

Article

Whole Genome Metagenomic Comparison of Microbial Communities and Activities in Solid and Liquid Rumen Microenvironments in Beef Steers

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Abstract

Previous research has established distinctions between microbial communities that associate with either the solid or liquid rumen microenvironments utilizing 16S rRNA sequencing. Whole genome metagenomic sequencing (WGS) facilitates comprehensive analysis of a microorganism's entire genome, producing strain level data and providing insights into microbial functionality. The objective of our study was to utilize WGS to characterize microbial species and enzyme profiles associated with the liquid versus solid rumen microenvironments in beef steers consuming forage. Four steers consuming forage were utilized in a 4 × 4 Latin Square experiment; on d 14 of each period, rumen contents were separated into solid and liquid samples and DNA was extracted then sequenced using WGS. Alpha and beta diversity measures revealed differences between solid and liquid microenvironments. There were also differences in enzyme profiles: carbohydrate-active enzyme classes were different across microenvironments, except glycosyl transferase. Further, the solid microenvironment was enriched in enzymes related to energy production/conversion and nutrient transport and metabolism, while the liquid microenvironment was enriched in coenzyme transport and metabolism enzymes. Species associated with cellulolytic activities were generally more abundant in the solid microenvironment while those associated with amylolytic activity were more abundant in the liquid. Genes related to methanogenesis were also different across microenvironments. Our findings offer valuable insights into the stratification of microorganisms across rumen microenvironments, expanding on existing literature with findings from next-generation sequencing and application to forage-based beef systems.

Keywords: beef cattle; metagenomics; methane; microbiome; rumen; sequencing

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1. Introduction

Ruminant animals have a symbiotic relationship with microorganisms where the rumen provides an ideal environment for proliferation [1,2] and products of microbial fermentation can be utilized to meet host animal energetic and other nutritional needs. The rumen is stratified into distinct and heterogenous microenvironments (i.e., liquid, solid, gaseous) [3] that microorganisms associate with depending on populations present, characteristics and composition of the feed, and other factors that dictate rumen conditions [4]. The solid and liquid rumen microenvironments contain mixed populations of microorganisms which are transient and have constant interactions with and mutual influences on each other [5].

The solid rumen microenvironment is characterized by its high substrate availability and, therefore, microbes present in this fraction are involved in fiber fermentation [4,6], which is critically important given the dietary strategy of ruminant animals. Conversely, the liquid microenvironment has lower substrate availability and free-floating microbial species that often detach from biofilms and adhere to feed particles; thus, there are fewer opportunities for metabolic reactions as microbes are likely to be washed out of the rumen with digesta [7].

Previous literature has consistently demonstrated that microbial composition differs between rumen microenvironments [5,8–11], reflecting spatiality associated with diet utilization and colonization within the rumen. Much of this research has employed 16S ribosomal ribonucleic acid (rRNA) sequencing, which utilizes polymerase chain reaction (PCR)-based amplification to identify taxa at the genus level [12,13]. However, 16S rRNA technology does not provide insight into the functional capabilities, genes, and/or pathways of microbial communities [14]. Therefore, these studies have established broad taxonomic partitioning between ruminal fractions but provide limited interpretation of microbial functionality and activities. For example, microenvironment-specific differences in microbial communities have been reported in lactating dairy cows [8], demonstrating enrichment of fibrolytic taxa in solid rumen fractions and soluble substrate specialists in liquid, but without functional gene characterization.

Metagenomic studies have begun to describe the functional potential of microbial communities within the rumen, including carbohydrate-active enzyme (CAZyme) activities in fiber-associated populations [15] and rumen microbial metagenome-assembled genomes (MAG; [16]). Further, some researchers have employed these analyses to explore the solid and liquid rumen microenvironments of cattle [17,18] and sheep [19]. However, whole genome metagenomic sequencing (WGS) comparisons of the taxonomic composition and functional potential of liquid- and solid-associated rumen microbial communities remain relatively limited, particularly in forage-fed beef cattle.

While previous studies have largely characterized rumen microenvironments in dairy cattle [8,11,20], beef production systems are centered around forage resources which differ substantially from total mixed rations (TMR) fed to dairy cows. These dietary differences alter ruminal fermentation dynamics underlying microbial community structure [1] and are likely to influence functional gene distribution across ruminal microenvironments. Therefore, microbial profiling across ruminal microenvironments observed in dairy production systems may not fully represent that of forage-fed beef cattle.

Whole genome metagenomic sequencing provides more thorough and comprehensive information about an organism's genetic makeup and generates strain- or species-level taxonomic data utilizing the whole genome, providing insights into the functionality of the genes present [13,21]. Therefore, metagenomic profiling that provides insights at a deeper taxonomic level, together with CAZyme and Clusters of Orthologous Group (COG) functional annotations across ruminal microenvironments, provide a more comprehensive view of how microbial functional potential is partitioned; this extends the current literature focused on profiling rumen microenvironments, especially for forage-fed beef cattle. Accordingly, the objective of our study was to characterize microbial species and enzyme profiles that are differentially associated with the distinct rumen microenvironments using WGS in beef steers consuming forage.

2. Materials and Methods

2.1. Experimental Design and Animals

Procedures were approved by the Institutional Animal Care and Use Committee at Texas State University (#8693). Four ruminally cannulated Angus steers were used in a

4 × 4 Latin Square design experiment such that each steer received each treatment over the duration of the trial.

Steers ($n = 4$, 539 ± 54 kg BW) were housed in individual stalls within a partially enclosed barn and offered ad libitum water, hay (coastal blend of kleingrass and medio bluestem) fed at 130% of the previous 5 d intake, and a trace mineral block in addition to alfalfa cubes provided at 200 g/kg of the previous 3 d hay intake (Table 1). Prior to the experiment, steers were adapted to stalls for 10 d. Each of the four periods lasted 28 d, including an 8 d adaptation to treatment, 5 d for measurements of intake and digestion, 1 d for determinations of ruminal fermentation and microbiome, 1 d sampling for subcutaneous adipose tissue, and 14 d of carryover for the ruminal microbiome to return to baseline as established in the literature [10].

Table 1. Chemical composition of basal diet.

Item	Hay	Alfalfa Cubes
% Dry Matter		
Organic matter	89.6	86.7
Neutral detergent fiber	69.9	35.4
Acid detergent fiber	49.3	27.8
Crude protein	3.44	17.9
Ether extract	1.70	2.21
Calcium	0.35	1.71
Phosphorus	0.04	0.20
Potassium	1.27	2.87

Treatments were a negative control containing no feed additive, and thymol at either 120, 240, or 480 mg/kg of hay and alfalfa cube intake from the previous day; treatments were not provided during the carryover period. Data related to treatment were not the focus of this study and are published elsewhere [22]; only data related to portioning of the rumen microbiome are presented here.

2.2. Sampling and Laboratory Analysis

On d 14 of each period at 4 h after feeding, ruminal contents were sampled (~1 kg) from four areas of the ventral and dorsal sac of the rumen for metagenomic analysis. This sampling time was selected based on past literature which indicates that fermentative activities are maximized in this relative timeframe after feeding [23]. Rumen contents were squeezed through four layers of cheesecloth to separate the solid and liquid fractions. Samples were snap-frozen in liquid nitrogen and stored at −80 °C before being thawed for subsequent DNA extraction and analysis.

After thawing, DNA was extracted from both solid ($n = 16$ from 4 steers) and liquid ($n = 16$ from 4 steers) rumen samples using the QIAmp PowerFecal Pro DNA kit (Qiagen, Valencia, CA, USA) protocol. Using Qubit fluorometry, DNA was tested for quantity and quality. The DNA samples were processed for Next Generation Sequencing (NGS) library preparations, and WGS sequencing was performed on a Novaseq NGS machine (Illumina, San Diego, CA, USA) using a 150 base pair (bp) sequencing strategy. For data sharing, raw sequencing reads were deposited in the NCBI Sequence Read Archive as Bioproject PRJNA1417001.

2.3. Bioinformatics

For taxonomic and abundance profiling, raw sequencing reads were mapped to two reference genome collections: Hungate1000 Collection [24] and Compendium of 4941 rumen metagenome-assembled genomes for rumen microbiome biology and enzyme discov-

ery ([16]; European Archive project ID PRJEB31266). The collections were downloaded from their respective data repositories and contigs in each were concatenated in one file as a reference genome using custom perl scripts. For sequencing reads mapping, a genome index for each reference genome was made using software Bowtie2 (v2.2.4) build function. Custom perl scripts were used to identify sequencing reads that uniquely map to one genetic loci without mismatch (alignment score = 0), or map to several loci but one of the loci exhibits perfect sequence matching (alignment score = 0). Sequencing reads matching these conditions were quantified using custom perl scripts.

