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Validation of a minimally-invasive method for sampling epithelial-associated microorganisms on the rumen wall

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The rumen microbiome provides approximately 70% of the required energy for the host by converting low-quality feedstuffs into usable energy for ruminants. The energy produced by the microorganisms is subsequently absorbed through the rumen epithelium and used towards growth and energy maintenance. There is evidence that ruminal epimural microbes directly interact with the rumen epithelium, acting as an intermediary communicator between the rumen liquid fraction and the host. Epimural microbiota have been demonstrated to be distinct from the ruminal liquid microbiome and perform unique roles within the rumen environment. However, methods to sample epimural communities from the rumen wall are limited and typically invasive, requiring animal fistulation or harvesting. To characterize the epimural communities present on the rumen wall, a novel and minimally-invasive surgical method was developed to swab the epithelium of the ventral sac of the rumen. The objective of this study was to validate this sampling method by comparing epimural and liquid fraction bacterial communities. During a 70-day feeding trial, Angus steers (n = 45) were sampled on day 35 using the novel surgery method and tubed on day 70 to sample rumen liquid content. Genomic DNA was used to generate amplicon libraries of the V4 region of the 16S rRNA gene. There were no differences between alpha diversity indices when comparing rumen versus epimural bacterial communities (P > 0.05). The Bray-Curtis dissimilarity was used to ordinate ASV counts, and then tested for differences between rumen and epimural communities using a PERMANOVA with 999 permutations (P < 0.05). Differential abundances of bacterial communities were tested using ANCOM-BC and MaAsLin2, where significance was determined by Q < 0.05 and overlap between both analysis methods. Within the 91 taxa that differed in abundance, 451 ASVs were found to be different between sample types (Q < 0.05). Unique ASVs associated with *Prevotella*, *Succinivibrio*, family-level *Eubacterium*, and family-level *Succinivibrio* were in greater abundance for the rumen epithelial-associated bacterial communities (Q < 0.05). The results demonstrate that the

novel method of sampling from the rumen wall can capture differences between epimural and ruminal fluid bacterial communities, thus facilitating studies investigating the interactions between epimural bacteria with the host.

KEYWORDS

rumen, epithelium, bacteria, epimural, beef

1 Introduction

The rumen epithelium plays a critical role in the uptake of short chain fatty acids, otherwise known as volatile fatty acids (VFAs), which provide approximately 70% of required energy to the ruminant host (Bergman, 1990). These VFAs are the primary fermentation product from the rumen microbiome, which consists of bacterial, archaeal, protozoal, and fungal communities. Due to the unique fermentative capabilities of the rumen, the rumen microbiome has been associated with the host phenotypes of feed efficiency and methane production (Roehe et al., 2016; Shabat et al., 2016; Li et al., 2017; Li et al., 2022; Andersen et al., 2023). Thus, the rumen microbiome plays a critical role in supplying energy for the host, which in turn alters production-relevant parameters.

The rumen microbiome has been characterized by three main types of ruminal microbes, primarily based on digesta type and tissues that the microbial communities are adherent to within the rumen. The common categories consist of the fiber-adherent, planktonic (liquid-associated), and epimural (papillae-associated) microbial communities. The fiber-adherent bacteria represent the majority of the ruminal bacterial biomass, approximately 70% (Millen et al., 2016), and serve the main role in digestion (McAllister et al., 1994; Larue et al., 2005). Planktonic bacterial communities constitute approximately 30% (Millen et al., 2016) of the bacterial biomass, synergistically working with solid-adherent bacteria for nutrient breakdown (McAllister et al., 1994). Overall, the differences in function and diversity between the fiber-adherent and planktonic fractions of the rumen have been well-studied across species (Larue et al., 2005; Cho et al., 2006; Mao et al., 2015; De Mulder et al., 2016; Liu et al., 2016; Schären et al., 2017). Epimural microbes, while making up only 1% of the total biomass (Czerkawski, 1986; Millen et al., 2016), directly interact with the rumen epithelium, potentially modulating host-microbiome interactions.

