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The Bovine Rumen Microbiome Revealed by Different Fractions of Rumen Contents

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The bovine rumen microbiome revealed by different fractions of rumen contents

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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 microbiota is made up of billions of microorganisms, primarily bacteria, that digest and ferment feed into volatile fatty acids and bacterial protein for the animal's energy and protein needs, respectively. Changes to the rumen microbiota 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 hay and fresh pasture wheat were used, which also highlights differences found between diets. Next generation sequencing was used to sequence the V4 region of bacterial 16sRNA. Results were analyzed via Mothur, an open source command-line used to analyze sequencing data in microbial communities, and visualized using R, a command-line software used for statistical analysis and graphical display. 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. The results of this study allow research scientists to pick the method of choice without sacrificing the accuracy of results.

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Meet the Student-Author

Ashlee Breakstone

I am from St. Louis, Missouri and graduated from Fort Zumwalt West High School in 2013. I graduated from the University of Arkansas in May 2017 with a major in Animal Science with a focus in microbiological research. My last two years at the University have been dedicated to my honors research project. I have also had the opportunity to be a part of numerous clubs such as Pre-Veterinary club and Block&Bridle club. For the past year, I have served as the President of the Arkansas Union Advisory Committee where I was able to improve policies and events in the Arkansas Union. I have also served as an Honors College Ambassador for the 2016–2017 academic year, which has given me access to numerous volunteer opportunities on and off campus. After graduation, I will enter a Master's Program for Molecular and Cell Biology at the University of Texas at Dallas. I hope for a long future in research and academia.

I would like to thank Dr. Jiangchao Zhao for serving as my honors mentor for this project and Robert Story and Charles Rosenkrans for serving as committee members. I would also like to acknowledge Jeff Pummill, Fanli Kong, and Robert Story for their help and assistance in the completion of this project.

Introduction

Within the livestock industry, ruminant species, such as cattle, make up a considerable component and present valuable resources to the United States. The economic value and substantial food source cattle provide are extensive. According to beefnutrition.org, a 3-oz. serving of lean beef provides more than 10% of the Daily Value of 10 essential nutrients (Cattlemen's Beef Board and National Cattlemen's Beef Association, 2017). The nutrients and high-quality protein found in beef could be crucial to the numerous nutritional issues Americans face (Cattlemen's Beef Board and National Cattlemen's Beef Association, 2017). Economically, the United States prospers from the beef and livestock industry. As of 2014, approximately $88.25 billion in farm gate receipts for cattle and calves were reported (National Cattlemen's Beef Association, 2016). In 2012, the livestock industry produced about $346 billion in total economic output and provided 1.8 million jobs (Dillivan and Davis, 2014). The production of cattle, regardless of end-product, is increasing with time. Researchers have begun to ask themselves whether the performance and production of cattle can improve. Due to advancements in technology and research, the knowledge needed to enhance the cattle industry has become more available.

The digestive system of a ruminant animal is highly complex in that it is made up of four separate stomach compartments: the rumen, reticulum, omasum, and abomasum. Of these four compartments, the rumen is possibly the most important and certainly the largest comprising the entire left side of the abdominal cavity and having the capacity to hold 40-60 gallons of material (Ishler et al., 1996). Around 150 billion microorganisms per teaspoon can be found in the rumen, ranging from prokaryotic species (bacteria and archaea) to eukaryotic species (protists and fungi) (Ishler et al., 1996; McCann et al., 2014; Weimer, 2015). The microorganisms found inside the rumen are a part of a mutually beneficial, host-microbe relationship (McCann et al., 2014). The microorganisms are provided essential nutrients needed for survival and consequently break down complex nutrients for the host that would otherwise be indigestible. These capabilities make the rumen the most important site for microbial activity and fermentation (Weimer, 2015).

Carbohydrates, both structural (fiber) and non-structural (sugars and starches), and proteins undergo microbial fermentation in the rumen. Volatile fatty acids (VFAs) are the primary end products resulting from carbohydrate fermentation. Volatile fatty acids play a crucial role in host energy demand, accounting for 50% to 70% of the energy production in cattle (Regents of the University of Minnesota, 2017). Another important function of the rumen is the capability to produce microbial protein from non-protein nitrogen sources and feed proteins. Microbial protein produced by microorganisms can be used for most

of the animal's protein needs, while the remainder is digested and absorbed in the abomasum and small intestine, respectively. The rumen and its working constituents (microorganisms) are necessary for digestion; therefore, loss of this function would lead to host productivity failure. Comparatively, improving rumen function may lead to significant improvements in digestive and fermentative performances; therefore, increasing animal growth and production.