For microbial enzyme analysis, raw sequencing reads underwent quality control using fastp (v0.23.2) with adapter trimming and removal of low-quality bases (average Phred score < 30). Host DNA contamination was eliminated by mapping reads to the bovine genome using Bowtie 2 (v2.5.3). De novo contig assembly was performed with MEGAHIT (v1.2.9). Open reading frame (ORF) prediction on the assembled contigs was conducted using Prodigal (v2.6.3) with the -p meta option to account for metagenomic data. Predicted coding sequences < 99 bp were discarded. The remaining protein sequences were clustered at a 95% identity threshold using CD-HIT to generate a non-redundant protein dataset, retaining representative sequences.

Functional annotation of the non-redundant protein dataset was achieved using eggNOG-mapper (v2.1.12), aligning sequences against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, COG [Categories: A—RNA processing and modification, B—Chromatin structure and dynamics, C—Energy production and conversion, D—Cell cycle control/cell division/chromosome partitioning, E—Amino acid transport and metabolism, F—Nucleotide transport and metabolism, G—Carbohydrate transport and metabolism, H—Coenzyme transport and metabolism, I—Lipid transport and metabolism, J—Translation/ribosomal structure and biogenesis, K—Transcription, L—Replication/recombination and repair, M—Cell wall/membrane/envelope biogenesis, N—Cell motility, O—Posttranslational modification/protein turnover/chaperones, P—Inorganic ion transport and metabolism, Q—Secondary metabolites biosynthesis/transport and catabolism, S—Function unknown, T—Signal transduction mechanisms, U—Intracellular trafficking/secretion/vesicular transport, V—Defense mechanisms, W—Extracellular structures, Y—Nuclear structure, Z—Cytoskeleton] [25] and CAZymes.

Custom Python 3.14 scripts were utilized to extract specific gene families, including methanogenesis-related genes, as well as proteins belonging to certain COG categories and CAZyme families, from the functional annotation output.

2.4. Statistical Analysis

Statistical analysis was conducted using R statistical software (version 4.3.2; IBM Corp., Cary, NC, USA). To generate relative abundances, raw read counts for each microbial strain were divided by total read counts for each sample. Within species, relative abundances of strains were totaled to determine species-level abundances. Relative abundances of species, COG categories, and CAZyme families were log₁₀-transformed prior to analysis to satisfy model assumptions of normality and homogeneity of variance and to appropriately model the proportional, compositional nature of relative abundance data.

Using the lmer model within the lme4 package, species-level relative abundances and the relative abundance of COG categories and CAZyme families were analyzed where fixed effects in the model were microenvironment (i.e., solid or liquid) and period with a random effect of steer. Differences in microenvironments were determined using Type III Analysis of Variance. Benjamini–Hochberg false discovery rate (FDR) correction was applied separately to species-level and enzyme class-level comparisons to control for Type I errors that can arise from the simultaneous testing of many taxa and enzyme classes.

Alpha diversity indexes and species richness were calculated using the vegan package. Differences in alpha diversity across microenvironment were determined using the same linear mixed model as described above. Bray–Curtis dissimilarity was also calculated using the vegan package, and community composition differences were evaluated using PERMANOVA. Differences in within-group multivariate dispersions were assessed using PERMDISP. Community composition was visualized using principal coordinates analysis (PCoA), plotted with the ggplot2 package, with 95% confidence ellipses drawn around each microenvironment group. Heatmaps of relative abundance for the most dominant taxa were generated using the pheatmap package, with taxa clustered by hierarchical clustering and samples grouped by microenvironment. Abundance values were row-scaled (z-score) to visualize relative differences across samples.

3. Results

3.1. Alpha Diversity Index

For alpha diversity index, there was a difference in species richness between microenvironments ($p \leq 0.01$; Table 2) where the liquid microenvironment had more species present than the solid (4665 versus 4663 species, respectively). There was not an effect of microenvironment on Shannon diversity ($p = 0.32$), which averaged 7.31. For Simpson index, there was a difference between microenvironments ($p = 0.02$) with the liquid microenvironment having greater diversity (0.9989) than the solid (0.9986). There was not a difference in total read count ($p = 0.94$) across microenvironments, averaging 219,076,300.

Table 2. Alpha diversity parameters of liquid and solid rumen microenvironments of steers ($n = 4$) consuming hay and alfalfa cubes.

	Microenvironment		SEM	<i>p</i> -Value
	Liquid	Solid		
<i>N</i>	16 (4 Steers)	16 (4 Steers)		
Richness	4665	4663	0.52	≤ 0.01
Shannon diversity	7.32	7.29	0.02	0.32
Simpson index	0.9989	0.9986	0.00	0.02
Total read count	218,738,580	219,414,020	10,900,000	0.94

3.2. Beta Diversity

To assess differences in beta diversity among microbial communities, Bray–Curtis dissimilarity was performed and visualized using principal coordinates analysis (PCoA) (Figure 1). Community composition differed between solid and liquid microenvironments (PERMANOVA, $R^2 = 0.80$, $p \leq 0.01$). Within-group dispersion also differed between fractions (PERMDISP, $p = 0.04$) with liquid samples exhibiting greater within-group dispersion than solid samples (mean distance to centroid: 0.084 vs. 0.067, respectively), indicating that solid communities were more compositionally similar to one another compared to liquid samples which demonstrated greater sample to sample variability in composition.

3.3. Species Abundance

The relative abundance of 171 microbial species differed across rumen microenvironment ($p \leq 0.05$); “high-abundance microbes” (19 species, $\geq 0.5\%$ relative abundance in either microenvironment) are presented in Table 3 and “low-abundance microbes” (152 species, $< 0.5\%$ relative abundance in both microenvironments) are presented in Table A1. A heatmap was also developed to visually represent the contrast between microenvironments according to the relative abundance of microbes (Figure 2).

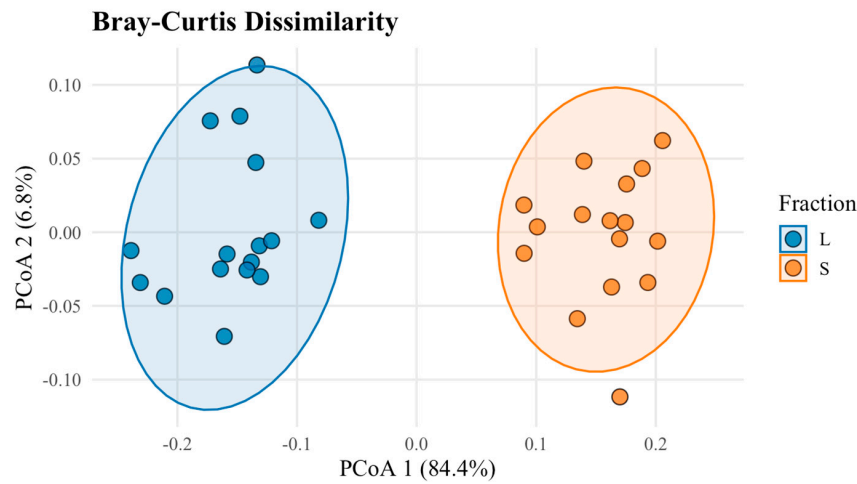


Figure 1. Principal coordinates analysis (PCoA) based on Bray–Curtis dissimilarity of microbial communities between solid ($n = 16$ from 4 steers) and liquid ($n = 16$ from 4 steers) rumen microenvironments of steers consuming forage ($n = 4$). Shaded ellipses denote 95% confidence regions. Community composition differed significantly between fractions (PERMANOVA, $R^2 = 0.80$, $p \leq 0.01$). Within-group variability also differed between fractions (PERMDISP, $p = 0.04$).

Table 3. Relative abundances of high-abundance microbes ($\geq 0.5\%$ relative abundance in either microenvironment) that differed across liquid and solid rumen microenvironments in steers ($n = 4$) consuming forage.