Early studies have identified that bacterial communities that adhere to the rumen wall are distinctly different from the liquid and solid fractions (Cheng and Wallace, 1979; McCowan et al., 1980; Sadet et al., 2007). Diet is the primary driver of planktonic and fiber-adherent communities (Tajima et al., 2001); however, the degree to which diet impacts epimural microbiota is unknown. There is evidence that diet can alter the epimural microbiome, especially in diets that induce acidosis (Sadet-Bourgeteau et al., 2010; Chen et al., 2011; Liu et al., 2013; Liu et al., 2015; Petri et al., 2020). Regardless of dietary impact, epimural microbes have been primarily implicated across ruminant species for their distinct functions in the rumen and their influence on oxygen scavenging (Cheng et al., 1979), urea hydrolysis (Cheng and Wallace, 1979), nutrient absorption (Mao et al., 2015; Zhao et al., 2017), and recycling of epithelial tissues (Dinsdale et al., 1980). Thus, epimural microbial communities may play significant roles in modulating health status and feed efficiency by protecting the ruminal epithelium and also in an intermediary role in nutrient uptake.

Many studies have focused on characterizing the planktonic and fiber-adherent communities, which include associations with health status (Khafipour et al., 2009; Zhang et al., 2022), production-relevant traits such as feed efficiency (Jami et al., 2014; Myer et al., 2015), and methane production (Wallace et al., 2019; Martínez-Álvaro et al., 2022). Yet only within the last decade have researchers begun to deeply investigate the abundance and functions of the epimural communities across ruminant species. This includes characterizing the epimural microbiome (Mao et al., 2015; De Mulder et al., 2016; Sbardellati et al., 2020) and examining the influence of ruminal acidosis challenges on the epimural microbiome (Penner et al., 2009; Petri et al., 2013; Wetzels et al., 2017; Li et al., 2019b). However, identifying the epimural microbiome across species and studies can be challenging due to technological differences, data processing, and analysis (Anderson et al., 2021; Pacífico et al., 2021). Further, the ruminal wall is difficult to access, thus resulting in many of these studies using small animal numbers.

Current methods to sample epimural microbiota from the rumen wall typically utilize animal fistulation or harvesting. To improve bovine rumen microbiome studies and recognize the limitations of terminal animal research, novel methods must be developed to effectively and comprehensively sample epimural microorganisms. Therefore, the objectives of this project were to assess an alternative, minimally-invasive technique for sampling microbiota from the rumen wall and validate the method's ability to capture differences between the rumen liquid and epimural bacterial communities.

2 Materials and methods

2.1 Animal ethics statement

This experiment was approved by the University of Tennessee Animal Care and Use Committee.

2.2 Experimental plan

To test and validate the method to collect epimural microbiota, 45 Angus steers were enrolled in a 70-day feeding trial. During this period, weight and feed intake was measured to determine health status as a response to the surgery method. Steers were kept on the same diet throughout the study, being a total mixed ration (TMR) of sudex (sorghum-sudangrass hybrid) and a growing Co-Op diet (Tennessee Farmer's Co). The TMR consisted of 14.72% crude protein and 66.19% TDN on a dry-matter basis.

2.3 Surgery method for rumen wall sampling

On day 35 of the 70-day trial, steers were restrained in a chute and a 2-in × 2-in square of hair coat was clipped using a #40 blade ventrally to the lumbar transverse processes and caudally to the last rib in the left paralumbar fossa. The skin was surgically prepared using alternate swabbing of povidone-iodine scrub and isopropyl alcohol. The area was locally anesthetized with subcutaneous and intramuscular infiltration of 10 mL of 2% lidocaine. Six to ten minutes following lidocaine administration, a 1.5-cm incision was made in the center of the prepped area using a #10 scalpel blade. A 10-mm diameter trocar-cannula unit was then inserted through the body wall until penetration of the lumen of the dorsal sac of the rumen. A swab was introduced through the cannula and oriented towards the rumen wall, arching and swabbing against the wall several times. The swab was removed and immediately placed into 1 mL of tris-EDTA buffer in a 15 mL conical tube and flash-frozen in liquid nitrogen. The trocar was retrieved from the body wall and the incision was closed by USP #2 suture material. Samples were stored at -80°C until further analysis. The surgery method for rumen wall sampling is depicted in Figure 1.

