hungate 1000 project and Global Rumen Census initiatives, are essential to improve the interpretation of sequence information, allowing for a more robust analysis. This information needs to be partnered with greater transparency, such as the efforts of alltrials.net and responsibledatascience.org, as nearly half of all rumen 16s rRNA data is not available publicly, and may be lost. This study, as it includes the full diversity of the available 16s rRNA gene based ruminal microbiome, may serve as a guideline for the design of future studies on rumen nutrition, and provide a framework to assess the significance of individual populations in the rumen. Future studies may consider comparing this dataset with 16s rRNA gene sequencing of *in vitro* populations, and those similar to Ziemer *et al.* (2000). Conducting a similar analysis of datasets using whole genome shotgun sequencing of the rumen microbiomes would be beneficial as well. Raw sequence and metadata can be found at Qiita.ucsd.edu (study ID: 10866). This platform will allow reanalysis of this dataset, and any additions, using the latest analytical technologies. It is the hope of the authors that this study will act as a springboard for further analysis of the subject, and the collaboration of rumen 16s rRNA gene data.

CHAPTER III

RESPONSES IN THE RUMEN MICROBIOME OF *BOS TAURUS* AND *INDICUS* STEERS FED A LOW-QUALITY RICE STRAW DIET AND SUPPLEMENTED PROTEIN

Introduction

Comparative studies of digestion and metabolism indicate *Bos taurus* are more responsive to protein supplementation while *Bos indicus* maintain greater levels of performance when consuming a low-quality diet (Habib *et al*., 2011). Protein is fractionated by its availability to the microbes and the host. Degradable intake protein (DIP) is used directly by microbes and is the most direct means of supplying nitrogen to the ruminal ecosystem. Undegradable intake protein (UIP) escapes the rumen to enter the small intestine, thereby increasing protein flow to the animal, resulting in improved forage utilization (Bandyk *et al*., 2001; Wickersham *et al*., 2009); indirectly, UIP can be made available to ruminal microbes by being metabolized to urea in the liver and subsequently be recycled to the rumen (Wickersham *et al*., 2004).

The underlying causes of sub-species differences in response to protein supplementation and consumption of low-quality diets remain unclear, but has been variously attributed to differences in energy requirements (Frisch and Vercoe, 1984), ruminal retention time, urea recycling capacity, and fermentation rate (Hunter and

Siebert, 1985). Additionally, we hypothesize that differences in composition and function of the rumen microbiome may contribute to these sub-species differences. To test these hypotheses, we conducted a study on *Bos taurus* and *indicus* fed a very low-quality rice straw hay with increasing levels of protein supplementation either DIP or UIP to identify shifts in the rumen microbial community using the 16s rRNA gene and 454 pyrosequencing technology. Developing a better understanding how the rumen microbiome adapts to the effects of source (UIP versus DIP) and level (low versus high) protein supplementation when fed low-quality forage could enable improved diet formulation, facilitate new probiotics discovery, and enhance our basic understanding of nitrogen metabolism.

Materials and Methods

The experimental protocol was approved by the Institutional Animal Care and Use Committee at Texas A&M University.

Experimental design

Five Angus steers (initial BW = 303 ± 10 kg), subspecies *Bos taurus,* and 5 Brahman steers (initial BW = 323 ± 28 kg), subspecies *Bos indicus,* were fitted with ruminal and proximal duodenal cannulas. Steers were then used in concurrent 5 × 5 Latin square experiments. Each steer received a subcutaneous vitamin injection (3 mL/animal; Vitamin AD Injection, Sparhawk Laboratories, Inc., Lenexa, KS) at the onset of the trial to prevent deficiencies. They were provided *ad libitum* access to fresh water and a trace mineral-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 (4.7% CP, 73% NDF) was processed through a tub grinder to facilitate feed delivery and was provided at 130% of the average intake of the previous 4-d.

Treatments consisted of an un-supplemented control (0 mg of N/kg BW daily) and isonitrogenous supplements: 2 levels of high UIP and 2 levels of low UIP (50 and 150 mg of N/kg BW daily). High UIP was 100% distillers' grains (27% CP, 74% UIP, and 88% TDN), while low UIP consisted of soybean meal, corn oil, urea, and soyhulls (27% CP, 74% DIP, and 88% TDN; Table 1). Treatments were given for 15 d periods, with a 9 d adaptation period in between treatment changes.

Microbiome sampling

On d 15 of each treatment period rumen contents were collected at h 0 and 4 after feeding for 16s rRNA gene analysis. Rumen contents were strained through 4 layers of cheesecloth to separate liquid and solid fractions. Samples were transferred into a 15-mL polypropylene centrifuge tube and snap frozen in liquid nitrogen. The samples were then transported to the laboratory and archived at −80°C. The DNA was extracted using the QIAamp stool DNA mini kit (Qiagen, Valencia, CA). Amplification of the V4-V6 segment of the 16S rRNA gene was conducted with barcoded primer tags and the universal eubacterial primers 530Fand 1100R, as previously described (Dowd *et al*., 2008). 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 analysis

Sequencing data was processed using the QIIME 1.9.0 pipeline (Caporaso *et al*., 2010) under default parameters. Sequences were depleted of barcodes and primers, then sequences with <150bp, ambiguous base calls, homopolymer runs exceeding 6bp were removed. Sequences were then de-noised and truncated using Denoise Wrapper and AmpliconNoise, with chimera removal at the average base pair quality score <25 (Caporaso *et al*., 2010; Quince *et al*., 2011). Operational taxonomic units were clustered using UCLUST at 97% similarity (Edgar, 2010). The Greengenes database (5.13) with PyNAST was used to align cluster representative sequences (McDonald *et al*., 2012; DeSantis *et al*., 2006). Singleton OTUs were removed. Taxonomic classification was performed using RDP Classifier (Wang *et al*., 2007). Samples were standardized to the lowest sequencing depth level (2,400 sequences per sample), then alpha, beta, and OTU richness was calculated. UniFrac-based Principle Coordinate Analysis (PCoA) was conducted to visualize the grouping of similar microbiome environments.

Resulting OTUs were then inputted into PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), http://picrust.github.com/picrust) (Langille *et al*., 2013). Functional predictions were made following the microbiome workflow described by the developers.

Statistical analysis

Relative abundances of prokaryotic taxa were analyzed with PROC MIXED on SAS 9.4. Terms included in the model were treatment, sub-species × treatment, fraction × treatment, time × sub-species, time, time × treatment, sub-species × fraction, sub-species, and treatment. Period and animal were random effects. Treatment and breed means were calculated using LSMEANS. Student's T-test, ADONIS, and Bonferroni were used to analyze differences between core microbiome/diversity indexes, PCoA, and functional predictions respectively.

Results

The 16s rRNA gene analysis of rumen contents generated a total of 2,229,152 reads from 200 samples (11,145 reads/sample), which were then used for downstream analysis, including a total of 97,826 operational taxonomic units (OTUs) at 97% similarity. These OTUs taxonomically separated into 26 phyla, 108 families, 255 genera, and 394 species. Some OTUs could not be assigned to the highest taxonomic resolution, in this case, the lower resolution assignment is reported along with the taxonomic rank in parentheses. Average sequence length for all sequences passing the quality filter was 499 bp. Diversity analyses included chao1 and Shannon indices, which did not differ (Table A2) across treatment, fraction, sub-species, or hour nor any of these interactions (Student's t-test: $P > 0.05$).

Core microbiome

Prokaryotic taxa ubiquitous across all samples were considered members of the core microbiome. There were 32 ubiquitous OTUs shared by all experimental treatments. Bacteroidales, particularly *Prevotella*, dominated this core microbiome. There were 361 unique OTUs that were shared across all experimental conditions, but were not found ubiquitously across all samples.

Thirty-six unique, but rare species were found in the solid fraction, but not the liquid fraction (Table A3). Collectively these 36 species accounted for 0.001% of relative abundance, and 9.1% of the total species diversity. Taxonomically, under the conditions of this experiment cattle subspecies *Bos taurus* and *Bos indicus* did not differ in their core microbiome (Student's t-test: $P > 0.05$). Across all OTUs, *Bos taurus* had 2 unique OTUs, while *Bos indicus* had 7 (Table A3).

Several unique species were associated with protein type and supplementation level (Table A3). However, similar what was observed with fraction, these taxa are found at very low abundance and not ubiquitously across each treatment. In samples collected at h 0 there were 32 rare, non-ubiquitous species compared to h 4 (Table A3).

PCoA analysis

Weighted and unweighted UniFrac PCoA exhibited separation of the prokaryotic community across the liquid and solid fraction (Figure A1). The solid fraction was more spatially heterogeneous based upon the unweighted and weighted PCoA (Figure A1). Separation was discernible with the solid and liquid fraction patterns (Figure A1). The

rumen bacterial community of *Bos taurus* displayed greater spatial heterogeneity compared to *Bos indicus* independent of weighting (Figure A1). Unweighted and weighted UniFrac distances indicated there was no difference in the rumen bacterial communities among diets (control, high or low UIP and DIP), and time (0 and 4 h) (ADONIS: Treatment $P = 0.052$, Time $P = 0.132$).

Effect on prokaryote taxa

Across all samples, *Bacteroidetes* and *Firmicutes* accounted for 65 and 28% of total bacterial abundance, respectively. An additional 24 phyla were identified. The top 10 in descending order of mean abundance for all samples were: *Bacteroidetes, Firmicutes, Spirochaetes, Fibrobacteres, Proteobacteria,* TM7*, Tenericutes, Chloroflexi, Actinobacteria, and Synergistetes* (Table A4). Additional phyla cumulatively accounted for less than 5% of observed sequences. The most abundant 20 bacterial families are listed in table A6 and the top 20 genera, representing 85% of the total microbiome, are listed in table 6.

Fraction

Of the experimental parameters, the most consistent differences in the rumen bacterial community were seen when comparing the liquid and solid fractions. Four out of the five most abundant phyla differed significantly between fractions ($P < 0.05$; Table A7). Bacteroidetes and Tenericutes were more prevalent in the liquid fraction. Firmicutes, Spirochaetes, Proteobacteria, Chloroflexi, Actinobacteria, and Synergistetes were all more abundant in the solid fraction ($P < 0.001$). Fibrobacteres and Tm7 did not

differ between fractions ($P > 0.05$). No significant differences were observed in any Archaea populations ($P > 0.05$).

When families were examined, Prevotellaceae, Ruminococcaceae, Sphingobacteriaceae, Lachnospiraceae, Bacteroidales (Order), Bacteroidaceae, Porphyromonadaceae, Spirochaetaceae, Erysipelotrichaceae, Veillonellaceae, Clostridiaceae, Leuconostocaceae, Cryomorphaceae, and Rikenellaceae all differed in their abundance between the solid and liquid fractions ($P \leq 0.01$; Table A4). The relative abundance differed significantly between solid and liquid fraction for 15 out of the 20 top genera (*Prevotella,* Prevotellaceae (family), Sphingobacteriaceae (family), *Saccharofermentans,* Bacteroidales (order), *Bacteroides*, *Treponema*, *Erysipelotrichaceae*, *Succiniclasticum*, *Blautia*, *Clostridium*, *Fibrobacter*, *Ruminococcus*, *Butyrivibrio*, *Barnesiella*, and *Xylanibacter*) ($P \leq 0.002$; Table A6).

Cattle sub-species

Cattle sub-species influenced the relative abundance of 7 out of the top 20 predominant families and 9 out of the top 20 predominant genera (*Prevotella,* Ruminococcus (family), Sphingobacteriaceae (family), Bacteroidales (order), *Pontibacter, Bacteroides, Succiclasticum, Barnesiella,* and *Xylanibacter*) ($P \leq 0.05$; Table 6). The average percent difference between the cattle sub-species was 12% at the genus and family level. The greatest differences were observed in Sphingobacteriaceae (family) 15.3%, Bacteroidales (order) 38.9%, *Pontibacter* 47.4%, and *Succiniclasticum* 17.4%.

Treatment

Firmicutes responded protein supplementation treatments with a maximum of 8.1% difference observed between the control and high UIP (27.1% vs. 29.5%; $P = 0.02$), however at higher resolution taxa no differences were observed. The families Prevotellaceae ($P = 0.05$) and Ruminococcaceae ($P = 0.004$) and the genera Prevotellaceae (family) ($P = 0.003$), a decrease in relative abundance was observed with protein supplementation over the control, however these differences in treatment did not conform to linear or quadratic response contrast models ($P > 0.09$). No differences in diversity or relative abundance of methanogenic and pathogenic taxa were observed attributable to type or level of protein supplementation protein supplementation ($P > 0.05$).

Time

Four of the five most abundance phyla differed across time ($P < 0.006$; Table A7). Relative abundance of Bacteroidetes decreased from h 0 to h 4 post-feeding, while Firmicutes increased at h 4 post-feeding ($P < 0.006$; Table A7). Proteobacteria and Chloroflexi increased after feeding ($P < 0.006$; Table A7). The relative abundance of Fibrobacteres was not influenced by time ($P>0.05$). Of the 20 most abundant families, 8 families exhibited significant differences between pre- and post-feeding ($P \leq 0.01$; Table A6). At the genera level, *Prevotella* and Sphingobacteriaceae decreased, while Ruminococcaceae and *Treponema* increased at h 4 post-feeding ($P \leq 0.001$; Table A6).

These changes accounted for a range of 6.0-21.5% difference in relative abundance and averaged 6.6% (Table A6).

16s rRNA gene inferred functional pathways

PICRUSt was used to conduct a functional analysis of the samples based on the 16s rRNA gene by proxy. The predominant level 2 KEGG pathways were amino acid metabolism (11.0%), replication and repair (10.8%), and carbohydrate metabolism (10.3%; Figure A2).

The solid fraction showed greater relative abundance of level 1 KEGG pathways for cellular processes, environmental information processing, and metabolism (Bonferroni, $P < 0.05$). Similarly, all level 2-pathway assignments were greater in the solid fraction except for the transport and catabolism category (Table A7). When KEGG assignments were compared across sub-species, no differences were observed, as was the case for supplementation treatment (Bonferroni, $P > 0.05$). All level 2-pathways were more abundant at h 4 post-feeding compared to pre-feeding (h 0) (change from h 0 to h 4 >8%, Bonferroni $P \leq 0.05$).