<i>n</i>	Microenvironment ¹		FDR <i>p</i> -Value
	Liquid 16 (4 Steers)	Solid 16 (4 Steers)	
Uncultured <i>Prevotellaceae</i> bacterium	18.0 ± 0.60	9.11 ± 0.44	≤0.001
Uncultured <i>Bacteroidia</i> bacterium	15.6 ± 1.03	19.1 ± 0.71	0.002
Uncultured <i>Bacteroidales</i> bacterium	13.2 ± 0.38	6.66 ± 0.30	≤0.001
<i>Ruminococcaceae</i> bacterium	8.42 ± 0.45	13.9 ± 0.43	≤0.001
Uncultured <i>Bacterium</i>	6.11 ± 0.40	0.78 ± 0.09	≤0.001
Uncultured <i>Clostridiales</i> bacterium	5.93 ± 0.24	13.4 ± 0.36	≤0.001
Uncultured <i>Methanobrevibacter</i> sp.	5.33 ± 0.40	6.26 ± 0.30	0.054
Uncultured <i>Bacteroidetes</i> bacterium	3.98 ± 0.16	1.66 ± 0.11	≤0.001
<i>Prevotella</i> sp.	3.05 ± 0.12	1.11 ± 0.06	≤0.001
Uncultured <i>Prevotella</i> sp.	3.01 ± 0.17	0.70 ± 0.06	≤0.001
Uncultured <i>Lachnospiraceae</i> bacterium	1.95 ± 0.12	8.33 ± 0.26	≤0.001
<i>Fibrobacter succinogenes</i>	1.69 ± 0.19	1.22 ± 0.15	0.015
Uncultured <i>Ruminococcus</i> sp.	1.68 ± 0.20	0.97 ± 0.03	≤0.001
<i>Butyrivibrio</i> sp.	1.33 ± 0.10	2.62 ± 0.22	≤0.001
Uncultured <i>Erysipelotrichaceae</i> bacterium	1.17 ± 0.10	0.55 ± 0.03	≤0.001
Uncultured <i>Coriobacteriaceae</i> bacterium	0.75 ± 0.06	2.09 ± 0.11	≤0.001
<i>Lachnospiraceae</i> bacterium	0.45 ± 0.02	1.00 ± 0.03	≤0.001
<i>Ruminococcus flavefaciens</i>	0.42 ± 0.03	1.01 ± 0.10	≤0.001
Uncultured <i>Sarcina</i> sp.	0.23 ± 0.03	1.42 ± 0.08	≤0.001
<i>Clostridiales</i> bacterium	0.10 ± 0.00	0.53 ± 0.03	≤0.001

¹ Presented as mean ± SEM.

Several high-abundance microbes were present at higher relative abundances ($p \leq 0.01$) in the solid rumen microenvironment (Table 3; Figure 3), including uncultured *Bacteroidia* bacterium (15.6% liquid, 19.1% solid), *Ruminococcaceae* bacterium (8.42% liquid, 13.9% solid), uncultured *Clostridiales* bacterium (5.93% liquid, 13.4% solid), uncultured *Lachnospiraceae* bacterium (1.95% liquid, 8.33% solid), *Butyrivibrio* sp. (1.33% liquid, 2.62% solid), uncultured *Coriobacteriaceae* bacterium (0.75% liquid, 2.09% solid), *Lachnospiraceae* bacterium (0.45% liquid, 1.00% solid), *Ruminococcus flavefaciens* (0.42% liquid, 1.01% solid), uncultured *Sarcina* sp. (0.23% liquid, 1.42% solid), and *Clostridiales* bacterium (0.10% liquid, 0.53% solid).

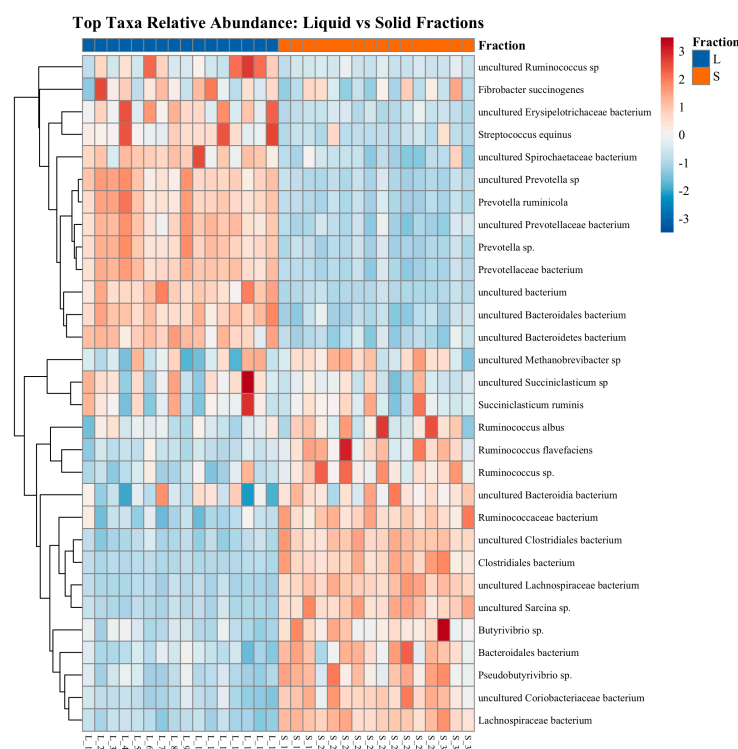


Figure 2. Heatmap outlining differential relative abundances of microbial species present in solid and liquid rumen microenvironments of steers ($n = 4$) consuming forage.

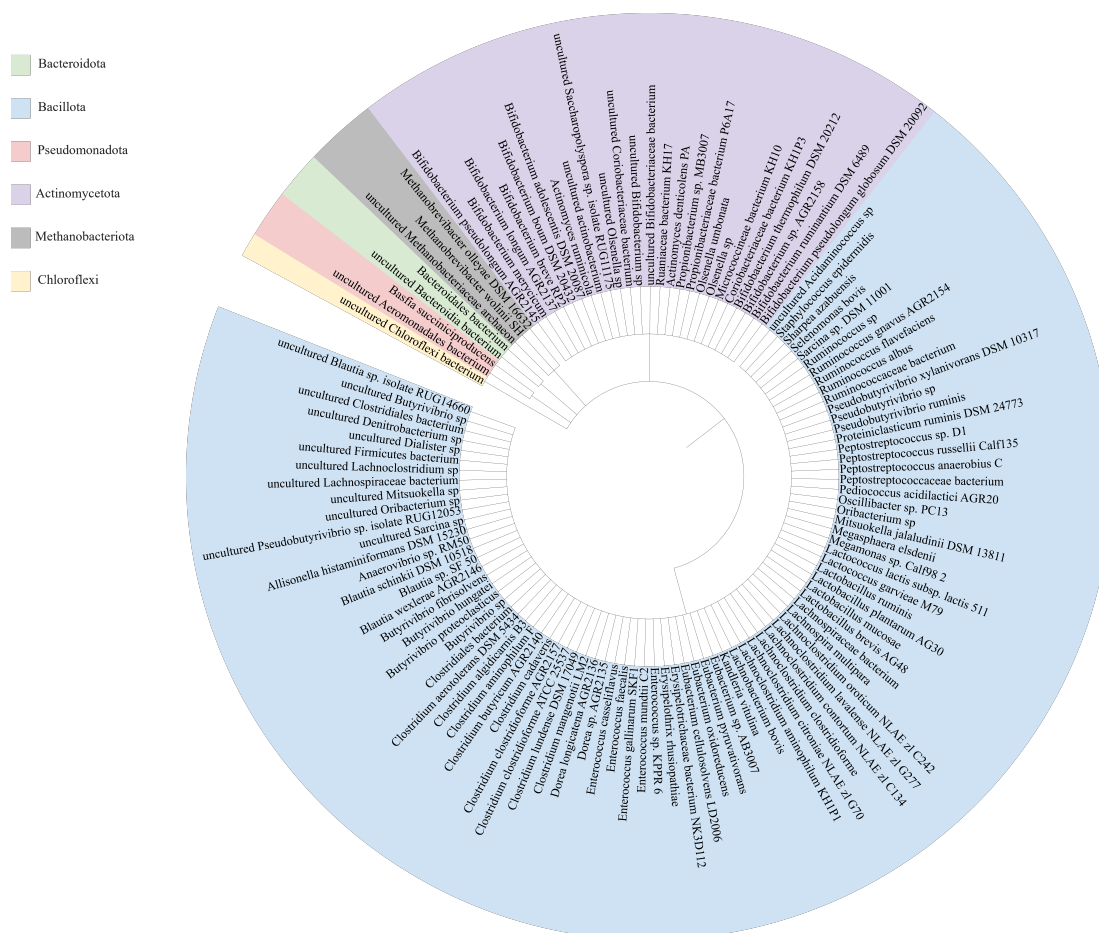


Figure 3. Phylogenetic tree of microbial species that were more abundant ($p \leq 0.05$) in the rumen solid microenvironment ($n = 16$ from 4 steers) versus the liquid ($n = 16$ from 4 steers) by phylum.