2.4 Surgery scoring

Steers were monitored daily post-operatively for signs of inflammation or discomfort. Two weeks after the surgery date, steers incision sites were measured in height and width of inflammation and swelling. During surgery site scoring, remaining sutures were removed and the presence or absence of sutures at the time of removal was noted. Surgery sites were then scored by veterinarians from the University of Tennessee Large Animal Clinical Sciences and ranged from 0 (no discharge and less than 20 × 20 mm site) to 3 (swelling greater than 60 × 60 mm) (Table 1). Any other indication of swelling or discharge was noted at that time.

2.5 Sampling of rumen fluid

On day 70 of the 70-day trial, steers were restrained in a chute and an orogastric tubing system was used to obtain rumen fluid. The initial extraction from within the ventral sac of the rumen was discarded to avoid saliva contamination. Following rumen fluid extraction, the tubing system was flushed with 70% ethanol and rinsed with water to reduce between-animal contamination. Approximately 50 mL of rumen liquid was collected per animal and flash-frozen in liquid nitrogen. Samples were stored at -80°C until further analysis.

2.6 Extraction, amplification, and sequencing of bacterial DNA

Genomic DNA was extracted from samples using a previously validated procedure by Yu and Morrison (Yu and Morrison, 2004). In brief, 0.2 g of sample were added to 2 mL bead beating tubes (Zymo Research, Orange, CA, United States) and 1 mL of lysis buffer (500 mM NaCl, 50 mM Tris-HCl pH 8.0, 50 mM EDTA, and 4% sodium dodecyl sulfate [SDS]) was added to tubes. Samples were homogenized at 21 Hz for three minutes and then incubated at 70°C for 15 minutes, with three inversions every five minutes. Samples were centrifuged for 10 minutes at 16,000 × g and 4°C. The supernatant was aspirated and placed into fresh 1.5 mL microcentrifuge tubes, 300 µL of lysis buffer was added to bead-beating tubes, and the steps above were repeated. Following mechanical and chemical lysis of cells, samples underwent isopropanol precipitation of nucleic acids. To each lysate tube, 260 µL of 10 M ammonium acetate was added and incubated on ice for five minutes. Supernatants were transferred to two 1.5 mL microcentrifuge tubes to add one volume of isopropanol. Following, samples were incubated on ice for 30 minutes. Samples were centrifuged for 15 minutes at 16,000 × g at 4°C to spin down the nucleic acid pellet. The supernatant was discarded and the resulting nucleic acid pellet was washed with 200 µL of 70% ethanol. The pellet was air-dried for five minutes at room temperature. Subsequently, 100 µL of Buffer TE was added to dissolve the pellet and aliquots were pooled. The QIAGEN DNeasy Blood and Tissue Kit was used for downstream purification (QIAGEN, Hilden, Germany). Proteinase K and Buffer AL were added to each sample per company protocol and samples were incubated at 70°C for 10 minutes. Following, 200 µL of ethanol was added. Samples were transferred to a QIAamp column and centrifuged for one minute at 16,000 × g. Buffers AW1 and AW2 were added at 500 µL sequentially, with flow through being discarded between each buffer after centrifugation. Columns were then dried at room temperature for one minute. Columns were then placed in a fresh 1.5 mL microcentrifuge tube for final elution. To elute DNA, 70 µL of Buffer AE was added to the column membrane, incubated at room temperature for 2 minutes, and then 30 µL of Buffer AE was added. Samples were centrifuged for one minute at 16,000 × g and 4°C. The quality of DNA was assessed using a 1% TBE agarose gel and quantity was measured using a DeNovix spectrophotometer (DeNovix Inc., Wilmington, DE, United States). Samples were diluted to 10 ng/µL before polymerase chain reaction (PCR) amplification.