Discussion

In this study, 16s rRNA gene sequences were used to characterize phylogenetic diversity and potential functional capability of the rumen microbiota of *Bos taurus* and *indicus.* To the best of our knowledge, no previous study has reported rice straw diet and protein supplementation dependent changes of the rumen microbes comparing these two sub-species of cattle. Rice straw is a unique low-quality forage in that it contains much

more silica (12-16%) compared to other straws, which typically have 3-5% silica (Jackson, 1977). Soluble silica in the rumen is known to inhibit cellulolytic digestion which leads to a depression in forage digestion, this is theorized to occur via a direct enzymatic depression by the silica and a reduction in the availability of minerals to the rumen microbes (Shimojo and Goto, 1989). Therefore, this study provides a better understanding of the rumen ecosystem comparing these two sub-species under a diet with low fermentation potential, which was previously lacking in the rumen microbial ecology literature.

The rumen core microbiome is the population of microbes that remain stable regardless of conditions. Deviations in and the size of the core microbiome may be indicators of changes in response to diet, time, host effect, or other experimental treatments (Li *et al*., 2012; Petri *et al*., 2013; McCann *et al*., 2014a; Omoniyi *et al*., 2014). For example, there were 32 OTUs across experimental parameters within the core microbiome of steers fed a low-quality rice straw diet in the current study. While, steers fed a higher-quality Bermuda grass diet were found to have 22 core OTUs (McCann *et al*., 2014a). Dairy cows fed a high-quality silage-based diet were found to have 26 unique OTUs (Li *et al*., 2012). Similarly, Petri *et al*. (2013) found 11 core OTUs in a high-grain diet and 38 in a high-forage diet. Likewise, Omoniyi *et al*. (2013) observed six core OTUs with goat fed a higher quality tree-based diet and 11 OTUs present in animals fed low-quality grass. The camel core microbiome consists of

approximately 746 OTUs, this study utilized very low-quality natural forages and woody shrubs (Gharechahi *et al*., 2015).

The effect of the low-quality, slowly fermentable rice straw diet was more pronounced in the solid fraction over the liquid fraction. As such, there was a total of 36 very rare, unique, non-ubiquitous species in the solid fraction that were absent in the liquid fraction similar to the results of (McCann *et al*., 2014b). This is best illustrated in the non-weighted PCoA, which exhibited greater spatial heterogeneity in solid fraction grouping as compared to the liquid. The solid fraction of rice straw diets represent a more biochemically diverse, nutrient dense and physiochemically stratified environment (Bae *et al*., 1997; Van Soest, 2006; Chen *et al*., 2008) than the liquid fraction; as such, there are an increased number of ecological niches available that can support greater taxonomic diversity and abundance. Correspondingly, there was an associated increase in metabolic pathway abundance, as reflected in the KEGG analysis (Table A7). The increase in OTUs in the solid fraction and across samples in low-quality diets compared to high-quality diets may be a natural consequence of a high forage or low-quality diet or could be a response to another feature of the diet such as an increase in secondary plant compounds or silica, which may support greater bacterial diversity (Bae *et al*., 1997; Van Soest, 2006; Chen *et al*., 2008).

While numerous significant differences across experimental parameters were found, the absolute change in the relative abundance of most bacterial taxa was modest, particularly in the context of the differences in protein type and levels among diets and

fractions. We had hypothesized that treatment, sub-species, and time would result in noticeable microbial community changes, however beta diversity metrics did not differ across these parameters (Table 2). Other studies examining the rumen microbiome with differing dietary conditions or protein supplementation have consistently found changes in relative abundance (>20%) and significant differences in diversity metrics across most taxa (Pitta *et al*., 2010; Fernando *et al*., 2010; Callaway *et al*., 2010). In retrospect, this is likely the result of the basal rice straw diet, which was very recalcitrant to digestion compared to previously reported straw and hay basal diets, with low carbohydrate diversity and CP, with associated elevated levels of indigestible silica, cutins and fiber, creating a matrix, which is resistant to initial microbial attachment and digestion (Jackson, 1977; Bae *et al* 1997; Van Soest 2006). Consequentially, total tract digestion (%) was low (52.8% Bi vs. 50.0% Bt, P =0.01) and no differences were observed in treatment (NRC, 2000; Weldon *et al*., 2013). These substrate attributes apparently limit attachment and biofilm consortia establishment to a comparatively narrow range of taxa. Thus, resulting in a more even bacterial community observed across all samples, in particularly the solid fraction, and effectively reducing the plasticity of the rumen microbial environment. As such, this diet likely selected for certain microbial community structures and functions, such that neither an increase in protein level or type, nor sub-species affectively altered the bacterial community. This is in line with previous studies also showing basal diet to be the major factor in influencing the microbiome (Carberry *et al*., 2012; Hernandez-Sanabria *et al*., 2012). Likewise, McCann

et al. (2014a) who similarly fed a low-quality oat straw diet and supplemented protein in the form of post-extraction algal residue did not distinguish PCoA separation with treatment.

A few significant differences were observed with dietary treatment. A high level of DIP supported the greatest number of rare species (15 unique, rare species in high DIP compared to 10 in control). This could be a reflection of DIP rumen availability improving rumen function and enhanced diversity as compared to no protein supplement or UIP, which largely escaped the rumen. Likewise, protein supplementation also appeared to increase the prevalence of mobility and chemotaxis pathways, which again indicates a response to the protein supplementation by rumen microbes. However, as previously mentioned, limited dietary effects on the relative abundance and diversity chances in bacteria taxa were attributed to the low digestion potential of rice straw.

Two of the greatest concerns to beef cattle producers and consumers is the safety of their product and the environmental repercussions (Galyean *et al*., 2011). This study detected no differences in potentially pathogenic taxa including *Salmonella*, *Escherichia*, and *Campylobacter* with the inclusion of distillers' grains, which previously had shown an increase due to the inclusion of certain distillers' grains (Jacob *et al*., 2007). This is in line with the work of Callaway *et al*. (2010) whom found no *E. coli* O157:H7 with increasing inclusion of dried distillers' grains. No differences were observed in the relative abundance or diversity in any methanogenic taxa. This was confirmed via

measurement of methanogen concentrations using quantitative PCR which showed no subspecies, treatment, or time differences observed (Bell, 2015).

Despite differing digestive phenotypes such as response to low quality feed and protein supplementation on the animal level, *Bos taurus* and *indicus* did not differ in the diversity of their core microbiome under our experimental conditions. While, *Bos taurus* has 2 unique OTUs and *Bos indicus* has 7, these OTUs do not represent novel taxa. This does contribute to the greater spatial heterogeneity in *Bos taurus* over *Bos indicus* reflected in the unweighted PCoA analysis, and why the weighed PCoA has no separation between the two biological types. There were no true diversity differences between the microbiomes of the two sub-species and dissimilarities are only seen in the relative abundance of some taxa on low-quality rice straw diets.

Other studies have cited large individual animal variation as a cause for a lack of treatment effects (Petri *et al*., 2012), in our experiment individual animals were seen to group together within fraction. The grouping of individual animals within sub-species and fraction may have been potentiated by the fact that the animals were previously housed in the same pens and had been co-enrolled in other studies (Bell, 2015; Weldon, 2013). Induction and transfer of microbial populations and function between groups of animals in adjacent pens has been documented (Majak and Cheng, 1984). Because these ten animals have interacted with each other so closely over long periods, it is possible that they may also have experienced a transfer of microbial function and diversity. This may also be a contributing factor to the lack of large treatment. Future studies comparing

sub-species of ruminants should utilize animals that did not share microbial populations through direct or indirect contact, however it will still be difficult to suss out the effect of individual animal phenotypes, location, and breed. However, the overall fermentation potential was so low in a rice straw diet that it likely masked experimental parameters by creating a nutrient limited environment that selected for a finite consortia of adapted rumen bacteria.

While, no significant differences in diversity were observed across sub-species, there were taxa that differed in their relative abundance between *Bos taurus* and *indicus*, which may have been modulated by certain ruminal host traits. For example, *Bos indicus* have greater rumen ammonia concentrations due to a faster digestion rate (Hunter and Siebert, 1985), and which was observed in this experiment as well (Weldon, 2013). It was been hypothesized that this may be partially due to their rumen bacteria being more efficient in utilizing dietary protein and recycled ammonia (Howes *et al*., 1963). Or physiological differences between sub-species such as ammonia concentration may select for changes in the abundance of bacterial taxa. In other words, it is unclear if the rumen microbiome is the cause or a consequence of differing rumen environments. However, due to the lack of data on the metabolic potential of most of the rumen microbe, it is difficult to associate individual taxa or functional groups with the physiological capacity of the animal. For example, *Pontibacter*, within the phylum *Bacteroidetes*, family *Cytophageae*, displayed large and significant sub-species differences (Table 6) with nearly double the relative abundance in *Bos taurus* as

compared to *Bos indicus*. However, nothing is known about its metabolic potential or role in the rumen, so we cannot posit on why or how it is more abundant in *Bos taurus*. Likewise, *Succiniclasticum* was significantly higher in *Bos taurus* (Table 6). This genus has members that exclusively derive energy from the conversion of succinate to propionate (Van Gylswyk, 1995). However, this was not reflected in the VFA profile as the molar percentage of propionate was not different across sub-species or treatments (Weldon, 2013). *Xylanibacter* was also significantly higher in *Bos taurus.* Although its function in the rumen is unknown, it is likely to be involved in the breakdown of plant polysaccharides (Li and Zhao, 2015). It has previously been established to be more abundant in the solid fraction, which was also found in this dataset (Petri *et al*., 2013). The genus *Barnesiella* was significantly higher in *Bos indicus*. Dietary starch is known to positively increase the relative abundance of *Barnesiella* in non-lactating Holsteins (Zened *et al*., 2013). It is unclear if the rumen of *Bos indicus* would create an environment more hospitable for this genus or other taxa via starch availability or other physiological differences across sub-species under current experimental conditions. Future studies could consider these taxa as probiotic candidate.

The majority of the most abundant taxa differed significantly between solid and liquid fractions, however these differences were relatively minor in terms of percent change with the exception of *Saccharofermentans*. *Saccharofermentans*, family *Clostridiaceae* phylum *Firmicutes*, were especially more abundant in the solid portion

compared to the liquid. This is in line with Petri *et al*. (2013), who found that these taxa were highly associated with a forage-based ration.

Prevotella was the most dominant genus across all communities and variables. *Prevotella*/Prevotellaceae were particularly sensitive to experimental conditions- changing significantly with sub-species, sub-species × treatment, fraction, fraction × treatment, fraction × sub-species, time × treatment, and time. *Prevotella* deceased in response to protein supplementation, was found in greater abundance in *Bos taurus* than *Bos indicus*, more abundant in the liquid fraction, and decreased between h 0 and h 4. Henderson *et al*. (2013) and Pitta *et al*. (2010) also found *Prevotella* to be more abundant in the liquid fraction. *Prevotella*/Prevotellaceae was found at a lower relative abundance in this dataset (30% on average) compared to other rumen microbiome analyses which reported upwards of 50% of the total bacterial abundance (Koike *et al*., 2003; Stevenson and Weimer, 2007; Bekele *et al*., 2010; Thoetkiattikul *et al*., 2013). Again, this is likely the result of the very low-quality rice straw diet and associated sub-ideal rumen available carbohydrate and protein.

Prevotella is a very diverse genus, as such; it is difficult to assign its metabolic strategies/roles (Edwards *et al*., 2004; Bekele *et al*., 2010). It is worth noting that *Prevotella bryantii* and *Prevotella ruminicola*, which dominate culture based research, only accounted for 0.6% and 3.8% of observed relative abundance respectively. Similarly, *P. bryantii* and *P. ruminicola* accounted for 0.0009% and 0.115% of total *Prevotella*, with unknown species accounting for over 80% of all observed diversity.

Previous studies showed *Prevotella* decreased with grain feeding and is more abundant in forage based diets, which may be related to sensitivity to pH (Mao *et al*., 2015). de Menezes *et al*. (2011) found *Prevotella* to be highest in a forage-based ration. However, if this were the case the relative abundance would be predicted to be greater in the solid portion. However, other studies have found the opposite trend, with *Prevotella* increasing with protein supplementation and a higher concentrate diet (Pitta *et al*., 2010; Callaway *et al*., 2010; Fernando *et al*., 2010; Thoetkiattikul *et al*., 2013; Pitta *et al*., 2014). Some members of *Prevotella* are highly associated with protein degradation, such as *Prevotella ruminicola*, which is one of the few groups that can degrade oligopeptides into amino acids. However, we observed a decrease in *Prevotella* and Prevotellaceae with both DIP and UIP protein supplementation. If *Prevotella ruminicola* were associated with a higher concentrate diet it would explain why the relative abundance was lower in our dataset compared to others. It is clear that the functional roles of *Prevotella* in the rumen will require additional studies as existing literature interestingly suggest that *Prevotella* are both cellulolytic and proteolytic (Edwards *et al*., 2004; Bekele *et al*., 2010).

Pre- and post-feeding had a significant impact on the majority of the most abundant phyla, but this trend was not reflected across many families and genera, or the community at large. Rather, only a few genera were observed to respond to time. This trend is reflected at the community level, i.e. unweighted and weighted PCoA, which does not show a significant grouping with h 0 and 4 post-feeding, indicating again that

only a few taxa responded to time. There was a decrease in some diversity metrics (chao1 and observed species) from time 0 to 4 h post-feeding, though this trend was not statistically significant. This is supported by the observation that more rare species were observed in the pre-feeding samples. The increase in abundance of KEGG pathways from h 0 to h 4 at first appears to conflict with the idea that feeding drops diversity. However, this may be an artifact of presenting data in terms of relative abundance. If there is a reduction in diversity then the relative abundance of the remaining pathways will be increased. The drop in diversity in all other metrics from h 0 from h 4 could be due to the underlying lack of readily fermentable carbohydrates and the consequential low metabolic potential, in addition, to the overall effect of dilution and rumen turnover.