The high-abundance microbes present at greater relative abundances ($p \leq 0.01$) in the liquid rumen microenvironment (Table 3; Figure 4) were uncultured *Prevotellaceae* bacterium (18.0% liquid, 9.11% solid), uncultured *Bacteroidales* bacterium (13.2% liquid, 6.66% solid), uncultured *Bacterium* (6.11% liquid, 0.78% solid), uncultured *Bacteroidetes* bacterium (3.98% liquid, 1.66% solid), *Prevotella* sp. (3.05% liquid, 1.11% solid), uncultured *Prevotella* sp. (3.01% liquid, 0.70% solid), *Fibrobacter succinogenes* (1.69% liquid, 1.22% solid), uncultured *Ruminococcus* sp. (1.68% liquid, 0.97% solid), and uncultured *Erysipelotrichaceae* bacterium (1.17% liquid, 0.55% solid).

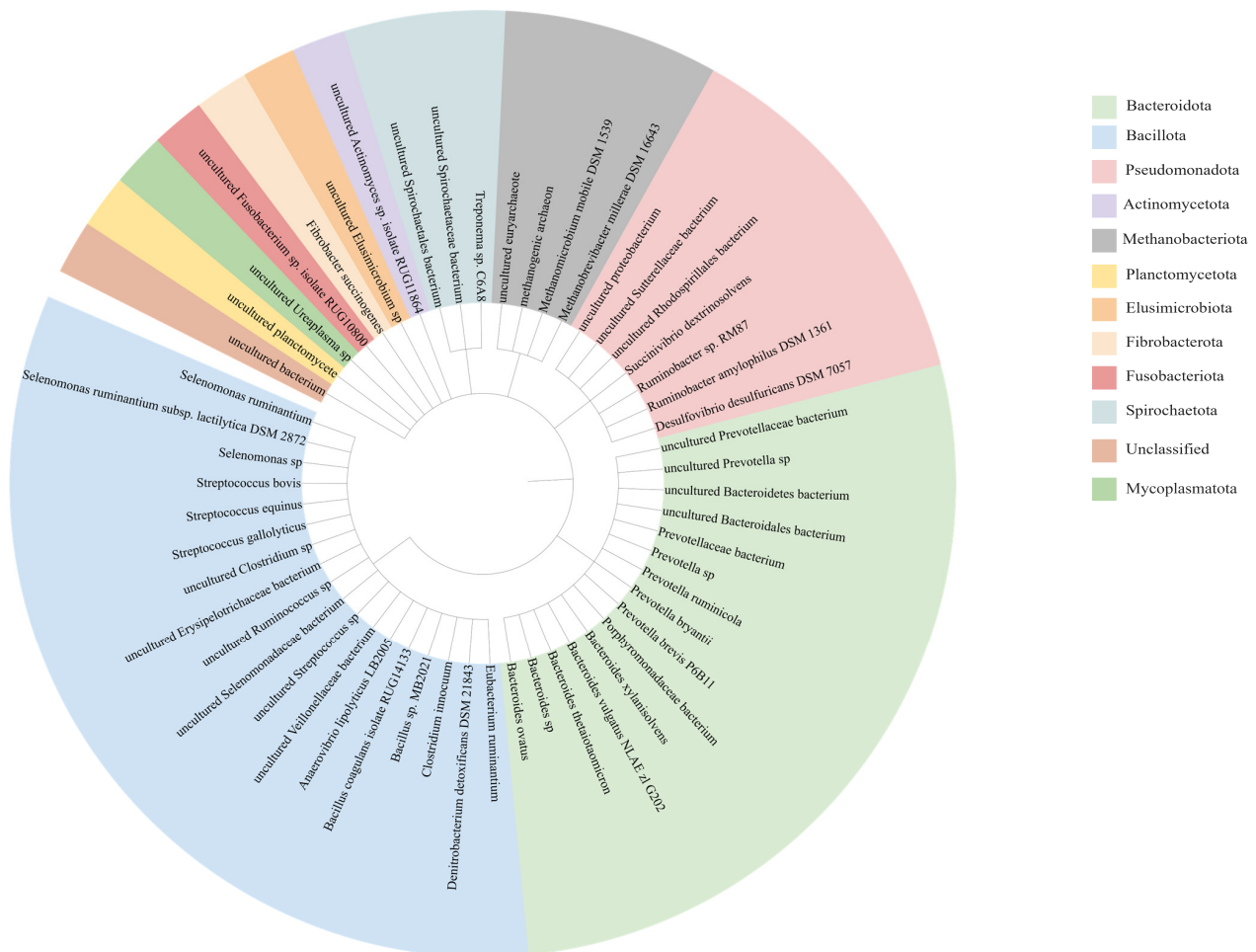


Figure 4. Phylogenetic tree of microbial species that were more abundant ($p \leq 0.05$) in the rumen liquid microenvironment ($n = 16$ from 4 steers) versus the solid ($n = 16$ from 4 steers) by phylum.

A large-scale analysis using COG of proteins was annotated for 35,504,340 and 45,237,342 non-redundant genes in the liquid and solid microenvironments, respectively; differences were detected between microenvironments for each category ($p \leq 0.05$; Figure 5). Most of the identified genes were categorized within the “unknown” group (Group S) and the “multiple” group, the latter accounting for genes with functionality in multiple metabolic pathways, regardless of microenvironment. A distinct distribution was observed between the microenvironments such that Group S was the most abundant category in the liquid microenvironment ($16.2\% \pm 0.07$), followed by the multiple COG group ($15.1\% \pm 0.37$). Conversely, in the solid microenvironment, the multiple COG group was predominant ($17.4\% \pm 0.08$), followed by the unknown category (i.e., Group S; 15.0 ± 0.03). Categories A, B, W, Y, and Z were detected and differed across microenvironments ($p \leq 0.03$), but relative abundances were $\leq 0.05\%$.

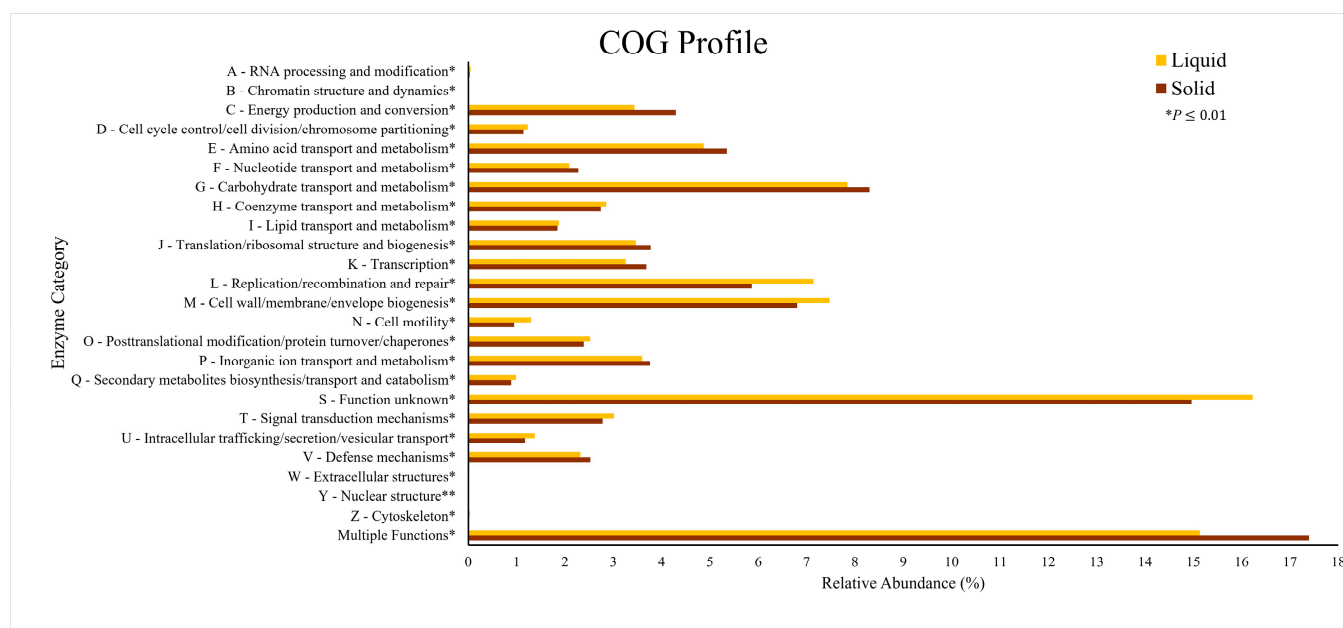


Figure 5. Relative abundance (%) of the functional gene categories (clusters of orthologous groups [COG]) between liquid ($n = 16$ from 4 steers) and solid ($n = 16$ from 4 steers) microenvironments. Categories denoted with an asterisk indicate a difference at $p \leq 0.01$ between microenvironments. Categories A, B, W, Y, and Z were detected but at abundances too low to be visible on this scale ($\leq 0.05\%$).