The rumen microbiota is made up of the millions of microorganisms harbored within the rumen, while the microbiome is made of the genes these cells harbor (Ursell et al., 2012). Bacteria are by far the most abundant and diverse, accounting for 95% of total microbiota (Brulc et al., 2009). The prevalence of bacteria and its consequent role in feed degradation and fermentation make it the highlight of most studies involving the rumen microbiome (Firkins and Yu, 2015). Past research methods involving the microbiome have used culture-dependent methods, such as isolation and cultivation of species. This is a very limited approach due to the immense number of bacteria that are not cultivable (Tajima et al., 1999). More recent microbiome research uses culture-independent methods which involve direct DNA and RNA sequencing and analysis. These novel approaches make it possible to uncover more information on the diversity and roles that bacteria and other microorganisms play in the rumen ecosystem.

The bacteria in the rumen are highly responsive to changes in diet, host genetics, and physiology, as well as geographical and environmental factors (Wu et al., 2012). The bacterial community can be affected in numerous ways regarding membership, composition (abundance), and diversity. The alpha diversity, the microbiome within a specific environment, and the beta diversity, the relationships of microbiomes between two or more different environments, can be affected and measured. The observed and measured differences in microbial ecology can have a direct and quantitative impact on animal function and health. Ultimately, the rumen microbiota controls the balance of fermentation products, such as VFAs and microbial protein, which determines the efficiency of nutrient fermentation and utilization; hence, the rumen microbiota is essential to the animal's well-being and productivity (Hernandez-Sanabria et al., 2012; Jami and Mizrahi, 2012; Jewell et al., 2015).

In the rumen, there are three interrelated environments associated with the microbial population. The liquid phase makes up about 25% of the microbial mass and consists of the free-living microbial groups in the rumen fluid. The largest portion, making up about 70% of the microbial mass, is the solid phase including all microbial groups attached or affiliated with food particles in the rumen. The microbes attached to the rumen epithelial cells and protozoa make up the last 5% of the microbial mass found inside the rumen (Ishler et al., 1996). Considering the microbial population's ability to modify according to several elements (diet, geographic location, genetics, etc.) and the effects these have on the animal, it is necessary to understand the ways in which the contrasting rumen fractions and fractionation methods might alter the view of the rumen microbiome. The research directed towards the different phases of the rumen contents is still new; past studies have determined that a substantial difference between the liquid and solid portions of the rumen exists and these differences could possibly reflect specialized functions related to digestion of feed (Pitta et al., 2010). Further investigation into the different rumen fractions is needed to provide additional insight into the microbiological functions that might be present.

The research and manipulation of the rumen microbiome has a strong influence on the livestock industry leading to possible changes in cattle growth, performance, and health. The significance of the rumen microbiome makes it a high priority in the field of research. Although fundamental variation in the rumen exists, a consistent sampling technique will improve the ability to detect microbiome differences among animals or treatments. Through the development of novel approaches and 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 five 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 the rumen microbiome.

Materials and Methods

The samples used for this study were obtained from 8 black angus steers involved in a coinciding study involving the comparison between hay and fresh pasture wheat diets. On week two of the study, rumen samples from four steers fed on wheat and four steers on hay were extracted using a separate, sterile oral stomach tube (5/8 inch outside diameter × 3/8 inch inside diameter × 10 ft, Valley Vet Supply, Marysville, Kansas). The steers used in this study were provided by the University of Arkansas System Division of Agriculture's Batesville Station. This portion of the study was performed and provided by Don Hubbell, Tom Hess, and Jiangchao Zhao.