Overall, it is apparent inhibition of microbial function and response is due to feeding rice straw, which was been previously established as very difficult to digest. As such, it was difficult to ascertain what, if any, effects the inclusion of increasing UIP or DIP at increasing levels of supplementation had on the rumen environment. This is supported by the cattle performance in that they lost weight over the course of the study (Weldon, 2013; unpublished data). It is reasonable to state in this study, a basal diet of rice straw was by far the dominant determining factor for the rumen microbiome composition and predicted function superseding the effects of host, protein supplementation, and time. While notable differences were observed between solid and liquid fraction prokaryotic diversity and prediction function, there were notable sub-species by treatment effects. The difference in relative abundance of taxa observed

across cattle sub-species may be a reflection of differing host rumen environments between subspecies *Bos indicus* and *taurus* such as their ability to utilize low quality forage and protein supplementation. Therefore, differences in breeds are likely a combination of the physiology and the bacterial populations, however more data is needed to connect taxa with host traits.

CHAPTER IV

MICROBIAL POPULATION AND FUNCTION IN THE RUMEN LIQUID AND SOLID FRACTIONS OF BEEF STEERS FED A CORN-BASED WET DISTILLERS' GRAINS, SORGHUM-BASED WET DISTILLERS' GRAINS OR A CAOH TREATED SORGHUM-BASED WET DISTILLERS' GRAINS FINISHING DIETS

Introduction

Distillers' grains are a co-product from ethanol production, which act as cost-effective, feed ingredient in cattle diet. As such they have become an integral supplement to the beef and dairy industries in the last 15 years. Research on the animal level has demonstrated that distillers' grains are an effective supplement leading to increases in average daily gain and other performance metrics (Klopfenstein *et al*., 2008). However, distillers' grains from sorghum feedstocks have a lower feeding value than corn-based distillers' grains (Owens *et al*., 2008; MacDonald, 2011). Chemical treatment of roughages with bases such as CaOH improves digestibility and consumption by beef cattle by breaking the cell wall matrix of hemicellulose and associated components, improving enzyme accessibility. We wanted to determine the changes in feeding value of CaOH-sorghum distillers' grains, sorghum distillers' grains and corn distillers' grains at 30% inclusion rates in SFC based finishing rations.

It is unknown if the improvement to ruminal digestion following CaOH treatment is due to a physiological response by the animal or an adaptive improvement by the

microbial population. We hypothesize that a 30% inclusion of chemically treated sorghum distillers' grains will result in a microbial population with a fermentative capacity more similar to that of the 30% inclusion of corn distillers' grains as compared to the untreated sorghum diet in SFC based finishing rations. To address rumen microbiome changes associated with CaOH treatment of SDG, we employed shotgun metagenomic sequencing, which facilitates the examination of the predicted functionality of the microbial population as well as taxonomic community composition. The study is paired with a companion whole-animal metabolism study thereby addressing knowledge gaps in host-microbiome interactions. Furthermore, because the field of rumen metagenomic is evolving and the rumen microbiome is still regarded as a 'black box;' it is unclear what effect the host has on the rumen and what level of phenotypic plasticity it has. For example, within animal groups receiving the same treatment, only 51% similarity across bacterial taxa in the rumen with regard to abundance and occurrence was found (Jami and Mizrahi, 2012) suggesting a high level of response to changes in the environment. Other studies have found greater level of similarity between samples from the same cow taken at different locations and time points than samples taken from different host animals within the same treatment (Li *et al*., 2009) highlighting the influence of host animal and low level of plasticity. Because of the repeated measure design, this study will provide further exploration on the influence and extent the host influences the microbiome and its associated plasticity.

Materials and Methods

All experimental protocols were approved by the West Texas A&M University-CREET Animal Care and Use Committee (approval # 03-01-14).

Six ruminally cannulated crossbred Angus steers (444 ± 4.0 kg of BW) were used in a replicated 3 × 3 Latin square design with 21 d periods consisting of a 17 d adaptation period followed by a 4 d collection period. Dietary treatments included steam-flaked corn finishing diet (57.1% ± 0.6) with either 30.0% ± 0.2 corn wet distillers' grains (CDG), sorghum (SDG), or calcium hydroxide treated sorghum WDGS (2.67% $Ca(OH)_2$ DM basis) (Supplemental Table 1). Sorghum stalks were included at 8.6±0.1% DM basis of all diets. Vitamins and minerals were provided to meet or exceed NRC (2000) requirements. Monensin and Tylosin were included at a rate to provide 22.2 and 5.0 mg/kg, respectively. Diets were offered once daily at 0700 in an amount to achieve *ad libitum* intake and water was offered on an *ad libitum* basis. Rumen samples were collected at 0600 and 1800 h on d 18 and 20, and at 1200 and 2400 h on d 19 and 21 strained through 4 layers of cheesecloth and immediately frozen. For analysis of animal performance, nutrient intake, and digestibility see Gentry, 2016.

DNA was extracted using PowerSoil DNA isolation kit (MoBio) following the manufacturer's user guide. Isolated genomic DNA was quantified and sample volumes were adjusted to achieve similar DNA concentrations (50 ng at a concentration of 2.5 ng/µl). Subsequently, the samples underwent the simultaneous fragmentation and addition of adapter sequences. These adapters are utilized during a limited-cycle (5

cycles) PCR in which unique index was added. Following the library preparation, the final concentration of the library was measured using the Qubit® dsDNA HS Assay Kit (Life Technologies), and the average library size was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies). The library was then pooled in equimolar ratios of 2nM, and 10.5pM of the library pool was clustered using the cBot (Illumina) and sequenced paired end for 300 cycles using the HiSeq 2500 system (Illumina).

Reads were analyzed using the metagenome rapid annotation subsystem technology (MG-RAST) server including removal of artificial duplicate reads, quality-based read trimming, length-based read trimming, and removal of bovine DNA. Reads that passed the quality filter were analyzed using the KEGG and M5nr subsystem for functional hierarchical and best hit classification where the maximum *e* value cut off and the identity cut off values were set as 1e-5 and 60%, respectively. Sequence data and information can be found in the MG-RAST server under the IDS 4665263.3 to 4665298.3. Statistical analyses were performed using PROC MIXED and ANOVA on SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Visualization of PCoA and Heatmaps were generated with R software (Team, 2014). QIIME was utilized for biodiversity metrics (Caporaso *et al*., 2010).

Results and Discussion

The primary aim of this study was to determine the influence of replacing 30% of the steam-flaked corn in a finishing ration with a) wet corn distiller's grains plus solubles (CDG), b) wet sorghum distiller's grains plus solubles (SDG) or c) CaOH

treated SDG (SGD-CH) on the associated microbiomes in the solid and liquid phases, relative composition and functional potential. Concurrently, microbiome relationships with diet nutrient composition, intra-ruminal metabolism and individual animals are explored. Six ruminally cannulated Angus cross steers, originating from a livestock auction, were enrolled in a 3×3 twice replicated Latin Square design based on the three experimental finishing diets and six animals. The nutrient concentration and chemical composition of the diets is presented in Table A1. Overall, energy and protein levels are near equivalent, therefore any observed differences in the rumen microbiota should result from ingredients as substrates and their corresponding rumen degradability or individual animal specific effects.

We compared both the relative abundance at the taxon level and functional gene content in the solid and liquid microbiomes across diets and fractions. The number of sequences per and post QC and gene hits in the metagenomic library (Table A2) did not differ across treatment or fraction. At the domain level, the microbiomes were comprised of 97.02% Bacteria, 0.90% Archaea, 0.93% Eukaryote, and 1.15% unassigned, which included 421/802, 57/84, and 103 genera/134 species respectively. Across experimental conditions, the most abundant phyla were Bacteroidetes (54.2%), Firmicutes (21.6%), Proteobacteria (12.9%), Actinobacteria (2.4%), Spirochaetes (1.4%), unassigned (1.2%), Fibrobacteres (1.1%), Euryarchaeota (0.9%) and the additional identified 37 phyla that represent 4.3% of the total relative abundance across all treatments.

The effect of diets on microbial rumen populations at the OTU level are illustrated by Bray-Curtis Dissimilarity matrix Principle Component Analysis (PCoA) in Figure A1a and b. No diet effects were spatially discernible. Accordingly, there were no differences in alpha diversity across treatments (Shannon: CDG 101.47, SDG 87.47, and SDG-CH 91.85) ($P = 0.19$); nor any other alpha diversity metrics examined. No fraction by treatment effects were observed ($P > 0.05$). Similarly, there were no genera that were unique to any treatment. There were 50 genera and 95 species across all domains that were not ubiquitous across all samples. Not unlike genera, no species was unique to any treatment; therefore, species richness was not influenced by treatment.

Similar to treatment observations, rumen fraction associated microbiomes (Figure A1b and c) did not exhibit separation. The absence of fraction, treatment and treatment × fraction interactions effects, ($P>0.05$) of the microbiome diversity, are attributed to all diets containing high levels of soluble carbohydrates and limited dietary fiber. This results in a more homogenous, comparably fermentable substrate ingesta matrix, which promotes more taxonomic evenness as compared to forage fed cattle (Hook *et al*., 2011; Mao *et al*., 2015) Congruently, alpha diversity did not differ ($P = 0.60$) between solid (Shannon: 94.5) and liquid (92.7) fractions. Therefore, we interpret that the observed differences in microbiomes result from changes in the relative abundance of specific taxa and metabolic potential rather than in the core microbiome, beta diversity, or the taxonomic community structure at the rumen ecosystem level.

KEGG-based annotations were used to predict the metabolic potential of these microbiomes. Figure A1e and f present the summary of functional annotation with a trend towards grouping with treatment under PC axis 1 and 2; this trend is not observed with PC 1 and 3. Differences in metabolic potential were also observed (Table A3) where 22% of KEGG level 2 pathways were significantly different across treatments. Therefore, these microbiomes, while similar at the taxonomic level, were less so in their metabolic potential, similar to the assemblages of Strickland *et al*. (2009) and Qin *et al*. (2010). However, the inverse has also been observed to be true, in which the ecosystem is taxonomically distinct, yet functionally similar (Taxis *et al.,* 2015). This is reflective of the thesis that any given microbial ecosystem has two ways to adapt to changes in their environment, through slower changes via alterations in the population taxonomy in two ways; 1) through slower changes via alterations in the population taxonomy, which happens within hours and days, or 2) through genes also known as gene selection which occurs in minutes. The idea that neither taxa nor functional patterns alone can fully capture or predict the effect on the host and its corresponding metabolism contributes to the challenge of consistently connecting taxa to phenotypes in this field. Recognizing that this study looked exclusively at DNA, which is not necessarily indicative of actual metabolic activities, a logical next step would be to observe the gene expression and to what degree this likens or diverges the metabolic network across animals, treatment and fractions.

On the bacteria phyla level, significant differences in relative abundance across dietary treatments included Tenericutes (0.207% SDG and SDG-CH versus 0.180% CDG, $P = 0.003$), Deinococcus-thermus (0.121% CDG versus 0.106% SDG and SDG-CH, $P = 0.005$)), and Synergistetes (0.109% CDG versus 0.084% SDG and SDG-CH, $P = 0.001$). *Mesoplasma*, *Ureaplasma, Acholeplasma,* and *Mycoplasma* are genera within Tenericutes that have been implicated in ruminant diseases (Nicholas *et al.,* 2008). It is unknown whether the 14.0% difference in Tenericutes in CDG versus sorghum-based treatments has implications toward animal health or why the sorghum would enrich for this taxa. Deinococcus-thermus is represented by the genera *Oceanithermus* (0.013% CDG versus 0.010% SDG and SDG-CH, 34.8%, $P = 0.001$) and *Meiothermus* (0.025% CDG versus 0.022%, 10.3%, $P = 0.027$) both of which were also elevated in CDG- although this taxa is consistently found in the metagenome of the rumen, very little is known about its function. However, a previous study also found it to be sensitive to changes in the rumen of cattle (Romero-Pérez *et al.,* 2011). In addition, not much is known about Synergistetes' role in the rumen, however it has been positively correlated with a high-grain diet (Petri *et al.,* 2013).

Archaea, at 0.9%, were on the low end of normal range- 0.5-3.0% (Lin *et al.,* 1997), most likely due to the high quality of the diet, which is known to select against this domain. Their abundance did not statistically differ across treatments, but were found to be more abundant in the solid fraction over the liquid. The archaea were predominantly represented by *Methanobacteria* (36.1%), *Methanomicrobia* (34.9%), and

Methanococci (12.5%). Archaea abundance was numerically greeter in the CDG treatment (non-significant), which is captured in a significant increase in genes associated with the methane metabolism pathway in CDG (0.475% vs 0.423%, P = 0.012, 10.9%). Previous estimates of methane production were significantly lower in animals eating sorghum rather than maize (63.48 and 103.00 kg CH4/head/ year respectively, $P < 0.001$), however this was comparing silage based diets rather than distillers' grain (Sarubbi *et al.,* 2014). Future studies comparing sorghum distillers' grain should consider including a measurement of methane *in vivo* as this may reveal a value added feature of feeding this co-product.

Viruses were relatively rare, representing 0.11% of the microbiome and, as expected, were dominated by caudovirales, the tailed bacteriophages (Fernando, 2012; Berg Miller *et al*., 2012), and did not differ among treatments. The viral community was predominantly *Bacteroides* phage B40-8 (26.4%), followed by *Salmonella* phage Vi II-E1 (11.8%), *Bacteriophage* APSE-2 (9.2%), *Bacillus* virus 1 (8.7%), *Pseudomonas* phage LIT1 (8.4%), *Clostridium* phage phiCTP1 (7.7%), and *Enterobacteria* phage N4 (7.6%). The inherent difficulty in identifying and culturing viruses tied with their extensive diversity has led to limited representation in public databases and therefore they may be underrepresented in the dataset. Nearly 15% of assembled reads could not be annotated to a known order, and 34% could not be annotated to species, indicating a significant amount of work is still required to understand the rumen virome.

