5-2017

# The Bovine Rumen Microbiome Revealed by Different Fractions of Rumen Contents

Ashlee L. Breakstone
*University of Arkansas, Fayetteville*

# The Bovine Rumen Microbiome Revealed by Different Fractions of Rumen Contents

Ashlee L. Breakstone

University of Arkansas

Primary Investigator: Dr. Jiangchao Zhao

Committee Members: Robert Story, Charles Rosenkrans, Jiangchao Zhao

Project assisted by Fanli Kong and Jeff Pummill

A thesis submitted to the Honors College at the University of Arkansas is partial fulfillment of the requirements for the degree Bachelor of Science in Animal Science with Honors

**ABSTRACT**

The bovine rumen microbiota is very important in terms of animal functionality and digestion. The fermentative capability of the rumen provides means for the digestion of complex plant material that is indigestible by humans. The rumen is the working ground for millions of microorganisms, primarily bacteria, to digest and ferment feed into volatile fatty acids and bacterial protein for the animal's energy and protein needs, respectively. Of significant importance is the rumen microbiomes ability to conform to certain factors such as genetics, feed, or geographic location. These changes can have a direct measure on animal growth, health, and performance. The possibility of productivity boosts in the cattle industry make the rumen microbiome a hot topic in the field of livestock research. A consistent and accurate method for the fractionation of rumen contents would improve the ability for researchers to detect differences found in rumen microbiomes among different animals and treatments. The objective of this study was to determine the view that five different sampling methods of rumen contents would have on the rumen microbiome. Steers fed on hay and fresh pasture wheat were used, which also highlight differences found between diets. Next generation sequencing was used to sequence the V4 region of bacterial 16sRNA. Results were analyzed via Mothur and visualized using R. The results of this study provided no significant differences between fractionation methods, however noteworthy differences were observed between the two diets. Due to the lack of differences between methods, the best method was chosen based on time efficiency and simplicity. However, this study allows research scientists to pick the method of choice without sacrificing the accuracy of results. The importance of this study provides a step towards the universalization of the methods for studying the rumen microbiome, therefore creating consistent results across multiple studies.

## TABLE OF CONTENTS

## INTRODUCTION

For years, ruminant species, specifically cattle, have been of great importance to the United States. The cattle industry is an important division of the United States presenting benefits to the economy, as well as providing a considerable source of food for human consumption. According to beefnutrtion.org, a 3-oz. serving of lean beef provides more than 10 percent of the Daily Value of 10 essential nutrients ("Beef: Big Nutrient Power in a Small Package,"). Several of the nutrients found in beef, including high-quality protein, could be key to the many nutritional issues that Americans face ("Beef: Big Nutrient Power in a Small Package,"). The dietary benefits cattle provide to humans is of high value, but their ability to convert indigestible plant products in the environment into digestible food sources is of equal importance. The advantages cattle provide as a food source do not stand alone: The United States economy prospers highly from the beef and livestock industry. As of 2014, approximately $88.25 billion in farm gate receipts for cattle and calves was reported ("Beef Industry Statistics," 2016). In 2012, the livestock industry produced about $346 billion in total economic output and provided 1.8 million jobs (Dillivan & Davis, 2014). The production and use of cattle, whether for milk or meat sources, is only increasing with time. For years now, research scientists have asked themselves whether the performance and production measures of cattle can be improved. As further advancements in technology and research are made, the knowledge needed to better cattle production has become more available.

The digestive anatomy and physiology of ruminants is highly complex, consisting of 4 stomach compartments. Of these compartments, the rumen is possibly the most important, serving as the primary site for pre-gastric degradation and fermentation. The rumen develops anatomically in size, structure, and microbial activity as a calf grows and undergoes a feed

change from liquid to dry. In mature cattle, the rumen is very large, filling up the entire left side of the abdominal cavity and having the capacity to hold 40-60 gallons of material (Ishler, Heinrichs, & Bánné Varga). Around 150 billion microorganisms per teaspoon are found within the rumen, with both prokaryotes (bacteria and archaea) and eukaryotes (protists and fungi) present (Ishler et al.; McCann, Wickersham, & Loor, 2014; Weimer, 2015). The microorganisms found in the rumen are utilized in a symbiotic host-microbe and microbe-microbe relationship, making the rumen the most important site for microbial activity and fermentation (McCann et al., 2014; Weimer, 2015).

The rumen provides a site where microorganisms can digest carbohydrates, fiber, and protein. Both structural (fiber) and non-structural (sugars and starches) carbohydrates can undergo microbial fermentation. Volatile fatty acids (VFAs) are the primary end products resulting from carbohydrate fermentation. Volatile fatty acids play a crucial role in energy production for the cow and can account for anywhere between 50 to 70 percent of the energy needs for an animal ("Ruminant Anatomy and Physiology," 2017). Another important function of the rumen is its capability to produce microbial protein from non-protein nitrogen sources and feed proteins. The microbial protein that is synthesized within the rumen can be used for most of the animal's protein needs, while the remainder comes from protein that is surpassed into the abomasum to be digested and consequently absorbed by the small intestine. Without the functioning capabilities of the rumen and its working constituents (microorganisms) the animal would lose all digestive functionality. Comparatively, improving rumen function may lead to considerable improvements in digestive and fermentative performances, therefore increasing animal growth and production.