Various methods of rumen sampling were used in this study to obtain five different fractions of rumen contents. Prior to each method, the contents were pulled from -80 °C and thawed overnight at 4 °C. Each sample was briefly spun under high speeds (vortexed) directly before each procedure to effectively mix the contents. The first fraction,

representing the whole digesta (meaning all of the ingested food and material found within the rumen), was collected via pulling a direct 100-µl sample of rumen contents. Also, representing the whole digesta, the next sample was obtained by homogenization of contents in a paddle blender (Stomacher 400, Seward Ltd., Worthing, West Sussex, U.K.) (2 min, normal speed). Following homogenization, a 100-µl sample of blended contents was pulled from the stomacher bag. The third fraction, representing the whole digesta, was collected using a centrifugal method. The contents were centrifuged in a bead-beating tube and the following supernatant, or liquid lying above the solid residue, was discarded, leaving the remaining solid-like contents for further sampling. The last two fractions, depicting the solid and liquid portions, were attained using a filtration method. The rumen contents were tightly squeezed through four layers of sterile cheesecloth. A 100-µl sample of filtered liquid was used for the liquid portion and the remaining solids were used for the solid fraction. The solid end-products had weights ranging from 200 to 300 mg. Each sample was transferred to -80 °C until use for further DNA extraction.

A physical bead-beating disruption method (where contents are put in a small tube with tiny micro-beads to disrupt cellls and release DNA) was used for microbial cell lysis (disintegration or rupture of the cell) 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. All extracted DNA was stored at -80 °C after quantification was performed using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Madison, Wisconsin). After quantification, the V4 region of 16SRNA was amplified and sequenced using the Illumina MiSeq System (Illumina, Inc., San Diego, California).

The sequencing reads from the bacterial DNA were aligned and analyzed using mothur v. 1.39.1 software package and followed the standard operating procedures of the MiSeq platform contributed by Pat Schloss (Kozich et al., 2013; Schloss et al., 2009). The diversity and composition of bacterial communities was determined at an operational taxonomic unit (OTU) level with a 97% similarity cutoff. The Shannon and Observed OTU (sobs) indices were utilized to measure community diversity and richness, respectively (Chao and Shen, 2003). The Bray-Curtis and Jaccard distance metrics were calculated to estimate the differences in community structure and membership for beta diversity (Bray and Curtis, 1957). The mantel test was used to determine the statistical correlation and significance between sampling methods. These distances were visualized by principle coordinate analysis (PCoA) and plotted using R (R version 3.3.2).

Results and Discussion

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 8662 to 19,931. The sequences were classified into 9147 OTUs. The coverage ranged from 93% to 98% with an average of 96%.

The results from this study will aid in future endeavors towards research in the bovine rumen microbiome. Although some minor differences were found, the substantiality of differences was inconsequential. In past studies, a large difference has been found between different fractions of rumen contents. The research done by Pitta et al. (2010) found that the genera *Prevotella* was dominant in all samples, but the liquid fraction of samples contained a greater dominance of *Prevotella* when compared to the solid and whole fractions. Similarly, upon examination of the top 20 OTUs per sample treatment, the solid fraction of this study was slightly lacking in *Prevotella* in comparison to the other samples which is also consistent with results found by Fouts et al. (2012) (data not shown). Despite this minor observation, there were no significant differences in genus and family levels when comparing sampling methods.

The two most abundant 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. (data not shown) (de Menezes et al., 2011). Significant differences in the relative abundance of each was found between diets with phylum *Firmicutes* showing a significant dominance in the hay fed steers (data not shown). These results may indicate that diet has a much greater effect on community membership than the sampling approach.

The Shannon measure of diversity takes into account both community richness, or number of observed species, and community evenness, or abundance of specific species, whereas the observed OTU index is solely the community richness. The measured diversity in opposing phases of the rumen contents has been conflicting. In studies performed by Kong et al. (2010) and Cho et al. (2006), it was found that the solid fraction contained a higher measure of diversity and a greater number of known bacteria (species richness). On the contrary, a study by de Menezes et al. (2011) determined that the bacterial diversity was higher in the liquid fraction. 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 fraction of the wheat diet. In this study, results across diets showed that the Shannon measure of diversity and the community richness (observed OTU) were significantly different ($P < 0.05$) (Fig.