Overall, eukaryote populations were comprised of 11.1% Ascomycota, 10.0% Strephtophyta, and 8.9% Apicomplexa, 5.5% Arthopoda, 2.6% Basidiomycota and 42.1% unclassified Eukaryota. The effect of extraction method chosen on the eukaryotic population is unknown, however it is likely this domain is also underrepresented (Ross *et al*., 2012). Class Litostomatea, previously established as a dominant protozoa (Shah *et al*., 2016), as well as the dominate genus *Isotricha,* were not detected. Fungal classes that were detected include: Microsporidia (higher in both sorghum diets than corn, 0.098% vs 0.033%, 148.8%, $P = 0.049$) and Ascomycota (no treatment differences), and Basidiomycota (no treatment differences) which have previously been detected in the rumen (Brulc *et al*., 2009). However, Chytridiomycota were notably absent, members of which are known anaerobic rumen fungi associated with plant cell wall hydrolysis (Trinci *et al*., 1994; Gordon and Phillips, 1998). This could perhaps be a result of the high fermentable carbohydrate and low fiber content of these diets.

Several animals had higher than average eukaryote levels, with up to 7.20% of total taxa being eukaryotes compared to the average of 0.93%. This may relate back to the effect of the individual animal on the rumen microbiome community, function, and conditions. These animals did come from comparable genetic backgrounds or rearing conditions. Host genetics have previously been shown to influence the rumen microbiome (Li *et al*., 2009; Weimer *et al*., 2010; Guo *et al*., 2015; Li *et al*., 2016) and were highly transferable depending on the environment (Majak and Cheng, 1984; Gonzalez-Recio *et al*., 2017; Roehe *et al*., 2016). This is illustrated in Figure A2a and b

in which the separation by individual animal is greater than the treatment effect (Figure A1a and b). The effect of individual animal genetics and background is lessened on the functional level, which does exhibit treatment effects (Figure A2c and d). This was also reflected in individual taxa, in which 15 phyla were found to differ significantly across animal, the most significantly different ones being of eukaryotic origins. Previous, studies have also found host genetics to have a significant influence on eukaryotic taxa abundance and diversity (Gonzalez-Recio *et al*., 2017). The mechanism behind the host influence on the microbial community is theorized to be a variety of biological factors including pH, saliva content and volume, passage rate, as well as size and structure of the rumen (Roehe *et al*., 2016)

There was significantly more butyrate in the CDG fed cattle compared to the SDG-CH and SDG fed. Typically, this would be associated with an increase in *Butyrivibrio*. However, this genus, and the species *Butyrivibrio proteoclasticus* B316 (formerly *Clostridium proteoclasticum*), were consistent across dietary treatments (average 1.42%, $P = 0.639$), although no other species of *Butyrivibrio* were detected. *Eubacterium,* however, was significantly higher in CDG than in SDG or SDG-CH (3.93% vs 3.15%, $P = 0.008$, 21.9%) (Figure A3a) and butyrate is one of the main fermentation products of this genus (Duncan *et al*., 2007; Hobson and Stewart, 2012). *Eubacterium ruminantium* and *cellulosolvens*, which are commonly associated with the rumen, were not detected. Instead, *Eubacterium rectale* and *E. eligens* were found. These isolates are hypothesized to be net acetate consumers during growth using the

butyryl coenzyme A-acetyl coenzyme A transferase pathway for butyrate production (Barcenilla *et al*., 2000). This may in part explain the lower acetate levels associated with a CDG as compared to SDG-CH and SDG, in addition to the higher butyrate. Accordingly, the relative abundance of the butyryl-CoA dehydrogenase gene is 35% greater in the rumen of CDG fed cattle ($P = 0.001$), although the butanoate metabolism pathway as a whole was unexplainably lower in CDG fed cattle ($P = 0.003$) (Figure A3b). *Clostridium* spp. are also associated with butyrate production and tended to have greater relative abundance in CDG as compared to SDG-CH and SDG (11.4%, $P = 0.060$). All detected *Clostridium* species were also elevated in CDG including *Clostridium botulinum*, *C. perfringens, C. ljungdah and C. sticklandii* ($P \leq 0.01$). No *Selenomonas* or *Megasphaera* were detected, which are also associated with butyrate production in the rumen environment. It is unclear why so many iconic rumen taxa were not detected from these metagenomes.

No difference was found in the relative abundance of the propionate metabolism pathway in any of the diets, however propionate levels were significantly higher in the CDG than SDG and SDG-CH containing diets (41.9 vs 35.4 mM, $P = 0.010$). One gene from the propionate metabolism pathway varied significantly among treatments: prpD; 2-methylcitrate dehydratase, which was 7-10 times more abundant in CDG than SDG and SDG-CH, respectively (0.01036 % in CDG, 0.00144% in SDG, 0.00057% in SDG-CH, $P=0.001$), however this gene is considered ancillary to propionate formation. *Selenomonas*, *Succiniclasticum*, *Succinomonas*, *Megasphaera*, *Ruminobacter*, and

Anaerovibrio, taxa typically associated with propionate production, were not detected (Russell, 2002; Hobson and Stewart, 2012). *Prevotella*, which is also associated with propionate production, did not differ among diets (Table 3 supplemental). *Clostridium*, another propionate producing taxa, did not differ among diets (Table A4). Eukaryotic taxa that have been associated with propionate production, such as *Isotricha* and *Dasytricha* spp., were also not detected under these methods (Ellis *et al*., 1991; Ellis *et al*., 1991a; Ellis *et al*., 1991b). Therefore, based on previously published research, it is challenging from a microbial community composition perspective to consider why propionate was elevated in the CDG diet.

Pelotomaculum was significantly more abundant in CDG than in SDG or SDG-CH diets (0.440% vs 0.352%, *P*=0.012, 22.22%). *Pelotomaculum* has been isolated via sequencing from the forestomach of alpacas who were fed alfalfa, and steers that were fed TMR, however the abundance was not determined (Pei *et al*., 2010; Hernandez-Sanabria *et al*., 2012). This taxa is a known propionate-oxidizing bacterium (Imachi *et al*., 2002), as such its significant increase in CDG fed steers may be a response to the increase in propionate. This species has been found to grow in co-culture with the methanogen *Methanothermobacter thermautotrophicus* (Imachi *et al*., 2002) that was also found to be positively correlated with the CDG treatment (0.008% vs 0.005%, *P*=0.027). *Lactobacillus* was significantly higher in CDG over SDG or SDG-CH (1.14% vs 0.88%, *P*= 0.001, 23.5%), which would contribute to the lower pH in CDG treatment (5.37 CDG versus 5.80 and 5.68 in SDG and SDG-CH respectively). It is well

established that this taxa increases with high-concentrate diets (Caldwell and Bryant, 1966; Latham *et al*., 1971; Latham *et al*., 1972) as rapidly fermentable foods cause a more favorable condition for *Lactobacillus* and decreases lactate-utilizing bacteria. *Streptococcus,* another lactic acid producing bacteria, was also positively correlated with CDG and the corresponding decrease in pH (0.71% vs 0.62%, *P*=0.002, 10.0%). *Corynebacterium* was significantly elevated in CDG compared to SDG and SDG-CH diets (0.081% vs 0.064%, 23.4%, *P*= 0.007). This genus has been positively associated with vitamin B production in the rumen (Al-Dilaimi *et al*., 2014).

There has been a concern whether the incorporation of distillers' grains into the finishing diets of steers would increase the intestinal carriage of pathogens such as *Campylobacter* and *E. coli*. *Campylobacter* was found on average at 0.19%, with a minimum of 0.12% and a maximum of 0.31%. Other studies have also found little or no *Campylobacter* in the rumen or duodenum of feedlot steers who were fed a distillers' grains based diet (Callaway *et al*., 2010; Anderson *et al*., 2014). Likewise, *Escherichia* was found on average at 0.19%, with a minimum of 0.06% and maximum 0.34%.

The KEGG pathway for nitrogen metabolism was more abundant in CDG and SDG-CH compared to SDG (0.255% vs 0.202%, *P*= 0.002, a 20.8% difference) (Figure 3b). Nitrate reductase within the nitrogen metabolism pathway was significantly elevated in CDG over SDG and SDG-CH (3.3% vs 1.8%, *P*=0.001, 46.2%). SDG steers had the lowest nitrogen intake (kg/d) to fecal output (kg/d) ratio, indicating that more nitrogen exited the system compared to CDG and SDG-CH. Nitrogen's apparent total tract

digestibility (%) was also reduced in SDG steers (Gentry, 2016). This may be a consequence of the reduced nitrogen metabolism pathways in the rumen of the microbiome of SDG steers. This in turn may result from the protein within these feeds being more available via the nature of corn and the chemical breakdown of sorghum with OH. This does not explain why ammonium was significantly elevated in CDG (5.82mg/dl) compared to SDG (3.64), but not to SDG-CH (3.65) (Table A1).

Desulfovibrio spp (*vulagris* and *desulfuricans*) are nitrate and sulphate-reducing bacterium which were elevated in CDG above sorghum-based treatments (0.32% vs 0.26%, 18.0%, *P*=0.003). This genus is thought to be responsible for most of the sulfite production in the rumen. *Desulfuromonas* and *Dethiosulfovibrio* were also significantly higher in CDG than SDG or SDG-CH (0.186% vs 0.168%, *P*=0.013, 10.17%)(0.156% vs 0.127%, p=0.001, 20.5%). Sulfur metabolism pathways were also elevated in CDG (0.352% vs 0.233%, *P*=0.001, 33.8%). This is most likely a response to the elevated sulfur levels in CDG (0.29%) as compared to the sorghum-based diets (0.17%). Sulfur is usually negatively correlated with methanogenesis (Li *et al*., 2013), however in the CDG diet methane metabolism was highest (0.475% vs 0.423%, *P*= 0.012, 10.9%). Again, future studies should consider including a measurement of methane *in vivo*.

The genus *Haemophilus*, a known rumen ureolytic taxa, with urea hydrolysis ability, was positively associated with SDG-CH (0.715% vs 0.542% (SDG-CH vs. average of CDG and SDG), *P*=0.003, 27.5%) (Garcia-Delgado *et al*., 1977; Jin *et al*., 2016). This is most likely a response to urea being higher in SDG-CH (0.33% versus

0.30% in CDG and SDG), which corresponds to a 2.85 g/d intake of urea above the other two treatments. It is unknown if a Bovine Respiratory Disease complex, which is strongly associated with *Haemophilus*, would increase with SDG-CH or urea supplementation.

An inherent difficulty with this study and many like it, is distinguishing the rumen microbiome from the microbial community that were associated with the feed. The feed, Stretophyta, was detected at very low levels (0.09%) although there was nothing intrinsically excluding this taxa with our methodologies. Several eukaryotic phyla that are not normally associated with the rumen environment were found at differing relative abundances across the three treatments including Arthropoda, Bacillariophyta, Basidiomycota, Apicomplexa, and Nematoda, this might be a reflection of the feed. Likewise, *Cyanothece* was higher in CDG (0.11% vs 0.098%, 10.8% difference, P=0.006) than sorghum-based treatments. Similarly, *Rhizobium*, a genus of nitrogen fixing bacteria and a non-rumen resident, was higher in CDG treatments (0.043 vs 0.033, 26.3% difference, P=0.001). However, *Zea* (the corn genus) (0.003% CDG vs 0.005% SDG and SDG-CH P= 0.146) and *Sorghum* (0.004% vs 0.008% P=0.120) were not significantly different across all treatments. *Sorghum bicolor* (0.006%) and *Zea mays* 0.004%) were detected at the species level, but again did not differ by treatment. Future studies, might consider including a metagenomic analysis of the feed itself to clearly differentiate.

Conclusion

No differences were observed in the total microbial community both in alpha and beta diversity. Likewise, the effect of fraction was less than expected. Intra-animal variation appeared to be a driving force in the taxonomic profile of this study. On the functional level, treatment was found to have a larger effect and it reflected in changes in the VFA profile, pH, and digestibility of the cattle.

Since the treated-sorghum microbial community trended towards corn and no statistical difference were observed in the overall microbiome of either treatment, it could be possible to substitute corn with treated- or untreated-sorghum in the finishing steer's diet from a microbial perspective. Further, these substitutions were not associated with increases in pathogens abundance. Beneficial characteristics were observed in the SDG and SDG-CH with regard to sulfur and methane metabolism and pH, which may be a result of changes in the microbe as a result of the diet.

CHAPTER V

CONCLUSIONS

Taking this dissertation as a whole, several trends on the overall controls of the rumen microbial ecosystem can be observed. The first being, that fractional differences across the liquid and solid fraction are greatest in lower quality diets as observed in Chapter III and become more homogenous with a higher quality diet as with Chapter IV. The effect of treatment did not exert a strong effect on the microbial ecosystem diversity, however changes in diet may cause differences on the functional level. Based on this work, diet may not be the strongest determinant of the microbial diversity as previously hypothesized. In this situation, individual animal seems to have the greatest pull. The repercussions of this conclusion are great as it implies producers have less control over the rumen microbial ecosystem and its corresponding influence on the animal than previously thought. This is confounding by the difficulty in separating biological differences and experimental parameters in this field as observed in Chapter II. Future work will hopefully include the expansion and exploration of the rumen microbiome repository at Qitta, culture work of the core rumen microbiome, and long-term studies to determine the plasticity and adaptive potential of the rumen microbiome.