Screening of contigs for CAZyme revealed 1,056,283 non-redundant genes in the liquid microenvironment and 1,375,942 in the solid microenvironment. All enzyme classes exhibited differences between microenvironments ($p \leq 0.01$), except for the glycosyl transferase (GT) class ($p = 0.74$; Figure 6). For both microenvironments, glycoside hydrolase (GH) was present at the greatest relative abundance (2.02% liquid, 2.07% solid). There was an enrichment in auxiliary activities (AA) and polysaccharide lyase (PL) classes in the liquid microenvironment, with fold changes of 2 and 1.5, respectively, compared to the solid microenvironment. In contrast, the solid microenvironment was enriched in carbohydrate esterase (CE) relative to the liquid with a fold change of 1.3.

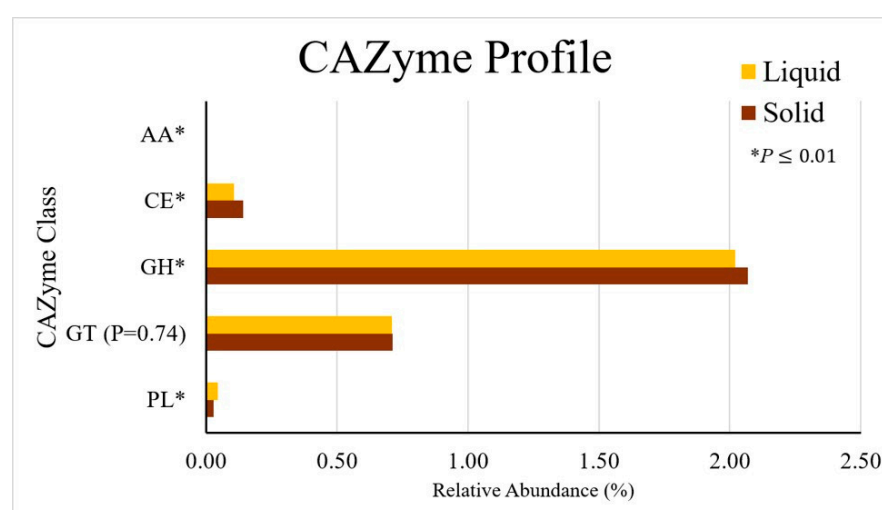


Figure 6. Relative abundance (%) of carbohydrate-active enzymes (CAZyme) between liquid ($n = 16$ from 4 steers) and solid ($n = 16$ from 4 steers) microenvironments. Categories denoted with an asterisk indicate a difference between microenvironments at $p \leq 0.01$. AA = auxiliary activities, CE = carbohydrate esterase, GH = glycosyl hydrolase, GT = glycosyl transferase, PL = polysaccharide lyase. Auxiliary activities (AA) were detected but at abundances too low to be visible on this scale ($\leq 0.02\%$).

Contigs were analyzed for genes related to methanogenesis, specifically those of the methyl-coenzyme M reductase (MCR) complex (*mcrA*, *mcrB*, *mcrC*, *mcrD*, and *mcrG*) and the particulate monooxygenase subunit A (*pmoA*). Differences in gene abundances ($p \leq 0.01$) were observed across the microenvironments where the liquid microenvironment contained a higher abundance of *mcrA*, *mcrB*, *mcrG*, and *pmoA* genes, with fold changes ranging from 1 to 2 compared to the solid microenvironment (Figure 7). The solid microenvironment exhibited a greater abundance of *mcrC* and *mcrD* genes, with fold changes of 1.2 and 2 relative to the liquid microenvironment, respectively ($p \leq 0.01$). Regardless of microenvironment, *mcrB* was present at the greatest relative abundance for MCR genes, 0.014% in the liquid and 0.010% in the solid microenvironment, followed by *mcrC*, which was present at 0.004% in the liquid and 0.005% in the solid microenvironment.

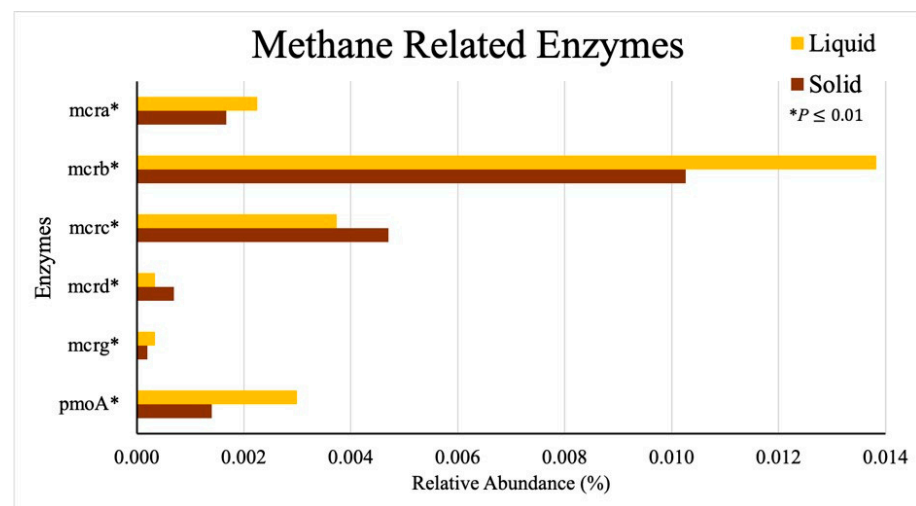


Figure 7. Relative abundance (%) of methane-related enzymes between liquid ($n = 16$ from 4 steers) and solid ($n = 16$ from 4 steers) microenvironments. Categories denoted with an asterisk indicate a difference between microenvironments at $p \leq 0.01$.

4. Discussion

This research utilized WGS to characterize taxonomic composition and genomic potential of microbial populations inhabiting two distinct rumen microenvironments (i.e., liquid versus solid) in beef steers consuming forage. It is important to note that the beef steers used in this research consumed ad libitum hay (70% neutral detergent fiber [NDF], 3.4% CP [crude protein]) and alfalfa cubes (35% NDF, 17.9% CP) provided at 200 g/kg of the previous 5 d intake. As the basal diet was low-quality (i.e., forage containing <7% CP), these findings may not be generalizable to other beef systems utilizing high-quality forages. However, forage quality can diminish as forage matures or enters dormancy and under certain forage management conditions [26]; therefore, the dietary conditions represented in the present study are relevant to forage-based U.S. beef production systems.

While prior research has established that rumen microenvironments are different in microbial composition and potential functionality, most studies have relied on 16S rRNA gene analysis and have focused on dairy cattle or other ruminant production systems [8,27–29]. In contrast to 16S rRNA analysis, the use of WGS in this context facilitates a more comprehensive assessment of both taxonomic profiling and metabolic capacity, therefore addressing a critical gap in understanding of microenvironment-specific microbial functionality in beef systems. Our findings revealed that the liquid and solid rumen fractions differed in both microbial species composition and metabolic pathway abundance in forage-fed beef steers.

The solid microenvironment was characterized by a higher relative abundance of uncultured *Bacteroidia* bacteria, *Ruminococcaceae* bacterium and uncultured *Clostridiales* bacteria. Although the most abundant sequence variants were grouped within the *Bacteroidia* class, this broader taxonomic group likely possesses a wide array of enzymes critical for degrading dietary structural polysaccharides (e.g., cellulose and hemicellulose). This specialized capacity for degrading plant cell matrices was supported by enrichments in COG categories related to energy production and conversion (category C), amino acid transport and metabolism (category E), and carbohydrate transport and metabolism (category G). Further, the solid microenvironment also contained elevated abundances of the CAZymes GH and CE which target ester linkages and glycosidic bonds of structural polysaccharides. These enriched metabolic pathways could reflect the higher energetic demands required for biofilm formation and attachment to the fibrous mat present in the rumen solid microenvironment [4]. It should be mentioned, however, that the differences in GH and CE between microenvironments were statistically significant, but might not have biological relevance as the fold-change for each was relatively minor, <2.5%.

Starch- and amylose-degrading bacteria, such as those belonging to the family *Prevotellaceae* and the genus *Prevotella*, are often more closely associated with the liquid versus solid rumen microenvironment [5,27,30]. This aligns with our current findings, where uncultured *Prevotellaceae* bacteria and uncultured *Bacteroidales* bacteria were doubly as abundant in liquid compared to the solid microenvironment, with relative abundances of 18% and 13% in liquid (respectively), distinguishing them as prolific members of this microenvironment. Species within the *Prevotellaceae* family express a broad range of proteins and CAZyme for degrading non-cellulosic plant structural fractions (e.g., pectins and hemicellulolytic xylans) and possess saccharolytic and proteolytic properties [31]. Moreover, *Prevotellaceae* synthesizes the volatile fatty acid propionate as one of its main functions [32]; as the metabolic pathway that produces propionate utilizes hydrogen, this functions as an alternative hydrogen sink that can directly compete with methane production [32].