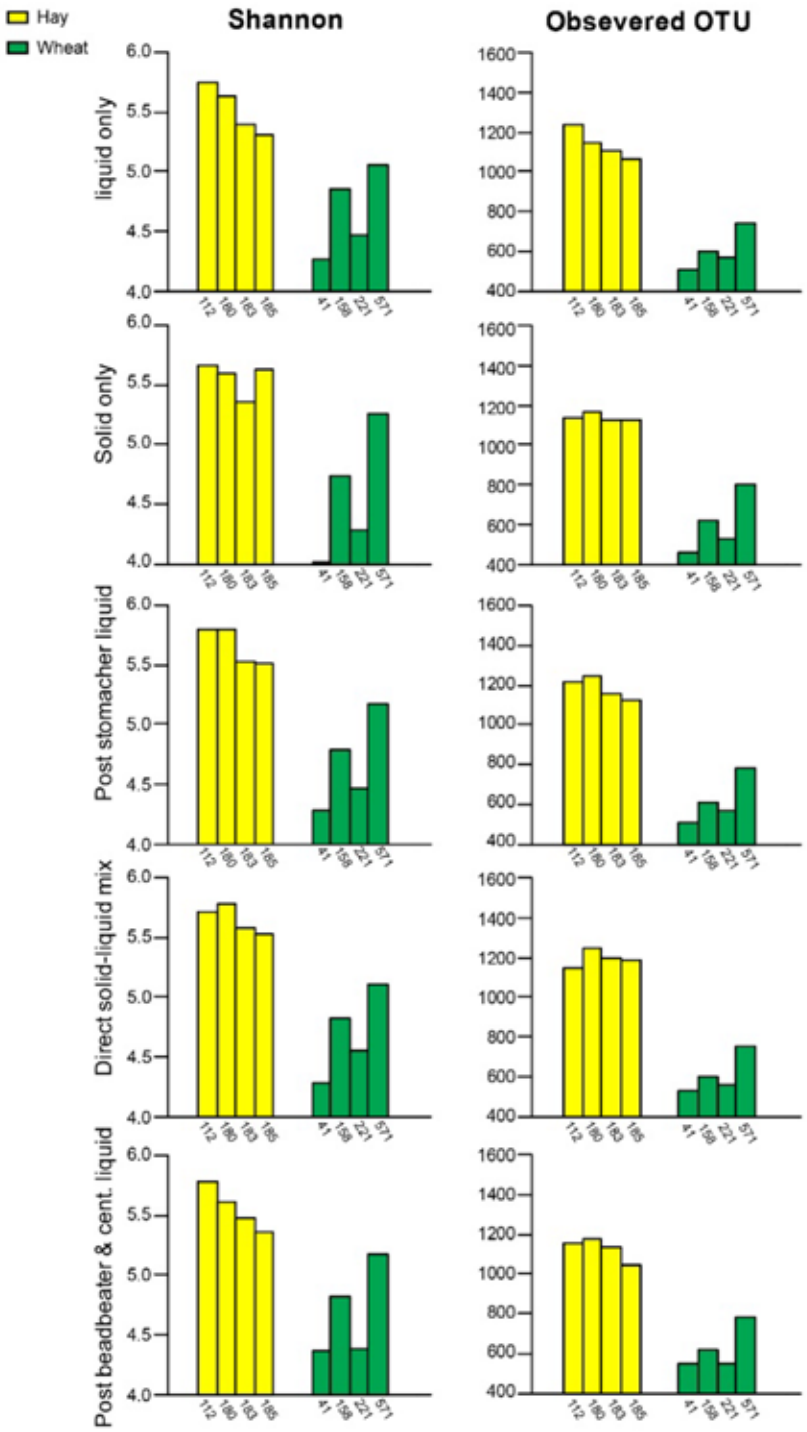

Fig. 1. The Shannon and Observed operational taxonomic unit (OTU) indices showing the alpha diversity (species richness and evenness) and species richness, respectively found in each diet and sample. Operational taxonomic units are individual and distinct organisms found in the sequences. The *x*-axis portrays the 8 steers with each number representing a specific animal and the two distinct colors portraying hay- and wheat-fed animals.