## LITERATURE REVIEW

The rumen microbiota consists of the millions of microorganisms harbored within the rumen, while the microbiome is made of the genes these cells harbor (Ursell, Metcalf, Parfrey, & Knight, 2012). Of these microorganisms, bacteria are by far the most abundant and diverse, accounting for 95 percent of the total microbiota (Brulc et al., 2009). Due to bacteria's primary role in feed degradation and fermentation, it remains the highlight of most studies involving the rumen microbiome (Firkins & Yu, 2015). Past studies have predominantly employed a culture-dependent method of sorts, however, only a small fraction of the microbial diversity in a particular ecosystem can be recovered via cultural methods (Amann, Ludwig, & Schleifer, 1995). Furthermore, the direct microscopic count of bacteria in the rumen ecosystem considerably exceeds the cultivable count (Tajima et al., 1999). Due to an introduction to culture-independent methods involving direct sequencing and analysis of the genome or transcriptome, it is possible to uncover more information on the diversity and roles that bacteria and other microorganisms play in the rumen ecosystem. These tools continue to reveal ways in which bacteria interact and contribute to rumen function.

The bacteria present in the rumen are highly responsive to changes in diet, host genetics, and physiology, as well as geographical and environmental factors (Robert, 2012). These factors can affect the bacterial community in numerous ways regarding structure, composition, and diversity. Both alpha diversity, the microbiome within a niche, and beta diversity, the relationships of microbiomes between two or more different niches, can be affected and therefore measured. These observed differences in the microbial ecology of the rumen can have a direct and quantitative impact on animal function and health. Various studies have shown the impact bacterial populations have on feed efficiency, growth, and performance of the host animal, yet,

much is still unknown with respect to how these factors can be modified to enhance animal production. The efficiency of nutrient utilization can be determined by the balance of fermentation products, VFAs and microbial protein, which can ultimately be controlled by the ruminal microbiota (Hernandez-Sanabria et al., 2012). Ruminal fermentation is necessary for animal growth and maintenance; hence, the rumen microbiota is essential to the animal's well-being and productivity (Jami & Mizrahi, 2012; Jewell, McCormick, Odt, Weimer, & Suen, 2015).

There are 3 interrelated environments associated with the microbial population found in the rumen. The first is a liquid phase, which makes up about 25 percent of the microbial mass. In the liquid phase, free-living microbial groups in the rumen fluid feed on soluble carbohydrates and protein. The second, and largest portion, making up about 70 percent of microbial mass is the solid phase. In the solid fraction, microbial groups are attached to or affiliated with food particles which work to digest insoluble polysaccharides (starch and fiber) and less soluble protein. The smallest phase, which represents the last 5 percent of microbial mass, is the portion of microbes attached to the rumen epithelial cells and protozoa (Ishler et al.). Considering the microbial population's ability to modify based on several elements, such as diet, geographic location or genetics, and the resulting effects this has on the animal, it is necessary to understand ways in which the contrasting rumen fractions might alter the view of the rumen microbiome.

The research directed towards the variance in phases of rumen contents is still new; past reports have determined that a substantial difference between the liquid and solid portions of the rumen exists. These differences likely reflect specialized niches related to digestion of soluble components and dietary fiber (Pitta et al., 2010). The primary phyla found within the rumen of all cattle irrespective of animal diet and age are *Firmicutes* and *Bacteroidetes*. Together these

phyla usually make up anywhere between 80 to 90 percent of the total sequences at the phylum level (de Menezes et al., 2011). This finding is synonymous with past research on the core structure and community of the rumen microbiome. In the same study performed by Menezes et al., (2011) the data showed dominance of the phyla *Fibrobacteres* and *Spirochaetes* in the solid fraction, whereas *Actinobacteria* was much more evident in the liquid phase. A SIMPER analysis revealed that the overall dissimilarity was 14.9% between liquid and solid phases. At the family level, the most prevalent found within the rumen of all cattle were the *Prevotellaceae*, the *Lachnospiraceae*, the *Ruminococcaceae*, and the *Fibrobacteriaceae*. In a study performed by Henderson et al., (2013) the liquid phase of the rumen contents had a higher relative abundance of the family *Prevotellaceae* and a lower relative abundance of the family *Lachnospiraceae* when compared with the total and solid rumen fractions (Henderson et al., 2013). They found that the differences were most noteworthy when the liquid samples were compared with solid and total rumen samples implying that the liquid phase may not be an accurate representation of the total rumen contents.

In a study performed by Pitta et al., (2010) changes in bacterial diversity among the solid, liquid, and whole rumen fractions of 14 ruminally cannulated steers, transitioned from bermudagrass hay (34 days) to grazing wheat forage (28 days), was demonstrated (Pitta et al., 2010). They found that *Prevotella* and *Rikenella* were the predominant genera found in all fractions of both diets. The proportion of the 2 genera was comparable to one another in the solid and whole fractions of bermudagrass, whereas *Prevotella* was more abundant in the liquid fraction. The transition to wheat created a shift towards *Prevotella* dominance in all fractions, however the liquid fraction still held the highest abundance of *Prevotella* regardless of diet. In another study performed by Fouts et al., (2012) it was observed that genera *Prevotella* and

*Tannerella* were overrepresented in the liquid fraction of 12 forage fed steers, and *Butyrivibrio* and *Blautia* were overrepresented in the solid fraction (Fouts et al., 2012). These findings coincide with past conclusions that *Prevotella* is more prevalent in the liquid fraction and *Butyrivibrio* is more abundant in the solid fraction. On the other hand, the *Tannerella* and *Blautia* results vary across studies which may be due to differences in geographical location, diet, time of sampling post feeding, and the genetic background or sex of the animals. A clear distinction between liquid and solid phases of the rumen contents exists, however the function of these specific niches is still unfamiliar.