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APPENDIX A CHAPTER I FIGURES AND TABLES

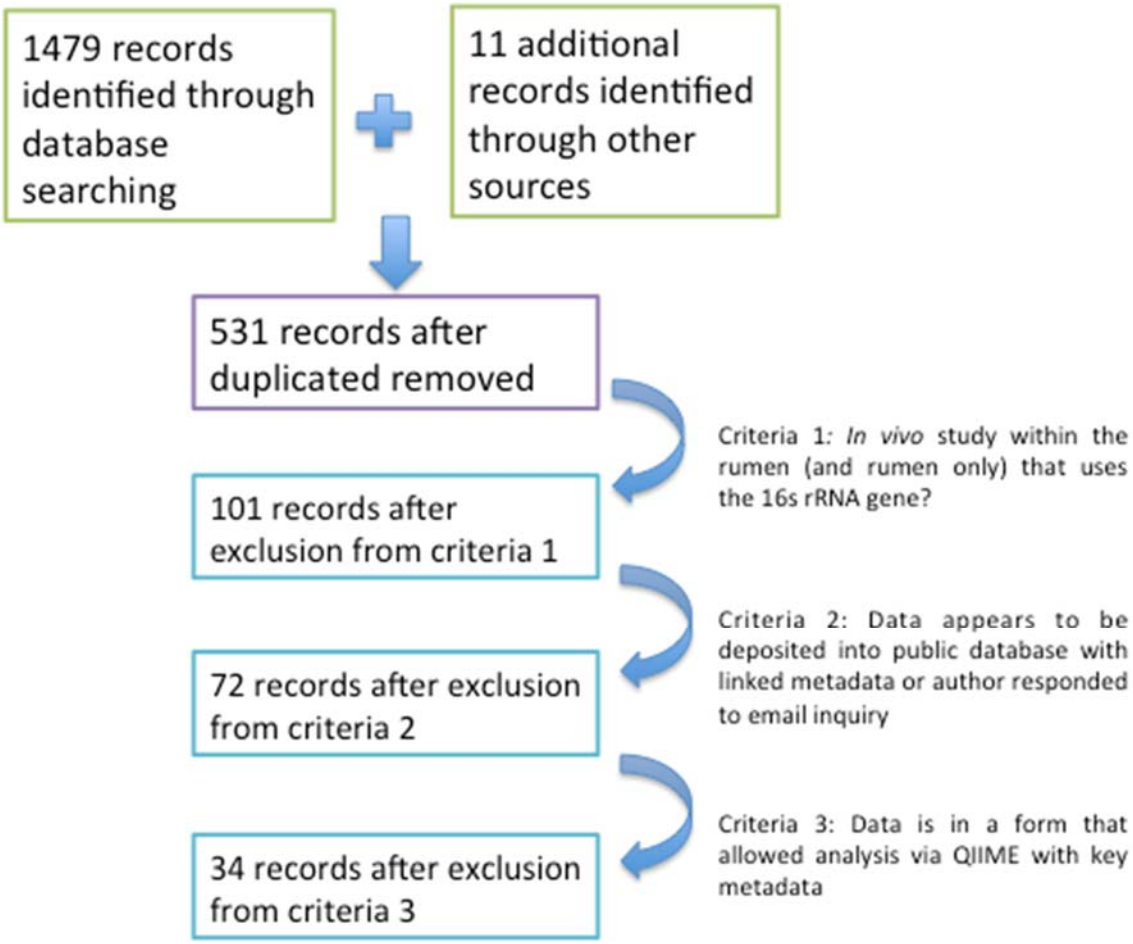

Figure A1: Flowchart and inclusion criteria for meta-analysis of ruminant 16s rRNA gene microbiome analysis

Table A1: Studies accessed in 16s rRNA gene dataset meta-analysis

Title	Pub Year	Data location
Rumen Bacterial Diversity Dynamics Associated with Changing from Bermudagrass Hay to Grazed Winter Wheat Diets	2010	SRA
Microbiome analysis of dairy cows fed pasture or total mixed ration diets	2011	Private server
Next Generation Sequencing to Define Prokaryotic and Fungal Diversity in the Bovine Rumen	2012	SRA
Nitrogen metabolism and rumen microbial enumeration in lactating cows with divergent residual feed intake fed high-digestibility pasture	2012	MG-RAST
The effect of brown midrib corn silage and dried distillers' grains with solubles on milk production, nitrogen utilization and microbial community structure in dairy cows	2012	Author contact
The effects of a probiotic yeast on the bacterial diversity and population structure in the rumen of cattle.	2013	EBI
Comparative analysis of microbial profiles in cow rumen fed with different dietary fiber by tagged 16S rRNA gene pyrosequencing.	2013	SRA
Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge.	2013	SRA
Temporal dynamics of fibrolytic and methanogenic rumen microorganisms during in situ incubation of switchgrass determined by 16S rRNA gene profiling	2014	SRA
An analysis of the ruminal bacterial microbiota in West African Dwarf sheep fed grass- and tree-based diets	2014	SRA
Bacterial communities in the rumen of Holstein heifers differ when fed orchardgrass as pasture vs. hay.	2014	SRA
High-Throughput DNA Sequencing of the Ruminal Bacteria from Moose (Alces alces) in Vermont, Alaska, and Norway	2014	SRA
Bacteria and methanogens differ along the gastrointestinal tract of Chinese roe deer (Capreolus pygargus)	2014	SRA
Bacterial Community Composition and Fermentation Patterns in the Rumen of Sika Deer (Cervus nippon) Fed Three Different Diets	2014	SRA
Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency	2014	MG-RAST
Microbial biodiversity of the liquid fraction of rumen content from lactating cows.	2014	SRA
Temporal dynamics in the ruminal microbiome of dairy cows during the transition period	2014	Author contact
Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential	2014	SRA
Relationship between the Rumen Microbiome and Residual Feed Intake-Efficiency of Brahman Bulls Stocked on Bermudagrass Pastures	2014	Author contact
Evaluation of composition and individual variability of rumen microbiota in yaks by 16S rRNA high-throughput sequencing technology.	2015	SRA
Exploring the sheep rumen microbiome for carbohydrate-active enzymes.	2015	MG-RAST and SRA
Illumina MiSeq Phylogenetic Amplicon Sequencing Shows a Large Reduction of an Uncharacterised Succinivibrionaceae and an Increase of the Methanobrevibacter gottschalkii Clade in Feed Restricted Cattle	2015	SRA
Characterization of the rumen lipidome and microbiome of steers fed a diet supplemented with flax and echium oil	2015	SRA
Examination of the Rumen Bacteria and Methanogenic Archaea of Wild Impalas (Aepyceros melampus melampus) from Pongola, South Africa	2015	SRA
Rumen Microbiome from Steers Differing in Feed Efficiency	2015	SRA
Response of the Rumen Microbiota of Sika Deer (Cervus nippon) Fed Different Concentrations of Tannin Rich Plants	2015	SRA
Prepartum and Postpartum Rumen Fluid Microbiomes: Characterization and Correlation with Production Traits in Dairy Cows	2015	SRA
Ruminal Bacterial Community Composition in Dairy Cows Is Dynamic over the Course of Two Lactations and Correlates with Feed Efficiency	2015	SRA

Table A1: Continued

Title	Pub Year	Data location
Associative patterns among anaerobic fungi, methanogenic archaea, and bacterial communities in response to changes in diet and age in the rumen of dairy cows	2015	Author contact
In-depth diversity analysis of the bacterial community resident in the camel rumen	2015	SRA
Rumen bacterial communities can be acclimated faster to high concentrate diets than currently implemented feedlot programs	2016	Private server
A comparison of rumen microbial profiles in dairy cows as retrieved by 454 roche and Ion Torrent (PGM) sequencing platforms	2016	Private server
Metagenomic analysis of rumen microbial population in dairy heifers fed a high grain diet supplemented with dicarboxylic acids or polyphenols	2016	Author contact

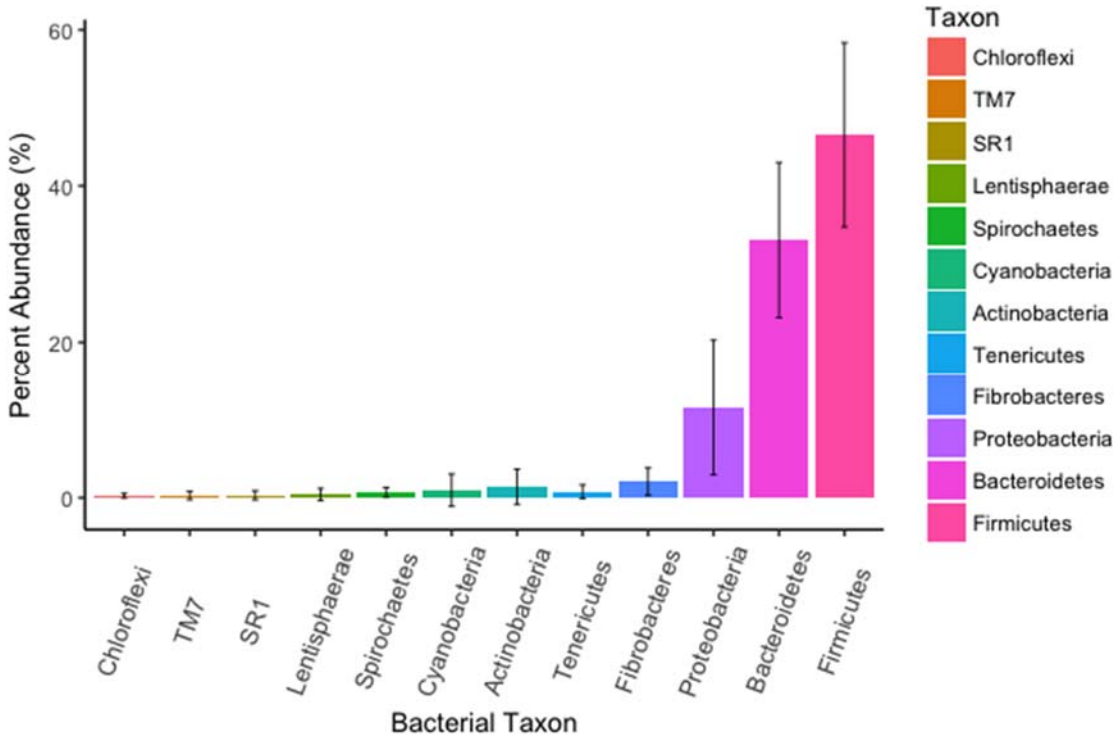

Figure A2: Relative abundance of top 12 bacterial phyla with 16s rRNA gene rumen census

Table A2: Summary of the 40 most abundant bacterial genera

Taxon	Percent Abundance	Prevalence (%)	Similarity to Culture Strains (%)
Unassigned	21.3	100	n/a
Prevotella	16.8	100	95.1
Clostridiales (order)	8.6	100	95.6
Ruminococcus	6.8	100	96.7
Lachnospiraceae (family)	6	100	95.9
Bacteroidales (order)	5	100	93.3
Butyrivibrio	2.9	100	95.1
Bacillus	1.6	81.8	95.6
Succinivibrionaceae (family)	1.5	90.9	88.9
vadinCA11	1.3	50	94
RF39 (order)	1.1	100	94.5
Succiniclasticum	1	100	95.8
S24-7 (family)	1	100	92.8
BS11 (family)	0.8	90.9	93.4
Treponema	0.7	100	91.4
Coprococcus	0.7	100	95.7
Mogibacteriaceae (family)	0.6	100	93.2
Fibrobacter	0.6	100	91.5
RF16 (family)	0.6	100	93.5
Streptococcus	0.5	90.9	96
Pseudobutyrivibrio	0.5	100	97.3
Succinivibrio	0.5	90.9	90.9
Clostridium	0.5	100	95.6
Bacteroides	0.5	81.8	95.9
CF231	0.4	100	93.5
YS2 (order)	0.4	100	91.8
F16 (family)	0.4	100	94.5
YRC22	0.4	100	93
Coriobacteriaceae (family)	0.4	100	94.2
Veillonellaceae (family)	0.4	100	94.1
Mogibacterium	0.4	100	95.2
Lactobacillus	0.3	77.3	95.2
Christensenellaceae (family)	0.3	100	95.3
RFN20	0.3	95.5	90.5
Carnobacterium	0.3	45.5	97
RFP12 (family)	0.3	86.4	90.7
Selenomonas	0.2	100	94.4
Paraprevotellaceae (family)	0.2	95.5	92.6
Bulleidia	0.2	100	93.5
Shuttleworthia	0.2	100	93.1

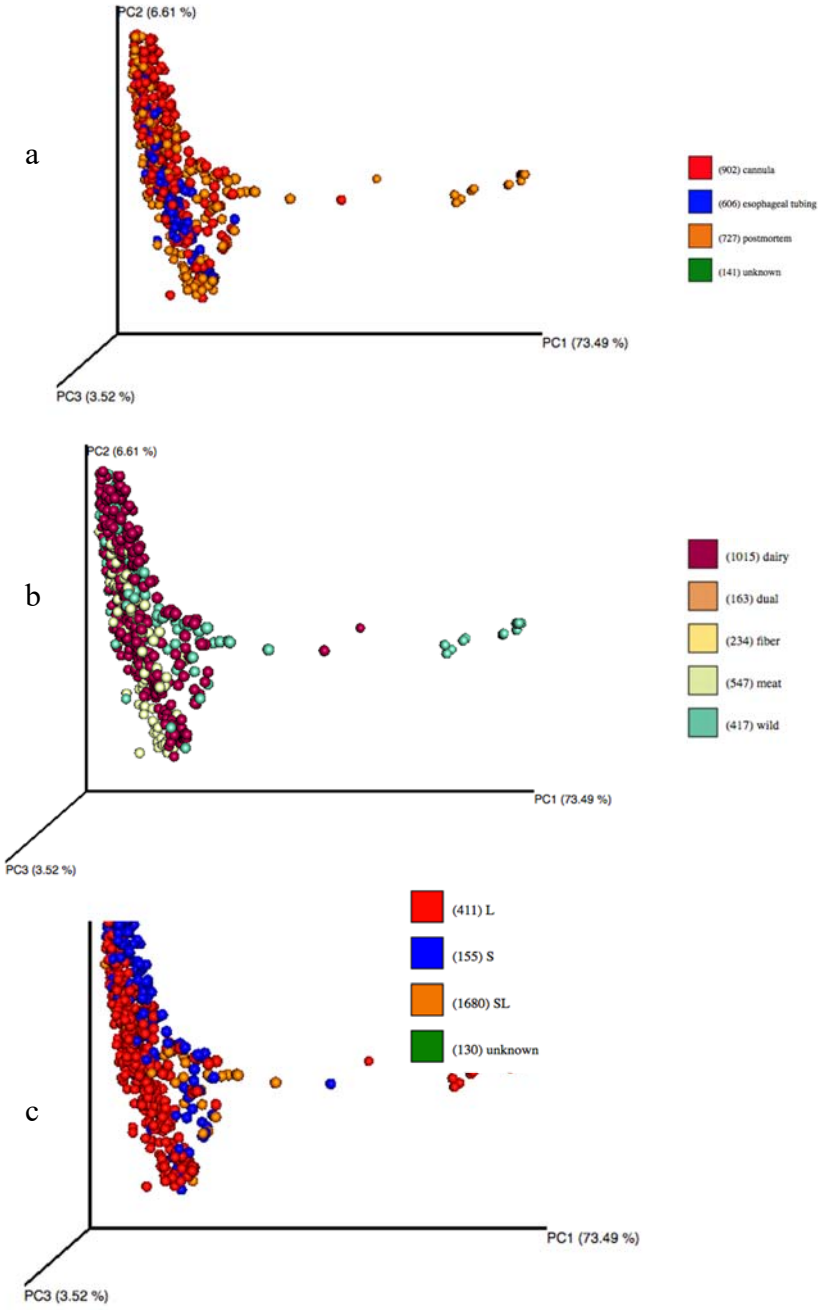

Figure A3: Rumen bacterial community beta diversity differentiation based of collection methodology (a), industry (b), and fraction (c) using weighted Unifrac distance