The liquid microenvironment houses microbes and activities involved in cross-feeding dynamics within the rumen, where solid-associated microbial species initially degrade cellulolytic fibers, releasing soluble oligosaccharides and pectins into the liquid fraction, where free floating microorganisms utilize PL to ferment them [27]. Our data demonstrate that PL was present in higher abundances in the liquid versus solid microenvironment, in agreement with past research. The liquid microenvironment was also enriched in COG category H, suggesting increased coenzyme transport and metabolism to support cofactor-dependent fermentation and methanogenesis [33,34]. The enrichment of coenzyme-related enzymes in the liquid rumen fraction is notable, as these enzymes are crucial precursors for bacterial energy synthesis. This creates a potential functional distinction between the two microenvironments: although the liquid phase contained a lower overall abundance of enzymes dedicated to energy production and conversion (COG category C), the higher concentration of precursors (COG category H) could indicate differences in metabolic strategy, suggesting that the liquid-associated community utilizes alternative, precursor-dependent pathways to generate or utilize energy. This was reflected by an abundance of hydrogenotrophic methanogens (i.e., *Methanomicrobium mobile* and *Methanobrevibacter millerae*) in the liquid microenvironment, indicating active hydrogen turnover.

Methanogenic archaea in the current study, including uncultured *Methanobrevibacter* sp., *M. ollyae*, *M. wolnii*, and uncultured *Methanobacteriaceae*, were present in greater relative abundances in the solid microenvironment. Further, the two distinct hydrogenotrophic methanogens *Methanomicrobium mobile* and *Methanobrevibacter millerae* were more abundant in the liquid, rather than solid, microenvironment, perhaps reflecting differences in hydrogen availability between the microenvironments. Bowen et al. [27] also observed greater abundance of the

genera *Methanobrevibacter* and *Methanosphaera* in the solid rumen fraction but suggested that liquid-associated methanogens were likely underestimated based on sampling technique.

Many methanogenic archaea are engulfed by protozoa in the liquid rumen microenvironment to provide greater access to hydrogen [35]; therefore, without assessing protozoal abundances in the rumen, it is difficult to draw conclusions about methanogenic activities in either microenvironment. We did not assess ruminal protozoa in the current work, which is a study limitation. Despite this, our enzyme data suggest potential differences in methanogenic activity between the microenvironments, as all genes of interest were different across liquid versus solid samples.

For the MCR complex, the liquid microenvironment had higher abundances of three of the five subunits. Further, the liquid microenvironment contained a higher abundance of the *pmoA* gene, a biomarker for methanotrophic bacteria that consume methane. The presence of this gene suggests that methanotrophic activity, which mitigates methanogenesis, may be more prevalent in the liquid microenvironment. In previous research where methanotrophic microorganisms were provided to steers, a decrease in methane production combined with an increase in the *pmoA* gene was observed, supporting the connection between methane reduction and this biomarker [36]. Although the solid microenvironment was not enriched in *mcrABG* structural subunits responsible for the final step of methanogenesis, the enrichment of *mcrC* and *mcrD* accessory genes indicates that this fiber-associated community possesses the genomic capabilities for assembling and activating the MCR complex and suggests that the solid rumen microenvironment is a site for a highly specialized and stable methanogenic population [37,38].

Alpha diversity was greater in the liquid versus solid microenvironment for both species richness and Simpson index, but not Shannon diversity. This finding contradicts much of the previous research utilizing 16S sequencing techniques, where greater alpha diversity has been observed for the solid rumen microenvironment using the Simpson [5] and Shannon indexes [27]. However, Pinnell et al. [39] reported that microbial communities in rumen liquid samples were richer and more diverse than solid and mucosal samples, aligning with our findings. Further, Bray–Curtis dissimilarity, used to assess beta diversity in our study, revealed a clear separation between the liquid and solid rumen microenvironments supporting that the two rumen fractions are distinct microbial habitats.

The notably high abundance of uncharacterized enzymes (COG category S) in our study underlines the knowledge gaps that persist in our understanding of the rumen microbiome and its functional metabolic pathways [40]. Both the liquid and solid microenvironments contain numerous unknown microbial species and hypothetical proteins that remain to be functionally characterized, underscoring the need for research to elucidate the roles of these uncharacterized proteins, which could provide a deeper understanding of the distinct metabolic processes and functional differences between the liquid and solid rumen microenvironments.

5. Conclusions

Employing WGS to describe the distinct microbial communities of rumen liquid and solid microenvironments and their functionalities in this study provided novel insights which contrast previous data from 16S rRNA studies and further our understanding of microbial communities and activities in cattle consuming forage. Study limitations include a lacking quantification of ruminal protozoa populations and activities and utilizing a Latin Square design which employs a relatively small number of biological experimental units. We recommend that further research should be conducted in cattle representing different species, physiological statuses, and environments (e.g., diets) utilizing WGS with varied experimental designs to understand if present findings can be extrapolated and applied to other scenarios.

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Abbreviations

The following abbreviations are used in this manuscript:

rRNA	Ribosomal ribonucleic acid
PCR	Polymerase chain reaction
CAZyme	Carbohydrate-active enzymes
MAG	Metagenome-assembled genomes
WGS	Whole genome metagenomic sequencing
TMR	Total mixed rations
COG	Clusters of Orthologous Groups
NGS	Next generation sequencing
bp	Base pair
ORF	Open reading frame
KEGG	Kyoto Encyclopedia of Genes and Genomes
FDR	False discovery rate
PCoA	Principal Coordinates Analysis
GT	Glycosyl transferase
GH	Glycoside hydrolase
AA	Auxiliary activities
PL	Polysaccharide lyase
CE	Carbohydrate esterase
MCR	Methyl-coenzyme M reductase
NDF	Neutral detergent fiber
CP	Crude protein
<i>pmoA</i>	Particulate monooxygenase subunit A

Appendix A

Table A1. Relative abundances of low abundance microbes (<0.5% abundance in either microenvironment) that differed across liquid and solid rumen microenvironments in beef steers ($n = 4$) consuming forage.