Past research regarding the diversity of bacterial species between the rumen content phases (solid and liquid) has been conflicting. In a study performed by Kong et al., (2010) they found that the Shannon measure of diversity present in the solid fraction was measured at 1.9 which was 3.5-3.8 times higher than either of the two fractions (liquid and loosely attached particles), regardless of diet type (Kong, Teather, & Forster, 2010). Similarly, in another study, the number of known bacteria was greater in the solid fraction of rumen (Cho et al., 2006). On the contrary, a study by de Menezes et al., (2011) used a rarefaction analysis to determine that the bacterial diversity was higher in the liquid compared to the solid fraction of rumen contents (de Menezes et al., 2011). McCann et al., (2014) analyzed the rumen content fractions of steers fed separate diets of hay and wheat and found that the liquid fraction of the hay diet contained the greatest number of bacteria compared to the lowest number in the whole digesta fraction of the wheat diet (McCann et al., 2014). This discrepancy could be explained by the apparent differences in bacterial diversity between hay and wheat diets, which found that steers on the hay diet had a greater bacterial diversity within the rumen contents regardless of fraction. Further investigation toward the rumen fractions is needed to provide additional insight into the

microbiological niches that might be present and the differences that exist between rumen phases.

**SIGNIFICANCE**

The research and manipulation of the rumen microbiome has a strong influence on the livestock industry, and equally mankind. One of the most prevalent studies involves the effects the rumen microbiome has on animal feed efficiency and production. The single largest expense in most commercial beef production enterprises is providing feed to cattle (Arthur, Archer, Herd, & Melville, 2001). Animals with lower feed efficiencies have a higher cost of production, consequently, any effort at improving the efficiency of feed utilization by animals will drastically reduce total costs on production sites (Arthur et al., 2001; Hernandez-Sanabria et al., 2012). Research in rumen microbiology provides a basis for manipulation of rumen microorganisms and the potential to advance ruminal fermentation, thus maximizing animal production. Changes in cattle growth, performance, and health are all possibilities resulting from the study of the rumen microbiome. Despite the importance of the previously mentioned implications, there is also a future in the mitigation of a considerable greenhouse gas, methane, and the potential to provide a rich source of enzymes for industrial processes and biofuel production (Durso, Wells, & Kim, 2015).

**OBJECTIVE**

The significance of the rumen microbiome makes it a high priority in the field of research. Although fundamental variation in the rumen microbiome is present, a consistent sampling technique will improve the ability to detect microbiome differences among animals or treatments. Accompanying the issue of uniformity, comes two additional factors: ease and accuracy. In the past only a few methods of rumen sampling have been employed in studies

involving the rumen microbiome. Of the previously used sampling methods, the most common involves the separation of rumen contents into liquid and solid fractions; the rumen contents are passed through four layers of sterile cheesecloth. This method often requires additional squeezing of the cheesecloth to obtain the maximum amount of liquid from the contents. The separated portions are then frozen at -80°C until further DNA extraction. An additional method, applied in only few studies, utilizes a metal sieve, rather than cheesecloth, to filter the rumen contents. Through the development of novel approaches, as well as comparisons between standard methods, the efficiency and accuracy of sampling the bovine rumen may improve. The objective of this study is to determine the effect, if any, that 5 different sampling methods have on the view of the rumen microbiome. The outcome of this study will provide a possible method(s) that produces the most stable and consistent view of rumen microbiome allowing for more efficient, and possibly more accurate sampling of the rumen contents.

## MATERIALS AND METHODS

### Animal Treatment

The experimental procedure used in this study was in accordance with the university's standards for animal care and research.

### Rumen Sample Collection and Storage

The samples used were obtained from 8 Black Angus steers involved in a coinciding study involving the comparison between hay and fresh pasture wheat diets. On week 2 of the study, rumen samples from 4 steers fed on wheat and 4 steers on hay were extracted using a separate, sterile oral stomach tube (5/8" O.D. x 3/8" I.D. x 10', Valley Vet Supply, Marysville, Kansas). Following immobilization of the head, a Frick speculum (Valley Vet Supply, Marysville, Kansas) was inserted into the mouth and over the base of the tongue. The beveled

end of the stomach tube was inserted through the speculum and slowly down the esophagus as the animal swallowed. Correct placement of the tube inside the rumen was confirmed by the distinctive odor of fermented gas detected coming from the other end of the tube. Internal rumen pressure produced enough sample contents to fill 2 sterile 50ml centrifuge tubes per animal. The samples were immediately put on dry ice for transport to the laboratory. Immediately following arrival at the laboratory, the samples were transferred to an ultralow freezer at -80°C for future microbiome analysis. The steers used in this study were provided by the Batesville Station, Division of Agriculture, University of Arkansas. This portion of the study was performed and provided by Robert Story and Jiangchao Zhao.