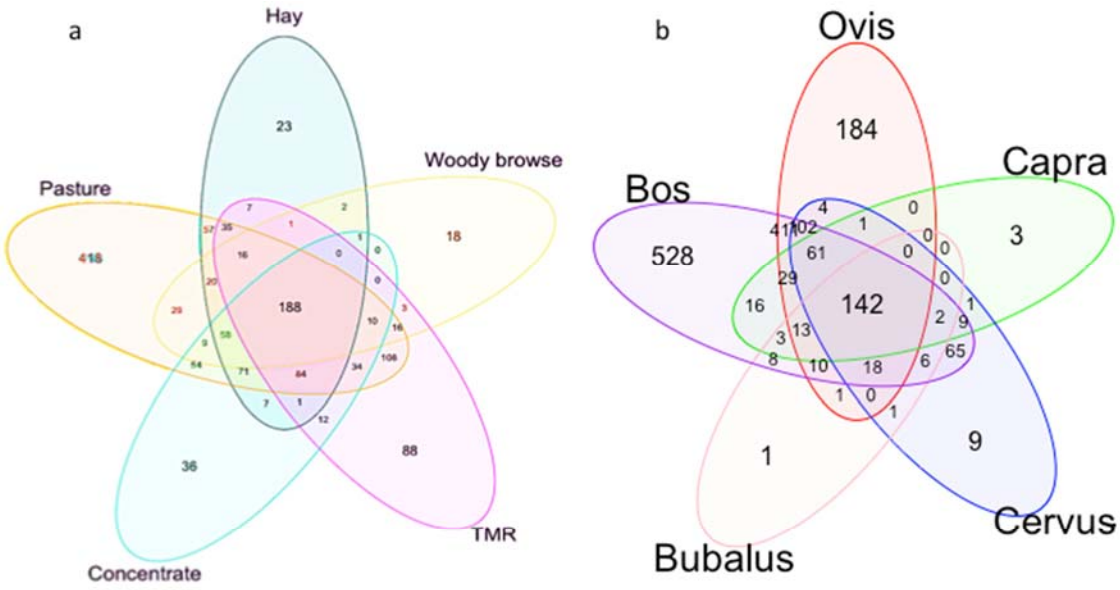

Figure A4: Venn diagram of the dominant OTUs across diet types (a) and major ruminant genera (b)

APPENDIX B CHAPTER II FIGURES AND TABLES

Table B1 Chemical composition of forage and supplements

Item	Rice straw[2]	L- DIP [1,3]	H-DIP [1,4]
	% DM basis		
OM	84.9	94.5	93.6
CP	4.7	26.7	26.9
TDN	NM	88.0	88.0
DIP	NM	28.0	72.0
NDF	72.8	41.8	35.0
ADF	52.3	19.0	12.4
Acid detergent insoluble ash	8.8	0.3	0.4

[1]Treatments: cattle were fed 0, 50 or 150 mg N/kg BW (CON, 50 L-DIP, 150 L-DIP, 50 H-DIP, or 150 H-DIP)

[2] *Ad libitum* access

[3] L-DIP= low degradable intake protein supplement (100% dried distillers' grains),

[4] H-DIP= high degradable intake protein supplement (69.5% wheat middling, 30% soybean meal, and 0.5% urea)

Table B2: Effect of experimental parameters on operational taxonomic unit richness and diversity at 97% similarity after rarefaction

Treatment[1]	Shannon[2]	Shannon Error	chao1[3]	chao1 Error
CON	6.256	0.155	579.4	119.8
50 H UIP	6.335	0.073	604.3	111.3
150 H UIP	6.298	0.13	630.7	142.1
50 L UIP	6.288	0.12	603.7	125.9
150 L UIP	6.344	0.108	640.2	127.1
Fraction				
L	6.323	0.118	641.6	135.0
S	6.285	0.128	581.6	111.8
Hour				
0.0	5.490	0.102	646.0	126.7
4.0	5.466	0.095	577.3	118.9
Sub-species				
Bos taurus	5.488	0.101	615.8	125.6
Bos indicus	5.469	0.096	607.4	129.3

[1]CON= supplemented, 50 H DIP = 50 mg N/kg BW 69.5% wheat middling, 30% soybean meal, and 0.5% urea, 150 H DIP = 150 mg N/kg BW 69.5% wheat middling, 30% soybean meal, and 0.5%, 50 L DIP = 50 mg N/kg BW 100% distillers' grains, 150 L DIP = 150 mg N/kg BW 100% distillers' grains

[2]Shannon= diversity indices as calculated by Shannon and Weaver, 1949

[3]chao1= species richness as calculated by Chao *et al*., 2013

Table B3: Core and unique OTUs across treatments in *Bos taurus* and *Bos indicus* steers (n=5) fed a low-quality forage

Measurement	Parameter	Unique, but not ubiquitous
Fraction	Liquid	0
	Solid	36
Treatment	Con	10
	60 H UIP	8
	60 L UIP	7
	120 H UIP	10
	120 L UIP	15
Sub-species	*Bos taurus*	2
	Bos indicus	7
Time	0h	32
	4h	0

Table B4: Effect of protein supplementation (treatment), fraction, sub-species (*Bos taurus* and *Bos indicus*), and time (0H and 4H post-feeding) on the relative abundance of the most abundant families

		Sub-species			Fraction			Time	
Family	Sub-species *P*-value	*Bos taurus*	*Bos indicus*	Fraction *P-value*	Liquid	Solid	Time *P*-value	0h	4h
Prevotellaceae [123]	0.04	43.82	42.28	0.01	44.01	42.19	0.001	44.05	42.15
Ruminococcaceae[1]	0.03	13.56	14.27	<.0001	10.90	16.99	0.01	13.56	14.33
Sphingobacteriaceae	0.04	7.77	8.91	<.0001	10.03	6.59	0.01	8.73	7.87
Lachnospiraceae	0.64	7.23	7.05	<.0001	4.09	10.25	0.04	6.85	7.49
Bacteroidales (order) [2]	<.0001	2.9	4.3	<.0001	5.72	1.44	0.14	3.42	3.73
Cytophagaceae	<.0001	4.18	2.57	0.27	3.25	3.45	0.55	3.39	3.31
Bacteroidaceae	0.003	2.4	2.73	<.0001	2.36	2.75	0.77	2.58	2.54
Porphyromonadaceae [2]	0.02	2.35	2.13	0.01	2.36	2.11	0.20	2.19	2.28
Spirochaetaceae [3]	0.19	2.14	2.28	<.0001	1.90	2.53	0.00	2.01	2.42
Erysipelotrichaceae	0.85	1.89	1.86	<.0001	3.19	0.54	0.35	1.81	1.93
Veillonellaceae [3]	0.01	1.71	1.41	0.001	1.71	1.41	0.09	1.62	1.50
Clostridiaceae	0.9	1.17	1.16	<.0001	0.86	1.48	0.28	1.14	1.20
Fibrobacteraceae [3]	0.34	1.09	1.19	0.19	1.07	1.23	0.08	1.05	1.24
Marinilabiaceae [3]	0.12	0.9	0.99	0.39	0.93	0.97	0.05	0.90	1.00
Leuconostocaceae	0.28	0.7	0.75	<.0001	0.84	0.60	0.44	0.71	0.73
Cryomorphaceae[2]	0.22	0.53	0.6	0.001	0.67	0.46	0.01	0.62	0.50
Rikenellaceae	0.46	0.6	0.53	<.0001	0.43	0.71	0.04	0.62	0.52

[1] Treatment ($P \leq 0.05$), Prevotellaceae CON 44.66%, 50 L UIP 41.99% 150 L UIP 43.18%, Ruminococcaceae CON 13.49% 50 L UIP 14.45% 150 L UIP 13.81% 50 H UIP 14.94% 150 H UIP 13.00, CON= supplemented, 50 L UIP = 50 mg N/kg BW 69.5% wheat middling, 30% soybean meal, and 0.5% urea, 150 L UIP = 150 mg N/kg BW 69.5% wheat middling, 30% soybean meal, and 0.5%, 50 H UIP = 50 mg N/kg BW 100% distillers' grains, 150 H UIP = 150 mg N/kg BW 100% distillers' grains

[2]Fraction × sub-species (P<0.05)

[3]Fraction × treatment (P<0.001)

Table B5: Effect of protein supplementation (treatment), fraction, sub-species (*Bos taurus* and *Bos indicus*), and time (0H and 4H post-feeding) on the relative abundance of the most abundant genera

	Sub-species			Fraction			Time	
Family	Sub-species *P*-value	*Bos taurus*	*Bos indicus*	Fraction *P-value*	Liquid	Solid	Time *P*-value	0h	4h
Prevotella [1] [2]	0.01	28.85	31.00	<.0001	32.81	28.17	<.0001	30.89	29.09
Prevotellaceae (family) [2] [3]	0.13	11.67	10.96	0.002	10.70	11.85	0.97	11.31	11.32
Ruminococcaceae (family) [1]	0.004	8.70	8.21	0.21	8.36	8.56	<.0001	8.10	8.82
Sphingobacteriaceae (family)	0.03	7.55	6.48	<.0001	8.46	5.54	0.02	7.35	6.64
Saccharofermentans	0.36	3.82	3.64	<.0001	1.39	6.10	0.97	3.74	3.75
Bacteroidales (order)	<.0001	4.30	2.90	<.0001	5.72	1.43	0.14	3.42	3.74
Pontibacter	<.0001	2.69	4.36	0.59	3.45	3.55	0.5	3.55	3.46
\0//p'y gtu-0[[[[[[[[[[[[[[[[[[[[[æˆ/B' acteroides	0.01	2.86	2.54	<.0001	2.43	2.94	0.69	2.71	2.66
Treponema	0.76	1.73	1.71	<.0001	1.10	2.38	0.001	1.54	1.91
Erysipelotrichaceae (family)	0.61	1.69	1.76	<.0001	2.98	0.45	0.47	1.67	1.76
Succiniclasticum	0.01	1.36	1.62	0.001	1.63	1.34	0.06	1.56	1.42
Blautia	0.2	1.41	1.49	<.0001	0.88	2.03	0.57	1.47	1.44
Clostridium	0.98	1.29	1.30	<.0001	0.93	1.66	0.22	1.26	1.33
Fibrobacter[4]	0.34	1.19	1.09	0.19	1.07	1.23	0.08	1.05	1.24
Ruminococcus	0.29	1.03	0.96	<.0001	0.53	1.48	0.72	1.00	1.01

[1]Sub-species × treatment (P<0.05)

[2] Fraction × treatment (P<0.05)

[3] Treatment (*P* = 0.0029) CON 11.70% 50 H UIP 11.47% 150 H UIP 12.97% 50 L UIP 10.04% 150 L UIP 10.40%, CON= supplemented, 50 H DIP = 50 mg N/kg BW 69.5% wheat middling, 30% soybean meal, and 0.5% urea, 150 H DIP = 150 mg N/kg BW 69.5% wheat middling, 30% soybean meal, and 0.5%, 50 L DIP = 50 mg N/kg BW 100% distillers' grains, 150 L DIP = 150 mg N/kg BW 100% distillers' grains

[4] Fraction × sub-species (P<0.05)

Table B6: Effect of protein supplementation (treatment), fraction, sub-species (*Bos taurus* and *Bos indicus*), and time (0H and 4H post-feeding) on the relative abundance of the most abundant phyla

			Fraction	
Phylum[1]	Relative Abundance	Fraction *P-value*	Liquid	Solid
Bacteroidetes [5]	65.44	<.0001	70.00	60.80
Firmicutes[2] [5]	28.13	<.0001	23.40	32.90
Spirochaetes[3] [5]	2.22	<.0001	1.90	2.50
Fibrobacteres[3]	1.14	>0.05		
Proteobacteria [5]	0.94	<.0001	1.40	0.50
Tm7	0.55	0.01	0.51	0.59
Tenericutes	0.48	<.0001	0.79	0.17
Chloroflexi [5]	0.2	<.0001	0.09	0.31
Actinobacteria	0.19	<.0001	0.11	0.27
Synergistetes	0.18	<.0001	0.11	0.24
Verrucomicrobia[4]	0.17	>0.05		

[1]No significant sub-species × treatment or fraction × treatment differences

[2] Treatment (*P = 0.025)* Control = 27.2%, 50 L UIP = 29.1%, 150 L UIP = 27.8%, H UIP = 29.5%, and 150 H UIP = 27.1% , CON= supplemented, 50 H DIP = 50 mg N/kg BW 69.5% wheat middling, 30% soybean meal, and 0.5% urea, 150 H DIP = 150 mg N/kg BW 69.5% wheat middling, 30% soybean meal, and 0.5%, 50 L DIP = 50 mg N/kg BW 100% distillers' grains, 150 L DIP = 150 mg N/kg BW 100% distillers' grains