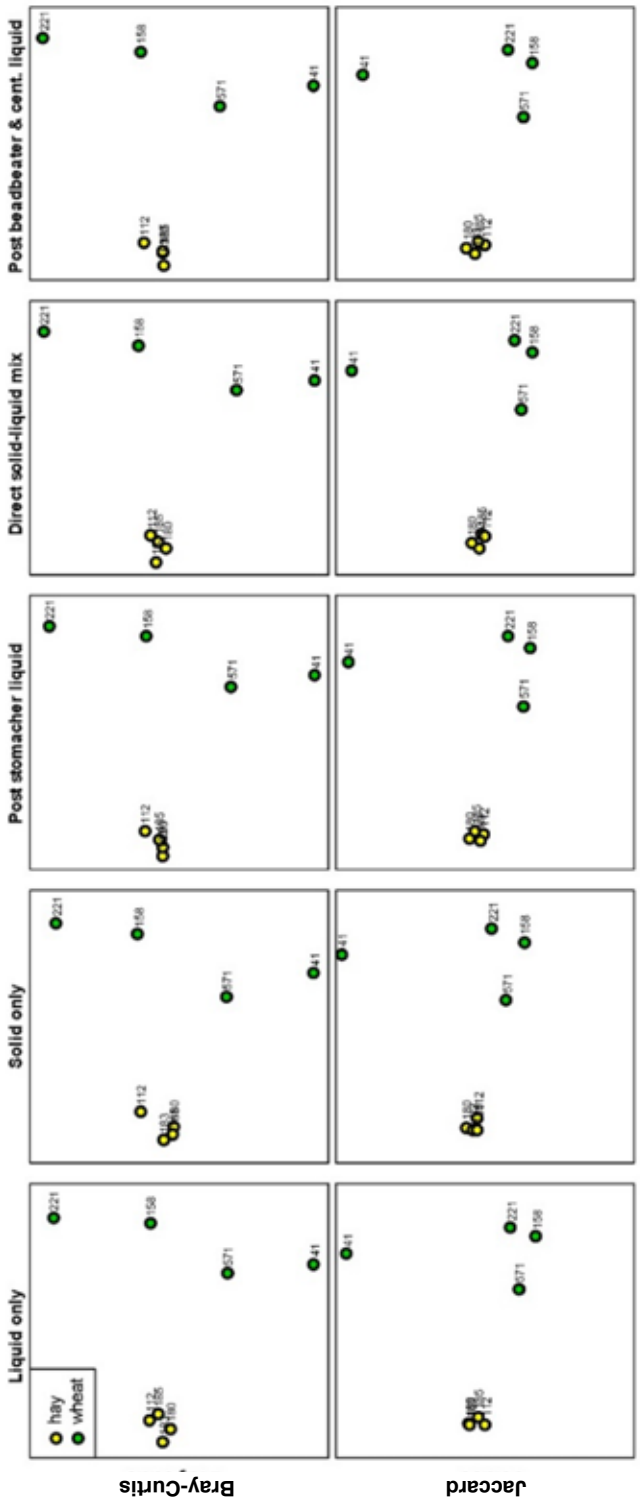

Fig. 2. Bray-Curtis and Jaccard distance matrices representing the beta diversity found between diets and samples. The Bray-Curtis coefficient considers community membership and structure, whereas the Jaccard coefficient only considers the community membership. Each numbered point represents a specific animal and the colors separate the hay- and wheat-fed animals. Across methods, the matrices are nearly identical reinforcing the inconsequential effects that the method has on the rumen microbiome.

1). Steers on hay diets had greater levels of diversity and richness when compared to steers on wheat diets, which is congruent with the results found by Pitta et al. (2010). When sample treatments were compared, there were no significant differences, keeping results neutral amid conflicting past results.

Distinct patterns in bacterial community structure and membership (beta diversity) were found between hay and wheat diets (Fig. 2). The wheat-fed steers had a much higher variability in comparison to the hay-fed steers. Comparison between methods showed insignificant differences between each treatment which is reflected by similar movements on the ordination plots. Using the mantel test, correlation statistics showed that each sample method in hay diets had a high correlation value at 0.8 and $P < 0.05$. 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.

Conclusions

No consequential distinctions were made among the five sampling methods chosen to characterize the rumen microbiome. Due to the lack of differences found among fractionation methods, 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 financial barriers to achieve equivalent results. However, the importance of this study indicates that any of the above-mentioned fractionation methods can be used, depending on user preference, without the certainty of the results being compromised. 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 (withdrawal of rumen contents by directly inserting a tube to the cow's abdomen through to the rumen) 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. This study and future considerations into the methods of rumen fractionation makes it possible for scientists with limitations in equipment, money, or time to use the rumen sampling method of choice, without sacrificing accurate results.

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