**Rumen Fractionation Methods**

Various methods of rumen sampling were used in this study to obtain 5 contrasting fractions of the rumen contents. Prior to sampling, the contents were pulled from -80°C and thawed overnight at 4°C. Each sample of rumen contents were briefly vortexed directly before each procedure to assure the contents were evenly integrated. The first fraction, representing the whole digesta, was collected via pulling a direct sample of rumen contents. A 100 µl sample was obtained for each direct fraction using a 300 µl notched pipette tip to avoid congestion caused by the density of solid material in the rumen contents. The next sample, also representing the whole rumen digesta, was obtained by homogenizing 1 ml of contents in a paddle blender (Stomacher 400, Seward Ltd., Worthing, West Sussex, UK) (2 min, normal speed). Following homogenization, a 100 µl sample of blended contents were pulled from the stomacher bag. The third fraction, representing the solid rumen contents, was collected using a centrifugal method. One ml of rumen contents was pipetted into a bead beating tube and centrifuged (1 min, 13000g). Succeeding centrifugation, the supernatant was extracted and discarded, leaving the remaining

solid contents for further sampling (avg. weight=). The last 2 fractions, depicting liquid and solid portions of rumen contents, were attained using a filtration method. Five ml of rumen contents was tightly squeezed through 4 layers of sterile cheesecloth. From the filtered liquid portion, a 100 µl sample was used, and any remaining pellet, with small cheesecloth debris, was used for the solid fraction of the contents (avg. weight=0.2g). Each sample was transferred to -80°C until use for further DNA extraction.

**DNA Extraction**

A physical bead-beating disruption method was used for microbial cell lysis and total DNA extraction. The extractions were performed using the MO BIO PowerLyzer PowerSoil protocol and DNA isolation kit (MO BIO Laboratories (a Qiagen company), Carlsbad, California), with few minor adjustments. The adjustments made were performed as follows: 100 mg of solid or 100 µl of liquid were initially used to begin the extractions; after solution C1 was added, the tubes were heated in a water bath at 65°C for 10 min (gently swirling halfway at 5 min); the samples were homogenized in a bead beater (2 min, 3500 rpm), let sit for 2 min, and the bead beating process was repeated a second time; following bead beating, the samples were centrifuged the next 3 times at 13,000 g (all other centrifugal steps were performed as written); after solution C2 and C3 were added the samples were incubated on ice for 5 min; and lastly, 50 µl of solution C6 was used. All extracted DNA was stored at -80°C after quantification was performed using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Madison, WI). After amplification, the DNA was sequenced using the Illumina MiSeq System (Illumina, Inc., San Diego, CA).

### Sequence Processing and Bioinformatics

The sequencing reads were denoised and analyzed using mothurv1.39.1 software package and followed the standard operating procedures of the MiSeq platform contributed by Pat Schloss (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013; Schloss et al., 2009). The sequences were aligned using the SILVA reference database before preclustering (Huse, Welch, Morrison, & Sogin, 2010). Chimeric sequences were removed based on the UCHIME algorithm (Edgar, Haas, Clemente, Quince, & Knight, 2011). Clean and high-quality data was assigned to operational taxonomic units (OTUs) with a 97% similarity cutoff. A representative sequence for each OTU was picked and assigned to taxonomic data using the Ribosomal Database Project (RDP) classifier (Wang, Garrity, Tiedje, & Cole, 2007). The diversity and composition of bacterial communities was determined at an OTU level. To reduce biases caused by sequencing efforts, the number of reads per sample was randomly subsampled to 8660 for diversity analysis.

The Shannon and Observed OTU (sobs) were utilized to measure community diversity and richness for alpha diversity (Chao & Shen, 2003). The Bray-Curtis and Jaccard distance metrics were calculated to estimate the differences in community structure and membership for beta diversity (Bray & Curtis, 1957). These distances were visualized by principle coordinate analysis (PCoA) and plotted using R (R version 3.3.2).

## RESULTS

### Sequencing Summary

The results were characterized by sequencing the bacterial 16S V4 hyper-variable region of the rumen microbiota. In total, 40 samples were described from 8 steers (4 hay, 4 wheat) with 5 different sample treatments per steer. A total of 532,735 high quality sequencing reads were obtained with an average of 13,318 reads per sample ranging from 8,662 to 19,931. The

sequences were classified into 9,147 OTUs. The reads of each sample were rarefied to 8,660 by random subsampling. The coverage ranged from 93% to 98% with an average of 96%.

**Alpha Diversity**

The bacterial community diversity and richness was measured using the Shannon Index and the Observed OTU index (Sobs), respectively. Between diets the Shannon measure of diversity and the community richness (Observed OTU) were significantly different ($P<.05$). The rumen microbiome of steers on hay diets showed much higher diversity and number of observed OTUs when compared to steers on wheat (Figure 1). The highest number of observed OTUs was found in the post stomacher liquid portion of a hay fed animal (tag 180) and the lowest number was found in the solid portion of a wheat fed animal (tag 41). Similar trends were found when comparing the Shannon diversity index. This tendency can be explained by the apparent differences found between hay and wheat diets. The alpha diversity between rumen sampling methods was similar when compared between treatments. No significant difference was found between methods, however the solid fraction tended to show slightly lower diversity when compared to the whole and liquid portions.