<i>n</i>	Microenvironment ¹		FDR <i>p</i> -Value
	Liquid	Solid	
	16 (4 Steers)	16 (4 Steers)	
<i>Pseudobutyrvibrio</i> sp.	0.21 ± 0.01	0.40 ± 0.03	≤0.001
<i>Bacteroidales</i> bacterium	0.18 ± 0.01	0.31 ± 0.02	≤0.001
<i>Ruminococcus</i> sp.	0.18 ± 0.01	0.24 ± 0.01	≤0.001
<i>Pseudobutyrvibrio ruminis</i>	0.10 ± 0.01	0.18 ± 0.01	≤0.001
<i>Ruminococcus albus</i>	0.13 ± 0.01	0.17 ± 0.02	0.006
Uncultured <i>Spirochaetaceae</i> bacterium	0.37 ± 0.02	0.17 ± 0.02	≤0.001
<i>Butyrvibrio proteoclasticus</i>	0.07 ± 0.01	0.16 ± 0.02	≤0.001
<i>Butyrvibrio hungatei</i>	0.07 ± 0.01	0.15 ± 0.01	≤0.001
<i>Butyrvibrio fibrisolvens</i>	0.08 ± 0.01	0.15 ± 0.01	≤0.001
Uncultured <i>Butyrvibrio</i> sp.	0.06 ± 0.01	0.13 ± 0.01	≤0.001
<i>Prevotellaceae</i> bacterium	0.29 ± 0.01	0.11 ± 0.01	≤0.001
<i>Lachnobacterium bovis</i>	0.04 ± 0.001	0.10 ± 0.004	≤0.001
<i>Methanobrevibacter millerae</i> DSM 16643	0.17 ± 0.02	0.10 ± 0.01	≤0.001
<i>Methanobrevibacter olleyae</i> DSM 16632	0.02 ± 0.002	0.10 ± 0.01	≤0.001
<i>Streptococcus equinus</i>	0.31 ± 0.04	0.09 ± 0.02	≤0.001
<i>Sarcina</i> sp. DSM 11001	0.01 ± 0.002	0.09 ± 0.005	≤0.001
<i>Ruminococcus gnavus</i> AGR2154	0.04 ± 0.001	0.09 ± 0.003	≤0.001
<i>Selenomonas ruminantium</i>	0.12 ± 0.01	0.09 ± 0.003	≤0.001
Uncultured <i>Selenomonadaceae</i> bacterium	0.16 ± 0.01	0.09 ± 0.005	≤0.001
Uncultured <i>Olsenella</i> sp.	0.04 ± 0.004	0.09 ± 0.005	≤0.001
<i>Prevotella ruminicola</i>	0.26 ± 0.01	0.07 ± 0.005	≤0.001
Uncultured <i>Chloroflexi</i> bacterium	0.02 ± 0.002	0.07 ± 0.003	≤0.001
<i>Oribacterium</i> sp.	0.05 ± 0.001	0.06 ± 0.003	≤0.001
<i>Peptostreptococcaceae</i> bacterium	0.03 ± 0.0008	0.06 ± 0.002	≤0.001
<i>Eubacterium pyruvativorans</i>	0.03 ± 0.0007	0.06 ± 0.002	≤0.001
<i>Bacteroides</i> sp.	0.14 ± 0.006	0.06 ± 0.005	≤0.001
<i>Lachnospirillum citroniae</i> NLAE-zl-G70	0.03 ± 0.0007	0.06 ± 0.001	≤0.001
<i>Blautia</i> sp. SF-50	0.03 ± 0.0006	0.05 ± 0.001	≤0.001
<i>Lachnospirillum lavalense</i> NLAE-zl-G277	0.02 ± 0.0006	0.05 ± 0.001	≤0.001
<i>Clostridium algidicarnis</i> B3	0.02 ± 0.001	0.05 ± 0.001	≤0.001
<i>Sharpea azabuensis</i>	0.03 ± 0.001	0.05 ± 0.001	≤0.001
Uncultured <i>planctomycete</i>	0.09 ± 0.006	0.05 ± 0.002	≤0.001
<i>Blautia schinkii</i> DSM 10518	0.02 ± 0.001	0.05 ± 0.001	≤0.001
<i>Megasphaera elsdenii</i>	0.02 ± 0.001	0.05 ± 0.001	≤0.001
<i>Oscillibacter</i> sp. PC13	0.02 ± 0.001	0.05 ± 0.001	≤0.001
<i>Porphyromonadaceae</i> bacterium	0.08 ± 0.002	0.05 ± 0.001	≤0.001
<i>Pseudobutyrvibrio xylanivorans</i> DSM 10317	0.03 ± 0.002	0.04 ± 0.005	≤0.001
<i>Clostridium aminophilum</i> F	0.02 ± 0.001	0.04 ± 0.001	≤0.001
<i>Bacteroides ovatus</i>	0.06 ± 0.002	0.04 ± 0.001	≤0.001
<i>Prevotella brevis</i> P6B11	0.11 ± 0.004	0.03 ± 0.002	≤0.001
Uncultured <i>proteobacterium</i>	0.16 ± 0.02	0.03 ± 0.001	≤0.001
<i>Streptococcus bovis</i>	0.09 ± 0.01	0.03 ± 0.006	≤0.001
<i>Bacteroides xylanisolvens</i>	0.06 ± 0.003	0.03 ± 0.001	≤0.001
<i>Bacteroides thetaiotaomicron</i>	0.06 ± 0.001	0.03 ± 0.001	≤0.001
<i>Eubacterium</i> sp. AB3007	0.01 ± 0.0004	0.03 ± 0.001	≤0.001
<i>Olsenella</i> sp.	0.01 ± 0.001	0.03 ± 0.002	≤0.001
<i>Megamonas</i> sp. CalF98-2	0.02 ± 0.0003	0.03 ± 0.001	≤0.001
<i>Bifidobacterium boum</i> DSM 20432	0.01 ± 0.0004	0.03 ± 0.001	≤0.001
<i>Bifidobacterium merycicum</i>	0.01 ± 0.0004	0.03 ± 0.001	≤0.001
<i>Bifidobacterium pseudolongum</i> globosum	0.01 ± 0.0003	0.03 ± 0.001	≤0.001
<i>Lachnospirillum clostridioforme</i>	0.01 ± 0.0003	0.02 ± 0.001	≤0.001
<i>Prevotella bryantii</i>	0.05 ± 0.002	0.02 ± 0.001	≤0.001

Table A1. Cont.

n	Microenvironment ¹		FDR <i>p</i> -Value
	Liquid	Solid	
	16 (4 Steers)	16 (4 Steers)	
<i>Denitrobacterium detoxificans</i> DSM 21843	0.01 ± 0.0004	0.02 ± 0.001	≤0.001
<i>Eubacterium ruminantium</i>	0.01 ± 0.001	0.02 ± 0.001	≤0.001
<i>Lachnospira multipara</i>	0.02 ± 0.001	0.02 ± 0.002	0.004
<i>Bifidobacterium adolescentis</i> DSM 20087	0.01 ± 0.0003	0.02 ± 0.001	≤0.001
<i>Bifidobacterium breve</i> RP2	0.01 ± 0.0003	0.02 ± 0.001	≤0.001
<i>Bifidobacterium thermophilum</i> DSM 20212	0.01 ± 0.0003	0.02 ± 0.001	≤0.001
<i>Selenomonas bovis</i>	0.01 ± 0.0003	0.02 ± 0.001	≤0.001
Uncultured <i>Dialister</i> sp.	0.01 ± 0.0002	0.02 ± 0.001	≤0.001
<i>Olsenella umbonata</i>	0.01 ± 0.001	0.02 ± 0.001	≤0.001
<i>Eubacterium oxidoreducens</i>	0.01 ± 0.0003	0.02 ± 0.001	≤0.001
<i>Erysipelotrichaceae</i> bacterium NK3D112	0.004 ± 0.0002	0.02 ± 0.001	≤0.001
<i>Lachnospirillum oroticum</i> NLAE-zl-C242	0.01 ± 0.0002	0.02 ± 0.0004	≤0.001
<i>Clostridium manganotii</i> LM2	0.01 ± 0.001	0.01 ± 0.001	0.007
<i>Coriobacteriaceae</i> bacterium KH1P3	0.01 ± 0.001	0.01 ± 0.001	≤0.001
<i>Clostridium clostridioforme</i> ATCC 25537	0.01 ± 0.0002	0.01 ± 0.0003	≤0.001
<i>Kandleria vitulina</i>	0.01 ± 0.0003	0.01 ± 0.0003	≤0.001
Uncultured <i>Streptococcus</i> sp.	0.05 ± 0.01	0.01 ± 0.004	≤0.001
Uncultured <i>Elusimicrobium</i> sp.	0.03 ± 0.003	0.01 ± 0.001	≤0.001
<i>Methanobrevibacter wolinii</i> SH	0.01 ± 0.001	0.01 ± 0.001	0.039
<i>Staphylococcus epidermidis</i>	0.01 ± 0.0002	0.01 ± 0.0003	≤0.001
<i>Dorea</i> sp. AGR2135	0.01 ± 0.001	0.01 ± 0.001	0.015
<i>Blautia wexlerae</i> AGR2146	0.01 ± 0.0002	0.01 ± 0.0002	≤0.001
Uncultured <i>Denitrobacterium</i> sp.	0.01 ± 0.0004	0.01 ± 0.0004	≤0.001
<i>Lactobacillus plantarum</i> AG30	0.01 ± 0.0002	0.01 ± 0.003	≤0.001
<i>Lactococcus lactis</i> subsp. <i>lactis</i> 511	0.01 ± 0.0002	0.01 ± 0.0003	≤0.001
Uncultured <i>Pseudobutyrvibrio</i> sp.	0.01 ± 0.001	0.01 ± 0.001	≤0.001
<i>Streptococcus gallolyticus</i>	0.02 ± 0.003	0.01 ± 0.002	≤0.001
<i>Treponema</i> sp. C6A8	0.02 ± 0.002	0.01 ± 0.001	≤0.001
<i>Eubacterium cellulosolvens</i> LD2006	0.01 ± 0.001	0.01 ± 0.001	0.002
<i>Enterococcus gallinarum</i> SKF1	0.01 ± 0.0002	0.01 ± 0.0002	≤0.001
<i>Lachnospirillum aminophilum</i> KH1P1	0.01 ± 0.0002	0.01 ± 0.0002	≤0.001
<i>Lactobacillus mucosae</i>	0.01 ± 0.001	0.01 ± 0.0003	≤0.001
<i>Pediococcus acidilactici</i> AGR20	0.01 ± 0.0002	0.01 ± 0.0002	≤0.001
<i>Enterococcus faecalis</i>	0.01 ± 0.0002	0.01 ± 0.0002	≤0.001
Uncultured <i>Spirochaetales</i> bacterium	0.07 ± 0.006	0.01 ± 0.0003	≤0.001
<i>Lachnospirillum contortum</i> NLAE-zl-C134	0.003 ± 0.0001	0.01 ± 0.0002	≤0.001
<i>Lactobacillus brevis</i> AG48	0.005 ± 0.0001	0.01 ± 0.0002	≤0.001
<i>Mitsuokella jalaludinii</i> DSM 13811	0.004 ± 0.0002	0.01 ± 0.0002	≤0.001
Uncultured <i>Aeromonadales</i> bacterium	0.008 ± 0.0004	0.01 ± 0.0002	0.008
<i>Bacteroides vulgatus</i> NLAE-zl-G202	0.01 ± 0.001	0.01 ± 0.0002	≤0.001
Uncultured <i>Sutterellaceae</i> bacterium	0.02 ± 0.004	0.01 ± 0.0001	≤0.001
Uncultured <i>Methanobacteriaceae</i> archaeon	0.003 ± 0.0002	0.004 ± 0.0004	≤0.001
<i>Clostridium clostridioforme</i> AGR2157	0.002 ± 0.0001	0.004 ± 0.0001	≤0.001
Uncultured <i>Veillonellaceae</i> bacterium	0.02 ± 0.003	0.004 ± 0.0004	≤0.001
<i>Selenomonas</i> sp.	0.01 ± 0.001	0.004 ± 0.0002	≤0.001
<i>Lactobacillus ruminis</i>	0.003 ± 0.0001	0.004 ± 0.0001	≤0.001
<i>Anaerovibrio</i> sp. RM50	0.003 ± 0.0001	0.003 ± 0.0001	0.011
Uncultured <i>Acidaminococcus</i> sp.	0.002 ± 0.0001	0.003 ± 0.0001	≤0.001
<i>Proteiniclasticum ruminis</i> DSM 24773	0.002 ± 0.0001	0.003 ± 0.0001	≤0.001
<i>Dorea longicatena</i> AGR2136	0.002 ± 0.0001	0.003 ± 0.0001	≤0.001
<i>Desulfovibrio desulfuricans</i> DSM 7057	0.003 ± 0.0001	0.003 ± 0.0001	≤0.001
<i>Enterococcus casseliflavus</i>	0.002 ± 0.00004	0.003 ± 0.0001	≤0.001
<i>Micrococcineae</i> bacterium KH10	0.002 ± 0.0002	0.003 ± 0.0002	≤0.001
<i>Actinomyces ruminicola</i>	0.002 ± 0.0001	0.003 ± 0.0002	≤0.001
Uncultured <i>Ureaplasma</i> sp.	0.003 ± 0.0002	0.002 ± 0.0004	0.027
<i>Selenomonas ruminantium</i> subsp. <i>lactilytica</i>	0.004 ± 0.0003	0.002 ± 0.0001	≤0.001