[3] Fraction × sub-species (*P*< 0.05), Spirochaetes: Angus × liquid 1.67%, Brahman × liquid 2.15%, Angus × solid 2.63%, Brahman × solid 2.44% and Fibrobacteres: Angus × liquid 0.89%, Brahman × liquid 1.25%, Angus × solid 1.29%, Brahman × solid 1.16%

[4]Sub-species (*P*=0.01), Angus 0.19% vs Brahman 0.13%

[5] Time (*P* < 0.006) Bacteroidetes 0h 66.7% vs 4h 64.1%, Firmicutes 0h 27.4% vs 4h 29.0%, Spirochaetes 0h 2.0% vs 4h 2.4%, Proterobacteria 0h 0.9% vs 4h 1.0%, Chloroflexi 0h 0.15% vs 4h 0.24%

Table B7: Functional analysis via PICRUSt of the most abundant pathways in the solid and liquid fraction of the rumen in *Bos taurus* and *Bos indicus* steers (n=5) fed a low-quality forage

Level 2 KEGG gene pathway	% Solid	% Liquid	% Difference
Replication and repair*	10.89	9.64	12.2
Amino acid metabolism *	10.26	9.94	3.2
Carbohydrate metabolism *	10.38	9.21	11.9
Membrane transport *	8.61	6.83	23.1
Translation *	7.67	6.84	11.4
Metabolism of cofactors and vitamins *	4.93	4.4	11.4
Glycan biosynthesis and metabolism	3.38	3.27	3.3
Folding, sorting, and degradation *	3.48	3.14	10.3
Lipid metabolism *	2.73	2.41	12.5
Transcription *	2.29	1.83	22.3
Metabolism of terpenoids and polyketides *	1.76	1.61	8.9
Metabolism of other amino acids *	1.64	1.51	8.3
Signal transduction *	1.6	1.3	20.7
Cell motility *	1.78	1.06	50.7
Biosynthesis of other secondary metabolites *	1.17	1.07	8.9
Xenobiotics degradation and metabolism *	1.16	1.04	10.9
Signaling molecules and interaction	0.55	0.54	1.8
Transport and catabolism	0.49	0.5	2.0

•Indicates p value < 0.05 based on Kruskal-Wallis ANOVA with Bonferroni corrections.

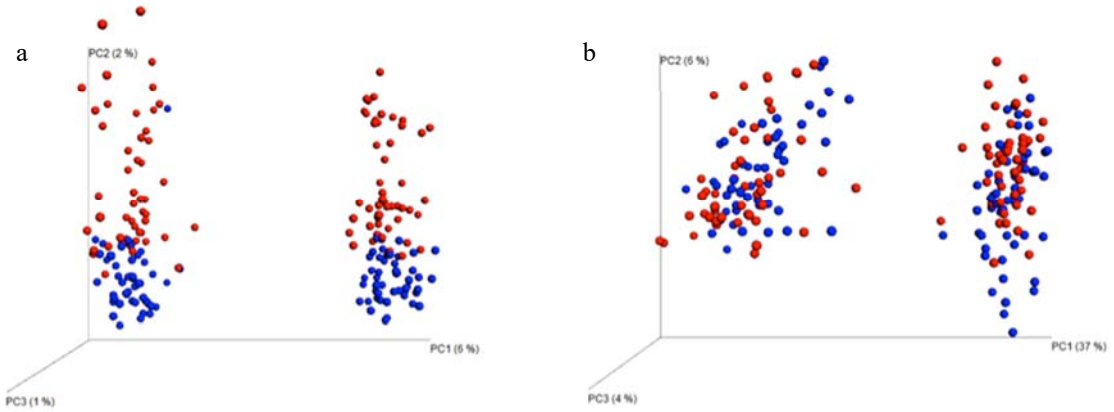

Figure B1: Rumen bacterial community beta diversity differentiation based of sub-species (*Bos taurus*-red and *Bos indicus*-blue, right cluster- liquid and left cluster- solid) using PCoA of unweighted (a) and eighted (b) Unifrac distance (ADONIS: Subspecies unweighted P=0.001 R^2=1.25, weighted P=0.003 R^2=2.07, Fraction unweighted P=0.001 R^2=5.18, weighted P=0.001, R^2=29.85)

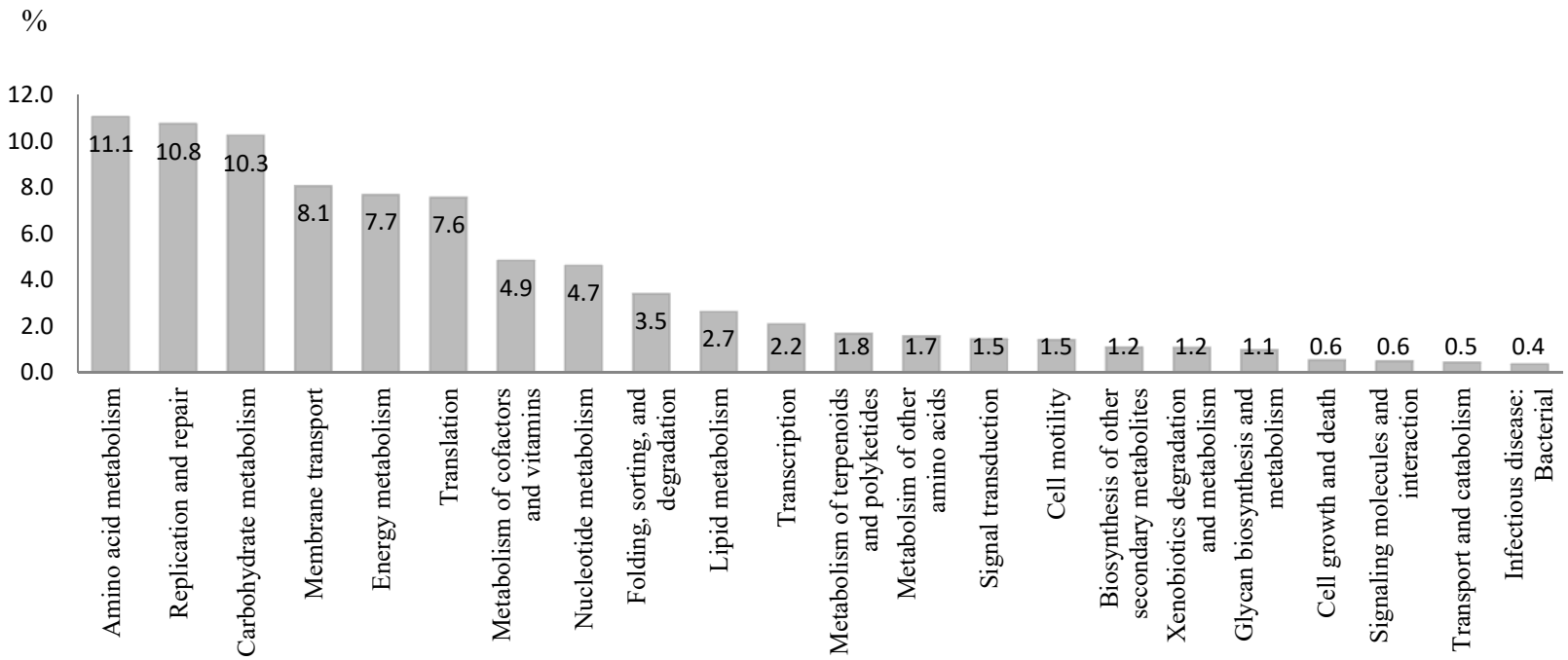

Figure B2: Functional analysis (KEGG PICRUSt) of pathways in the rumen of *Bos taurus* and *Bos indicus*

APPENDIX C CHAPTER III FIGURES AND TABLES

Table C1. Dry matter composition (%) and nutrient analysis of finishing diets fed to steers

	Dietary Treatment[1]		
Item	CDG	SDG	SDG-CH
Ingredient			
Steam-flaked corn	56.6	56.77	58.04
Corn WDG	30.09	-	-
Sorghum WDG	-	29.74	-
Treated sorghum WDG	-	-	29.68
Sorghum stalks	8.52	8.69	8.72
Urea	0.3	0.3	0.33
Limestone	2	2	0.75
Supplement[2]	2.49	2.5	2.47
Analyzed values			
CP, %	17.31	17.86	18.2
NDF, %	17.98	17.02	17.64
ADF, %	7.87	10.82	10.78
EE, %	6.32	6.29	5.6
Ca, %	0.77	0.8	0.73
P, %	0.41	0.43	0.43
S, %	0.29	0.17	0.17
Fermentation characteristics[3]			
Total, mM	141.5^{a}	116.2^{c}	126.6^{b}
Acetate, μmol/ml	38.5^{b}	46.7^{a}	46.0^{a}
Propionate, μmol/ml	41.9^{a}	34.8^{b}	36.0^{b}
Butyrate, μmol/ml	13.7^{a}	10.2^{b}	10.3^{b}
pH	5.37^{c}	5.80^{a}	5.68^{b}
$NH_{3,}$ mg/dL	5.82^{a}	3.64^{b}	3.65^{b}

[1]CDG = 30% corn wet distillers grains plus solubles diet, SDG = 30% sorghum wet distillers grains plus solubles diet, SDG-CH = calcium hydroxide treated sorghum wet distillers grains plus solubles diet (2.67% $Ca(OH)_2$ DM basis).

[2] Provided vitamins and minerals to meet or exceed NRC (2000) requirements. Monensin and Tylosin were included at a rate to provide 22.2 and 5.0 mg/kg, respectively.

[3] Treatment means without a common superscript differ [a,b,c] ($P \leq 0.05$)

Table C2. Summary of shotgun pyrosequencing data obtained from steers (n= 3, replicated) fed a finishing diet supplemented with corn (CDG), sorghum, or calcium hydroxide treated sorghum (SDG-OH) distillers' grains in the solid and liquid fraction.

	Treatment			Fraction	
Parameters	CDG	SDG-CH	SDG	Liquid	Solid
No. of sequences per sample	10355833	10380000	10099167	10278333	10278333
Avg. length of sequences, bp	164.5	165.5	165.7	165.26	165.3
Past QC sequence count	8174614	8130447	7959940	7942738	8233929
Predicted Protein Features	3442615	3442615	3464769	3312624	3587376
Identified Protein Features	1199080	1189080	1199674	1136393	1255495
Identified Functional Categories	597400	596484	603283	567411	630700
DRISEE Error	0.05	0.15	0.09	0.1429	0.05484
Archaea %	0.89	0.82	0.82	0.78[a]	0.90[b]
Bacteria %	97.43	97.18	96.51	96.80	97.30
Eukaryote %	0.40	0.81	1.46	1.24	0.53
Unassigned %	1.29	1.00	1.02	1.96	2.17
% that were significantly different (function KO level 3)		12.6% [25:199]		17.6% [35:199]	
% that wre singnificantly different (taxonomy genus)		12.7% [74:581]		28.6% [166:581]	

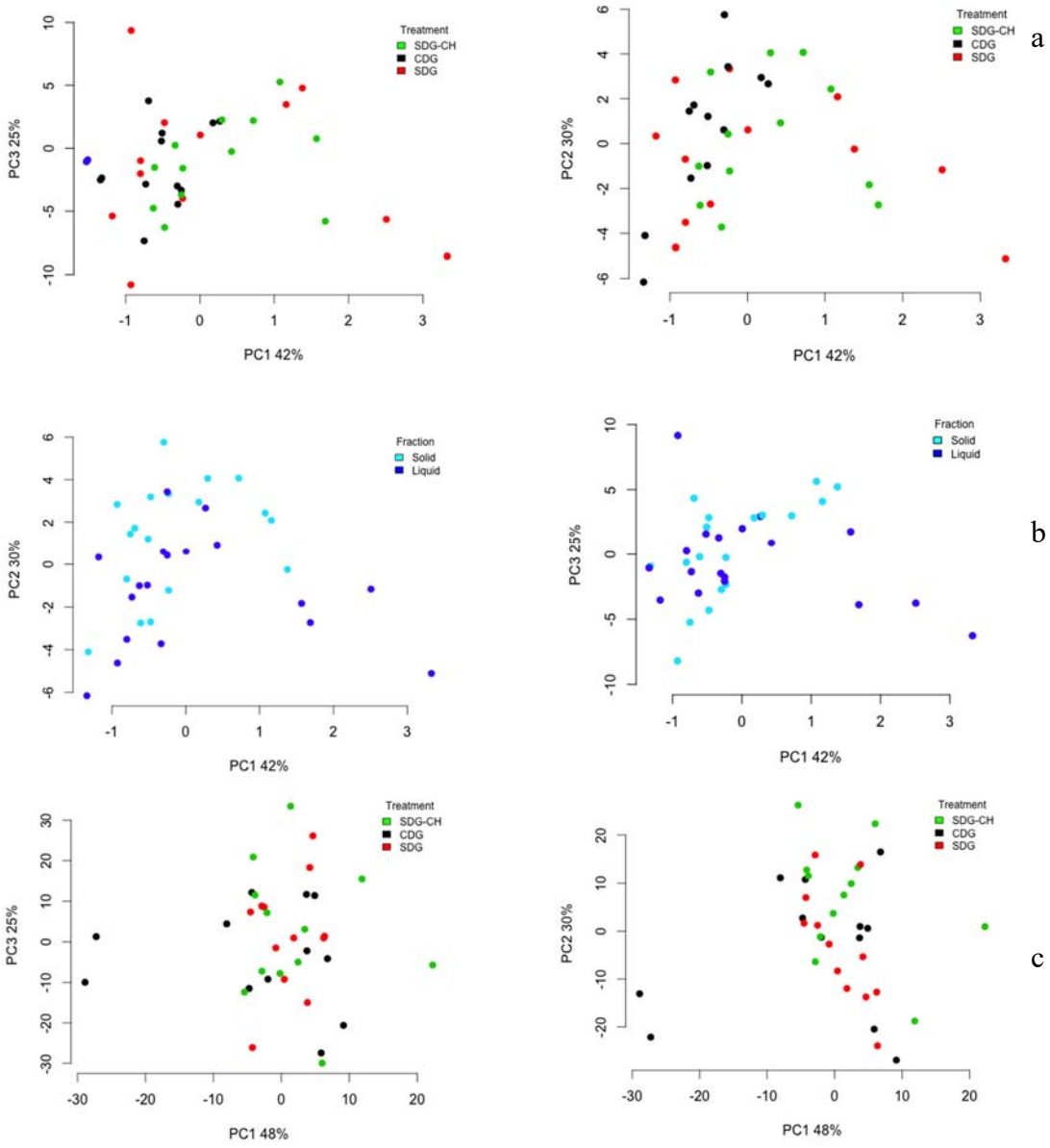

Figure C1: Principal coordinates analysis (PCoA) based on (a) OTU relative abundance of the microbial community composition in steers fed sorghum (SDG), calcium hydroxide treated sorghum (SDG-CH), or corn based distiller's grains (CDG) supplement at 30% with a finishing diet, (b) colored by fraction (solid and liquid) and (c) hierarchical classification colored by treatment. The first three principal coordinated (PC1, PC2, and PC3) from the principal coordinate analysis of weighted UniFrac are plotted for each sample. The variance explained by the PCs indicated on the axes.