**Beta Diversity**

The beta diversity was visualized using PCoA plots based on Jaccard and Bray-Curtis matrices. Distinct patterns were found in bacterial community structure and membership between hay and wheat diets. The steers fed wheat diets showed a much higher variability in comparison to the steers fed hay, which remained associated within each sample treatment (Figure 2). Comparison between methods proved to show insignificant differences in community structure and membership between each treatment which is reflected by similar movements on the ordination plots (Figure 2). Correlations statistics between each method were also shown by the

Mantel test comparing Bray-Curtis distance matrices. In the hay diets, the correlation measures comparing each sample method was greater than 0.8 with a P value of less than 0.05. Furthermore, the correlation measures found between methods in the hay diets were more variable, with the lowest correlation found between the solid only and liquid only fractions, however these results were insignificant ($P>0.05$). In conclusion, the sampling methods did not produce any significant differences in rumen bacterial community structure or membership.

**Community Composition**

The relative abundance of the top 20 OTUs per sample treatment was examined (Figure 3). The microbial communities between hay and wheat were distinct showing significant differences in the distributions of OTUs. The distribution of OTUs between sample methods was more similar, however distinct features between the solid portion when compared to the whole digesta and liquid portions can be found.

**DISCUSSION**

The results from this study will aid in future endeavors towards research in the bovine rumen microbiome. In past studies a large difference has been found between different fractions of rumen contents. Although some minor differences were found, the substantiality of differences was irrelevant. The research done by Pitta et al., (2010) found that the genera *Prevotella* was dominant in all samples, but a shift towards wheat, rather than hay, created a shift in the dominance of *Prevotella*. Further, the liquid fraction of samples contained a higher dominance of *Prevotella* when compared to the solid and whole fractions. On the contrary, the results from this study showed a slight dominance of *Prevotella* in the hay fed animals, but this could potentially be explained by the separation of diets, rather than a shift between diets. Similarly, the sample method portraying the solid fraction of this study was slightly lacking in

*Prevotella* in comparison to the other samples. Fouts et al., (2012) similarly found a dominance of *Prevotella,* apart of the family *Prevotellaceae,* in the liquid fraction and *Butyrivibrio,* apart of the family Lachnospiraceae, in the solid fraction. However, I found no significant differences in genus and family levels when comparing sampling methods.

The top two phyla found within all rumen microbiomes was *Firmicutes* and *Bacteroidetes* which is consistent with most past studies confirming that these two phyla are a part of the core rumen microbiome regardless of diet, age, fraction, etc. Significant differences in the relative abundance of each was found between diets. The phylum *Firmicutes* showed a significant dominance in the steers fed hay. These results may indicate that diet has a much higher effect on community composition than the chosen sampling method.

The community diversity and richness presented in my results have been of similar conclusions made in the past. The research on diversity present in different fractions of rumen contents has been conflicting. Kong et al., (2010) and Cho et al., (2006) both found that the solid fraction of rumen contents had a higher number of known bacteria, known as species richness. On the contrary, McCann et al., (2011) and de Menezes et al., (2014) found higher measures of diversity in the liquid fraction of rumen contents. The results from this study remained neutral, showing no differences in diversity between sampling methods. Again, significant differences of diversity were found between diets rather than sampling methods. Steers on hay diets had much higher levels of diversity and richness when compared to steers on wheat diets, which is congruent with the results found by Pitta et al., (2010).

In conclusion, no consequential distinctions were made between the five sampling methods chosen to characterize the rumen microbiome. Due to the lack of differences found

between fractionation methods, I can safely say that the direct method is the preferred choice. This method is the most user-friendly and time efficient, making it possible for researchers across multiple contexts each with different time limitations, equipment, or money barriers to achieve equivalent results. However, the importance of this study proves that any of the above-mentioned fractionation methods can be used depending on user preference without the certainty of the results being given up. One limitation may have been in the method of rumen collection, via the stomach tube, which is considered the liquid portion of rumen contents by some researchers. Future research utilizing rumen cannulation and the comparison of sampling methods is needed to thoroughly understand the results of this study. This step towards universalizing sampling approaches used in the study of the rumen microbiome is important for researchers everywhere. The bovine rumen and its microorganisms are a prevalent topic in the field of livestock research due to the potentials of improving ruminal fermentation and animal production. This study and future considerations into the methods of rumen fractionation makes it possible for scientists with lacking equipment, money, or time to use the rumen sampling method of choice, without sacrificing accurate results.

# References

Amann, R. I., Ludwig, W., & Schleifer, K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev, 59*(1), 143-169.

Arthur, P., Archer, J., Herd, R., & Melville, G. (2001). *Response to selection for net feed intake in beef cattle.* Paper presented at the Proceedings of the Association for the Advancement of Animal Breeding and Genetics.

Beef Industry Statistics. (2016).

Beef: Big Nutrient Power in a Small Package. *Beef Nutrients.*

Bray, J. R., & Curtis, J. T. (1957). An ordination of the upland forest communities of southern Wisconsin. *Ecological monographs, 27*(4), 325-349.

Brulc, J. M., Antonopoulos, D. A., Miller, M. E., Wilson, M. K., Yannarell, A. C., Dinsdale, E. A., . . . White, B. A. (2009). Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. *Proc Natl Acad Sci U S A, 106*(6), 1948-1953. doi:10.1073/pnas.0806191105

Chao, A., & Shen, T.-J. (2003). Nonparametric estimation of Shannon's index of diversity when there are unseen species in sample. *Environmental and ecological statistics, 10*(4), 429-443.