Table A1. Cont.

<i>n</i>	Microenvironment ¹		FDR <i>p</i> -Value
	Liquid	Solid	
	16 (4 Steers)	16 (4 Steers)	
Uncultured <i>Blautia</i> sp. isolate RUG14660	0.001 ± 0.00003	0.002 ± 0.0001	≤0.001
<i>Clostridium innocuum</i>	0.002 ± 0.0001	0.002 ± 0.0001	≤0.001
Uncultured <i>euryarchaeote</i>	0.004 ± 0.001	0.002 ± 0.0002	≤0.001
<i>Succinivibrio dextrinosolvens</i>	0.003 ± 0.0004	0.002 ± 0.0002	0.004
<i>Basfia succiniciproducens</i>	0.002 ± 0.0001	0.002 ± 0.0001	≤0.001
<i>Allisonella histaminiformans</i> DSM 15230	0.001 ± 0.0001	0.002 ± 0.00003	≤0.001
<i>Ruaniaceae</i> bacterium KH17	0.001 ± 0.0001	0.002 ± 0.0001	≤0.001
<i>Enterococcus mundtii</i> C2	0.001 ± 0.00002	0.001 ± 0.00003	≤0.001
<i>Actinomyces denticolens</i> PA	0.001 ± 0.0001	0.001 ± 0.0001	≤0.001
Uncultured <i>Mitsuokella</i> sp.	0.001 ± 0.0001	0.001 ± 0.00003	≤0.001
Uncultured <i>Bifidobacteriaceae</i> bacterium	0.001 ± 0.00002	0.001 ± 0.00004	≤0.001
<i>Lactococcus garvieae</i> M79	0.001 ± 0.00003	0.001 ± 0.00004	≤0.001
<i>Peptostreptococcus anaerobius</i> C	0.001 ± 0.00002	0.001 ± 0.00004	≤0.001
<i>Enterococcus</i> sp. KPPR-6	0.001 ± 0.00004	0.001 ± 0.00003	≤0.001
<i>Propionibacteriaceae</i> bacterium P6A17	0.001 ± 0.0001	0.001 ± 0.0001	0.006
<i>Methanomicrobium mobile</i> DSM 1539	0.12 ± 0.02	0.001 ± 0.0002	≤0.001
<i>Erysipelothrix rhusiopathiae</i>	0.001 ± 0.0001	0.001 ± 0.0001	≤0.001
<i>Peptostreptococcus russellii</i> Calf135	0.001 ± 0.00002	0.001 ± 0.00004	≤0.001
Uncultured <i>Oribacterium</i> sp.	0.001 ± 0.00003	0.001 ± 0.00004	≤0.001
Methanogenic archaeon	0.01 ± 0.001	0.001 ± 0.0002	≤0.001
<i>Peptostreptococcus</i> sp. D1	0.001 ± 0.00002	0.001 ± 0.00003	≤0.001
Uncultured <i>Bifidobacterium</i> sp.	0.001 ± 0.00003	0.001 ± 0.00004	≤0.001
Uncultured <i>Saccharopolyspora</i> sp.	0.001 ± 0.0001	0.001 ± 0.0002	0.002
Uncultured <i>Firmicutes</i> bacterium	0.0002 ± 0.00001	0.001 ± 0.0001	≤0.001
<i>Propionibacterium</i> sp. MB3007	0.001 ± 0.0001	0.001 ± 0.0001	0.011
<i>Clostridium aerotolerans</i> DSM 5434	0.0003 ± 0.00002	0.001 ± 0.00002	≤0.001
Uncultured <i>actinobacterium</i>	0.0004 ± 0.00002	0.001 ± 0.00002	≤0.001
<i>Clostridium cadaveris</i>	0.0002 ± 0.00001	0.001 ± 0.00002	≤0.001
Uncultured <i>Lachnospirillum</i> sp.	0.0002 ± 0.00001	0.001 ± 0.00002	≤0.001
<i>Anaerovibrio lipolyticus</i> LB2005	0.001 ± 0.0001	0.001 ± 0.0001	≤0.001
<i>Bifidobacterium</i> sp. AGR2158	0.001 ± 0.00002	0.001 ± 0.00002	0.014
<i>Clostridium butyricum</i> AGR2140	0.0003 ± 0.00001	0.001 ± 0.00001	≤0.001
<i>Bifidobacterium pseudolongum</i> AGR2145	0.0002 ± 0.00002	0.0004 ± 0.00003	≤0.001
<i>Bifidobacterium longum</i> AGR2137	0.0002 ± 0.00001	0.0003 ± 0.00001	≤0.001
<i>Ruminobacter amylophilus</i> DSM 1361	0.001 ± 0.00004	0.0003 ± 0.00002	≤0.001
<i>Bifidobacterium ruminantium</i> DSM 6489	0.0001 ± 0.00001	0.0002 ± 0.00001	≤0.001
Uncultured <i>Rhodospirillales</i> bacterium	0.001 ± 0.0002	0.0002 ± 0.00001	≤0.001
Uncultured <i>Actinomyces</i> sp. isolate RUG11864	0.001 ± 0.00003	0.0002 ± 0.00001	≤0.001
<i>Clostridium lundense</i> DSM 17049	0.0001 ± 0.00001	0.0002 ± 0.00002	0.005
<i>Ruminobacter</i> sp. RM87	0.0003 ± 0.00003	0.0002 ± 0.00001	≤0.001
Uncultured <i>Clostridium</i> sp.	0.0001 ± 0.00001	0.0001 ± 0.00001	0.014
<i>Bacillus coagulans</i> isolate RUG14133	0.0001 ± 0.00002	0.0001 ± 0.00001	≤0.001
Uncultured <i>Fusobacterium</i> sp.	0.0003 ± 0.0001	0.00001 ± 0.00001	≤0.001
<i>Bacillus</i> sp. MB2021	0.0001 ± 0.00001	0.00001 ± 0.00001	≤0.001

¹ Presented as mean ± SEM.

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