Table C3: Top KEGG Level 2 Functional assignments as determined by MG-RAST

	Treatment (Relative abundance %)				*P*-values	
KEGG Level 2[1]	CDG[2]	SDG	SDG-OH	SEM	Treatment	Fraction
Amino Acid Metabolism	21.87	21.51	21.50	0.0037	0.166	0.035
Carbohydrate Metabolism	14.79	14.97	14.97	0.0027	0.521	0.076
Translation	10.67	11.02	10.86	0.0021	0.033	0.005
Replication and repair	7.21	7.21	7.13	0.0006	0.488	0.428
Membrane Transport	6.66	6.45	6.75	0.0026	0.388	0.472
Metabolism of Cofactors and Vitamins	5.60	5.60	5.66	0.0006	0.403	0.035
Nucleotide metabolism	5.40	5.43	5.46	0.0004	0.508	0.13
Energy Metabolism	3.48	3.13	3.17	0.0006	0.001	0.628
Signal Transduction	3.18	3.14	3.10	0.0008	0.251	0.004
Folding sorting and degradation	3.18	3.28	3.21	0.0004	0.074	0
Glycan biosynthesis and metabolism	2.88	2.99	3.01	0.0010	0.022	0.016
Lipid metabolism	2.28	2.08	2.09	0.0005	0	0.667
Biosynthesis o fOther Secondary Metabolites	2.26	2.34	2.29	0.0010	0.666	0.448
Transcription	2.25	2.34	2.32	0.0004	0.112	0.097
Cell growth and death	1.99	2.03	1.99	0.0009	0.723	0.516
Cell motility	1.81	1.48	1.76	0.0029	0.23	0.333
Metabolism of Terpenoids and Polyketides	1.37	1.37	1.37	0.0002	0.964	0.67
Infectious diseases	0.87	0.86	0.86	0.0003	0.831	0.008
Transport and catabolism	0.79	1.01	0.86	0.0014	0.087	0.071
Metabolism of other amino acids	0.48	0.52	0.52	0.0003	0.075	0.002
Xenobiotics Biodegradation and Metabolism	0.30	0.31	0.29	0.0002	0.782	0.09
Environmental Adaptation	0.27	0.29	0.31	0.0001	0.008	0.837

[1] Less than 0.2% excluded

[2] CDG = 30% corn wet distillers grains plus solubles diet, SDG = 30% sorghum wet distillers grains plus solubles diet, SDG-CH = calcium hydroxide treated sorghum wet distillers grains plus solubles diet (2.67% $Ca(OH)_2$ DM basis).

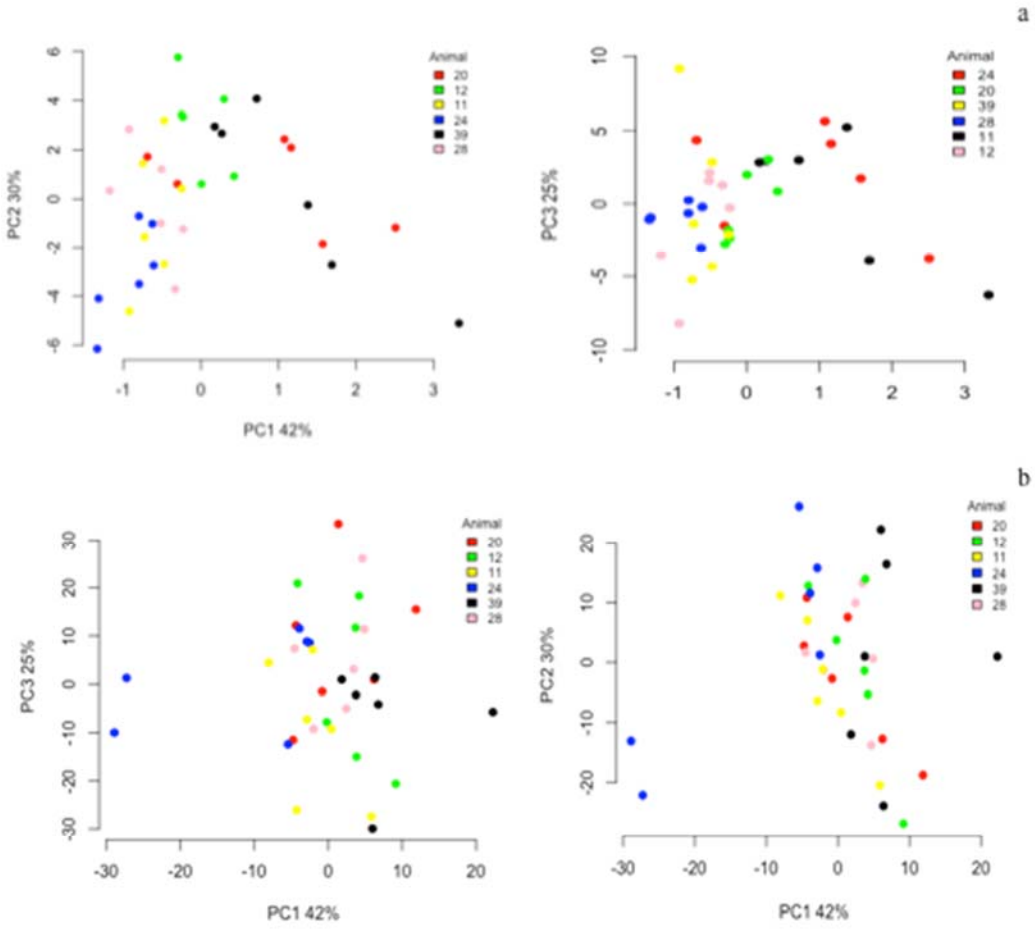

Figure C2: Principal coordinates analysis (PCoA) based on (a) OTU relative abundance of the microbial community composition in steers fed sorghum (SDG), calcium hydroxide treated sorghum (SDG-CH), or corn based distiller's grains (CDG) supplement at 30% with a finishing diet and (b) hierarchical classification colored by individual animal. The first three principal coordinated (PC1, PC2, and PC3) from the principal coordinate analysis of weighted UniFrac are plotted for each sample. The variance explained by the PCs indicated on the axes.

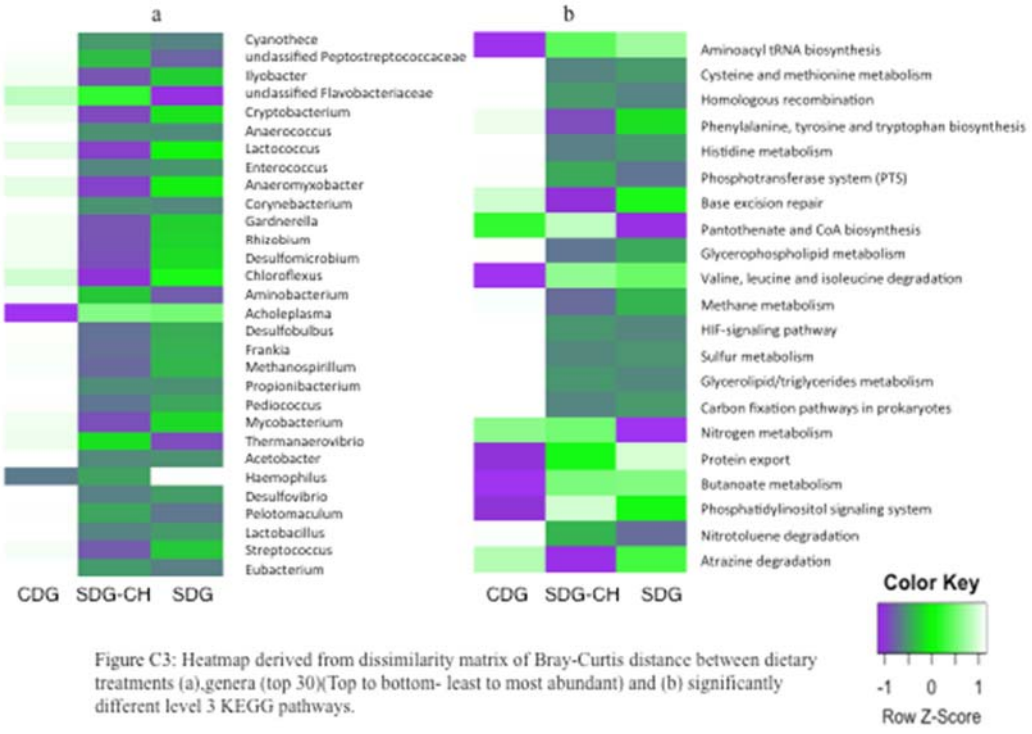

Figure C3: Heatmap derived from dissimilarity matrix of Bray-Curtis distance between dietary treatments (a),genera (top 30)(Top to bottom- least to most abundant) and (b) significantly different level 3 KEGG pathways.

Table C4: Top 45 genera as determine by MG-RAST with shotgun pyrosequencing data obtained from steers (n= 3, replicated) fed a finishing diet supplemented with corn (CDG), sorghum (SDG), or calcium hydroxide treated sorghum (SDG-OH) distillers' grains

	Relative Abundance (%)				*P*-values	
Genera	CDG	SDG	SDG-OH	SEM	Treatment	Fraction
Prevotella	28.03	29.93	30.32	0.0219	0.297	0.19
Bacteroides	17.01	16.88	15.72	0.0095	0.281	0.919
Clostridium	5.77	5.19	5.15	0.0063	0.165	0.013
Eubacterium	3.93	3.19	3.13	0.0058	0.026	0.017
Parabacteroides	2.82	2.89	2.72	0.0024	0.523	0.66
Butyrivibrio	1.45	1.44	1.37	0.0024	0.639	0.001
Acidaminococcus	1.39	1.42	1.34	0.0024	0.887	0.397
unassigned	1.21	1.15	1.13	0.0007	0.361	0.704
Lactobacillus	1.14	0.87	0.89	0.0014	0.001	0.087
Aeromonas	0.97	1.10	1.16	0.0049	0.584	0.02
Porphyromonas	0.95	0.92	0.92	0.0012	0.843	0.409
Bacillus	0.90	0.83	0.83	0.0009	0.257	0.035
Ruminococcus	0.89	0.90	0.94	0.0015	0.776	0.002
Fibrobacter	0.87	1.01	1.31	0.0043	0.121	0.001
Paludibacter	0.81	0.85	0.85	0.0013	0.635	0.087
Shewanella	0.74	0.79	0.87	0.0027	0.467	0.044
Streptococcus	0.71	0.60	0.63	0.0006	0.004	0.021
Bifidobacterium	0.66	0.62	0.59	0.0007	0.615	0.05
Tolumonas	0.65	0.75	0.77	0.0033	0.637	0.022
Flavobacterium	0.64	0.69	0.65	0.0009	0.572	0.74
Treponema	0.63	0.57	1.01	0.0046	0.248	0.012
Desulfitobacterium	0.62	0.52	0.53	0.0006	0.056	0.128
Veillonella	0.60	0.54	0.49	0.0010	0.457	0.312
Vibrio	0.51	0.55	0.63	0.0021	0.368	0.039
Alkaliphilus	0.49	0.44	0.43	0.0005	0.111	0.053
Desulfotomaculum	0.46	0.39	0.38	0.0006	0.096	0.273
Caldicellulosiruptor	0.37	0.33	0.32	0.0004	0.066	0.052
Pseudomonas	0.36	0.37	0.41	0.0011	0.635	0.075
Chitinophaga	0.36	0.34	0.32	0.0002	0.06	0.517
Spirochaeta	0.36	0.27	0.43	0.0017	0.232	0.071
Spirosoma	0.35	0.35	0.32	0.0003	0.123	0.629
Geobacter	0.35	0.31	0.34	0.0002	0.102	0.148
Slackia	0.35	0.34	0.34	0.0002	0.678	0.001
Ethanoligenens	0.34	0.34	0.33	0.0003	0.866	0.752
Geobacillus	0.32	0.27	0.27	0.0004	0.054	0.028
Desulfovibrio	0.32	0.26	0.27	0.0002	0.001	0.678
Thermoanaerobacter	0.31	0.26	0.26	0.0003	0.09	0.171
Paenibacillus	0.29	0.25	0.25	0.0003	0.056	0.04
Pedobacter	0.28	0.30	0.27	0.0004	0.272	0.372
Haemophilus	0.27	0.28	0.37	0.0008	0.01	0.132
Actinobacillus	0.27	0.28	0.32	0.0008	0.35	0.061
Methanobrevibacter	0.27	0.27	0.26	0.0003	0.946	0.051
Dyadobacter	0.26	0.25	0.23	0.0002	0.263	0.807
Pelotomaculum	0.25	0.20	0.19	0.0002	0.019	0.125
Brachyspira	0.24	0.23	0.25	0.0003	0.472	0.042