Cho, S. J., Cho, K. M., Shin, E. C., Lim, W. J., Hong, S. Y., Choi, B. R., . . . Yun, H. D. (2006). 16S rDNA Analysis of Bacterial Diversity in Three Fractions of Cow Rumen. *Journal of Microbiology and Biotechnology, 16*(1), 92-101.

de Menezes, A., Lewis, E., O'Donovan, M., O'Neill, B., Clipson, N., & Doyle, E. (2011). Microbiome analysis of dairy cows fed pasture or total mixed ration diets. *Federation of European Microbiological Sciences, 78*, 256-265.

Dillivan, K., & Davis, J. (2014). Economic Benefits of the Livestock Industry.

Durso, L., Wells, J. E., & Kim, M. S. (2015). Diversity of microbiomes in beef cattle. *Encyclopedia of Metagenomics: Environmental Metagenomics*, 129-138.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., & Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics, 27*(16), 2194-2200. doi:10.1093/bioinformatics/btr381

Firkins, J. L., & Yu, Z. (2015). RUMINANT NUTRITION SYMPOSIUM: How to use data on the rumen microbiome to improve our understanding of ruminant nutrition. *J Anim Sci, 93*(4), 1450-1470. doi:10.2527/jas.2014-8754

Fouts, D. E., Szpakowski, S., Purushe, J., Torralba, M., Waterman, R. C., MacNeil, M. D., . . . Nelson, K. E. (2012). Next generation sequencing to define prokaryotic and fungal diversity in the bovine rumen. *PLoS One, 7*(11), e48289. doi:10.1371/journal.pone.0048289

Henderson, G., Cox, F., Kittelmann, S., Miri, V. H., Zethof, M., Noel, S. J., . . . Janssen, P. H. (2013). Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. *PLoS One, 8*(9), e74787. doi:10.1371/journal.pone.0074787

Hernandez-Sanabria, E., Goonewardene, L. A., Wang, Z., Durunna, O. N., Moore, S. S., & Guan, L. L. (2012). Impact of feed efficiency and diet on adaptive variations in the

bacterial community in the rumen fluid of cattle. *Appl Environ Microbiol, 78*(4), 1203-1214. doi:10.1128/AEM.05114-11

Huse, S. M., Welch, D. M., Morrison, H. G., & Sogin, M. L. (2010). Ironing out the wrinkles in the rare biosphere through improved OTU clustering. *Environ Microbiol, 12*(7), 1889-1898. doi:10.1111/j.1462-2920.2010.02193.x

Ishler, V. A., Heinrichs, A. J., & Bánné Varga, G. *From feed to milk : understanding rumen function*.

Jami, E., & Mizrahi, I. (2012). Composition and similarity of bovine rumen microbiota across individual animals. *PLoS One, 7*(3), e33306. doi:10.1371/journal.pone.0033306

Jewell, K. A., McCormick, C. A., Odt, C. L., Weimer, P. J., & Suen, G. (2015). Ruminal Bacterial Community Composition in Dairy Cows Is Dynamic over the Course of Two Lactations and Correlates with Feed Efficiency. *Appl Environ Microbiol, 81*(14), 4697-4710. doi:10.1128/AEM.00720-15

Kong, Y., Teather, R., & Forster, R. (2010). Composition, spatial distribution, and diversity of the bacterial communities in the rumen of cows fed different forages. *FEMS Microbiol Ecol, 74*(3), 612-622. doi:10.1111/j.1574-6941.2010.00977.x

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., & Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol, 79*(17), 5112-5120. doi:10.1128/AEM.01043-13

McCann, J. C., Wickersham, T. A., & Loor, J. J. (2014). High-throughput Methods Redefine the Rumen Microbiome and Its Relationship with Nutrition and Metabolism. *Bioinform Biol Insights, 8*, 109-125. doi:10.4137/BBI.S15389

Pitta, D. W., Pinchak, E., Dowd, S. E., Osterstock, J., Gontcharova, V., Youn, E., . . . Malinowski, D. P. (2010). Rumen bacterial diversity dynamics associated with changing from bermudagrass hay to grazed winter wheat diets. *Microb Ecol, 59*(3), 511-522. doi:10.1007/s00248-009-9609-6

Robert, W. (2012). The bacterial community composition of the bovine rumen detected using pyrosequencing of 16S rRNA genes. *Metagenomics, 2012*.

Ruminant Anatomy and Physiology. (2017). *Feeding the Dairy Herd.*

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., . . . Weber, C. F. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol, 75*(23), 7537-7541. doi:10.1128/AEM.01541-09

Tajima, K., Aminov, R. I., Nagamine, T., Ogata, K., Nakamura, M., Matsui, H., & Benno, Y. (1999). Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. *FEMS Microbiology Ecology, 29*(2), 159-169.

Ursell, L. K., Metcalf, J. L., Parfrey, L. W., & Knight, R. (2012). Defining the human microbiome. *Nutrition reviews, 70*(suppl 1), S38-S44.

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol, 73*(16), 5261-5267. doi:10.1128/AEM.00062-07

Weimer, P. J. (2015). Redundancy, resilience, and host specificity of the ruminal microbiota: implications for engineering improved ruminal fermentations. *Front Microbiol, 6*, 296. doi:10.3389/fmicb.2015.00296

**FIGURES**

1.)

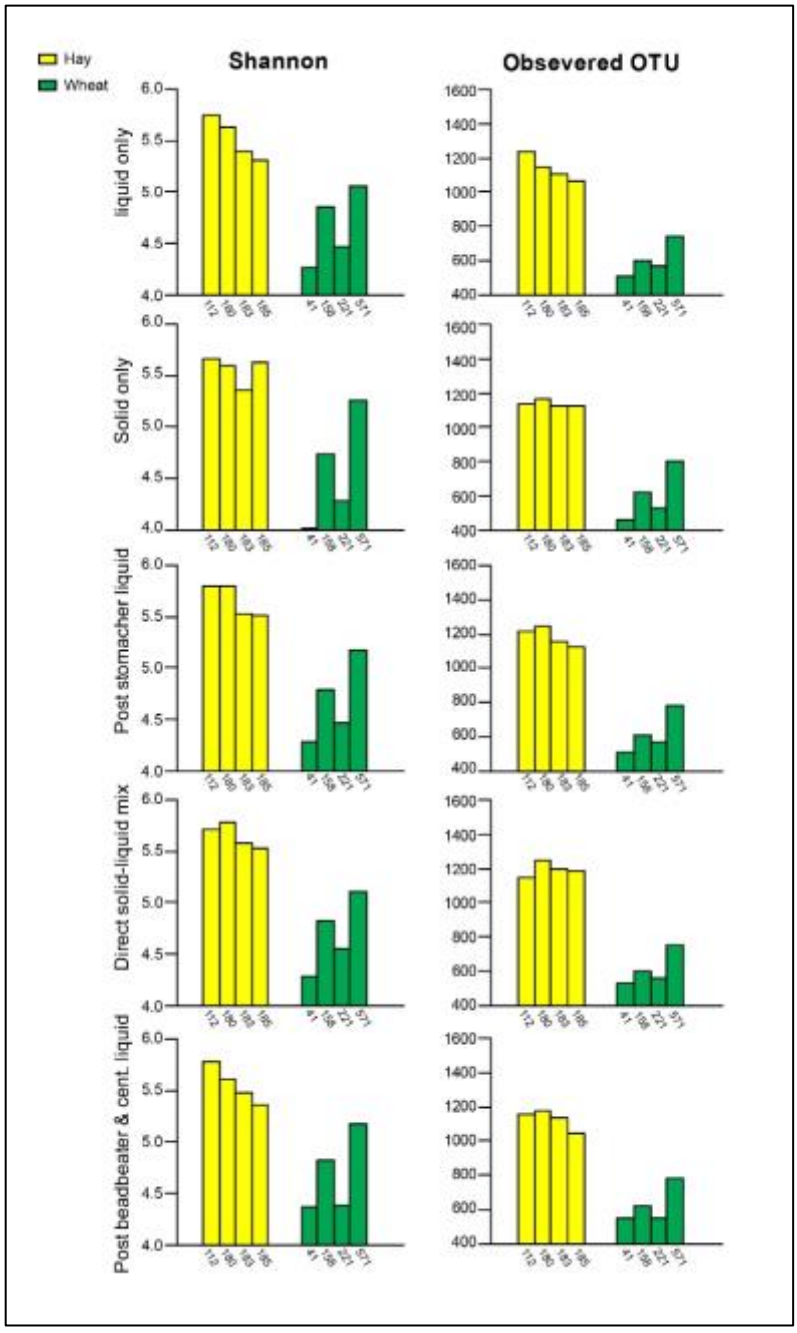


Figure 1 Shannon diversity index and Observed OTU index across treatments.

2.)

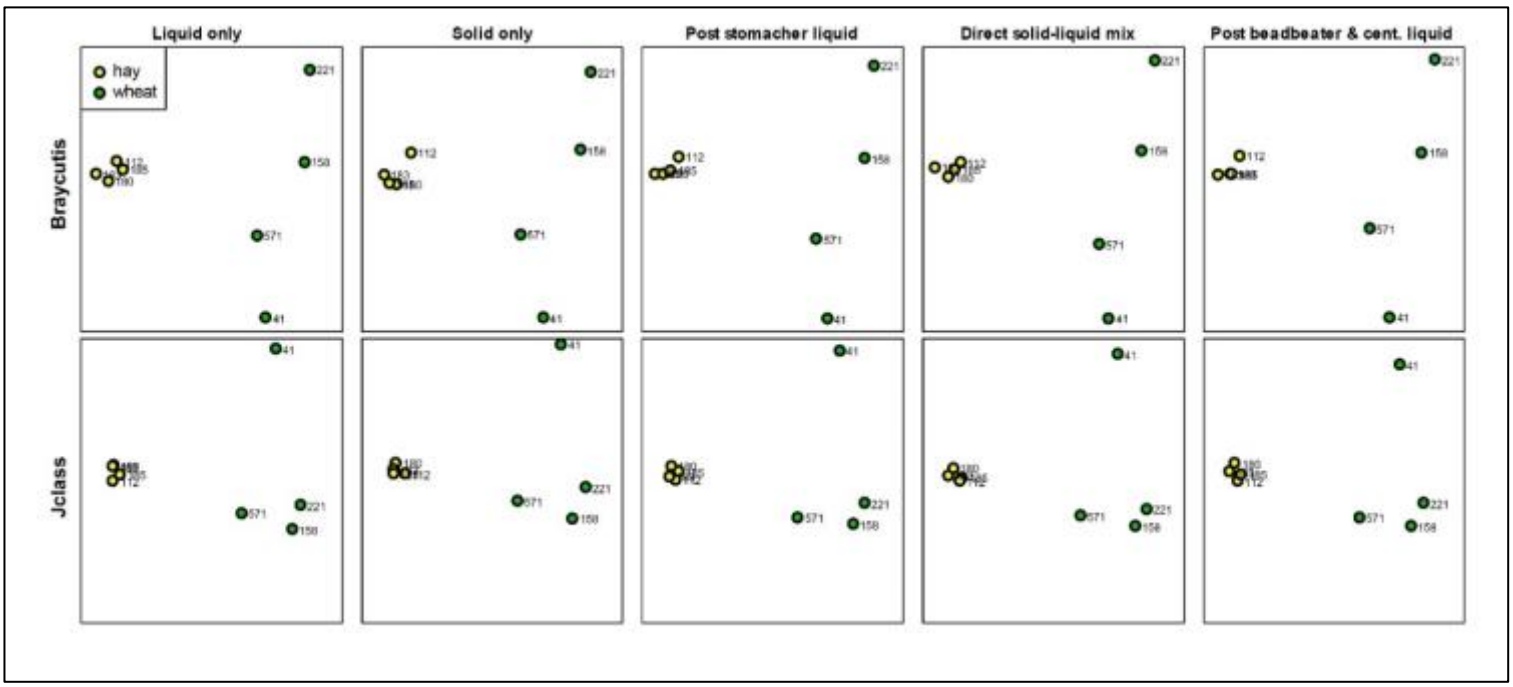


Figure 2 Braycurtis and Jaccard distance matrices showing bacterial composition and community differences across treatments.

3.)

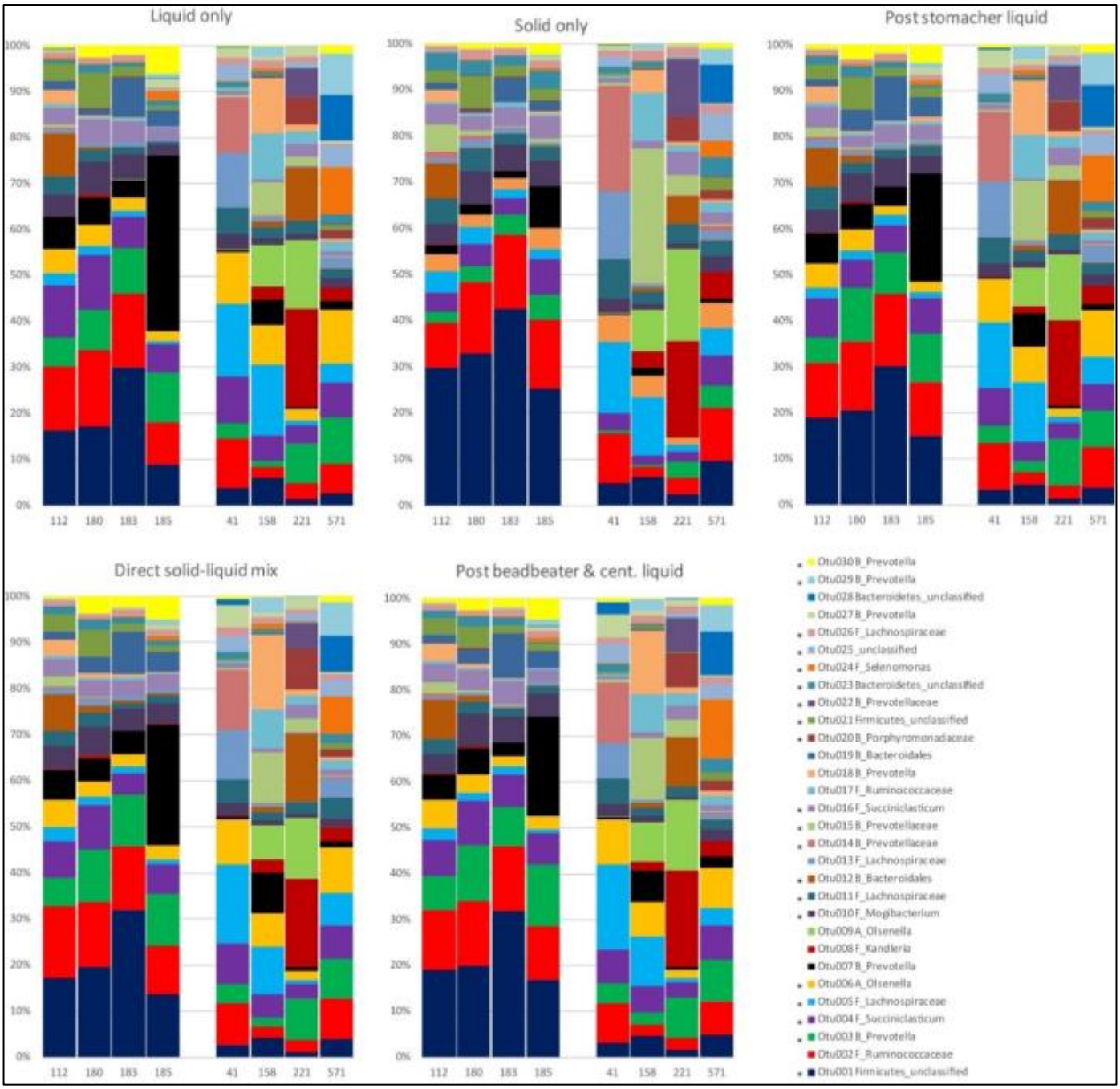


Figure 3 Relative abundance of top 20 OTUs found per method.