Amplicon sequencing of the 16S rRNA region was performed at the University of Tennessee Genomics Core, following their standard operating procedures of a two-step PCR. The V4 region

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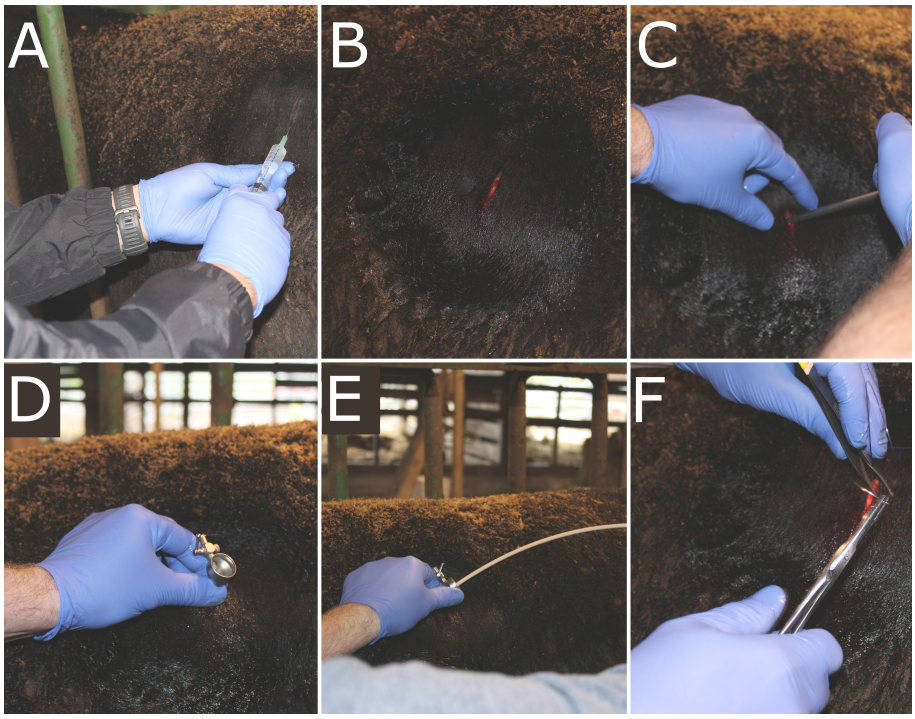

FIGURE 1
Surgery method for sampling from the rumen wall. **(A)** Incision site was prepped using alternating povidone iodine scrub and 70% isopropyl alcohol, and lidocaine was administered. **(B)** A 1.5-cm incision was made, followed by **(C)** insertion of a 10-mm trocar. **(D)** Once the trocar was fully inserted into the lumen of the dorsal sac, **(E)** the swab was introduced and arced to swab against the ventral sac of the rumen wall. Once the swab and trocar were removed, **(F)** the incision site was closed via USP #2 sutures.

of the 16S rRNA gene was amplified from the extracted DNA using primers 515Fb (GTGYCAGCMGCCGCGGTAA) (Parada et al., 2016) and 806Rb (GGACTACNVGGGTWTCTAAT) (Apprill et al., 2015), modified with adapters for Illumina MiSeq sequencing. The initial PCR consisted of 2 × KAPA HiFi HotStart ReadyMix Taq (Roche, Indianapolis, IN, United States), 1.5 µM each primer, and 2.5 µL template DNA. The reaction consisted of 3 min at 95°C, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. Successful PCR amplification was confirmed via gel electrophoresis on a 2% agarose gel. The PCR product was then purified using 20 uL of AMPure XP beads (Agencourt, Beverly, MA, United States) and ethanol washes, then eluted in 50 uL of Tris-HCl. Nextera XT indexes (Illumina, Inc., San Diego, CA, United States) were then added to the PCR products using a second, reduced-cycle PCR, such that each sample had a unique combination of forward and reverse indexes. This reduced reaction consisted of 3 min at 95°C, followed by 8 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The products were purified again using 56 uL of AMPure XP beads, with ethanol washes and a final elution in 25 uL Tris-HCl. Samples were quantified on a NanoDrop spectrophotometer (Fisher Scientific International, Inc., Hampton, NH, United States) and pooled to approximately equal concentrations. Final product sizes and concentrations were confirmed on an Agilent Bioanalyzer (Santa Clara, CA, United States) using the standard sensitivity kit. The final library was then diluted to 4 pM, and combined with 20% of a 10 pM PhiX library control (Illumina, Inc., San Diego, CA, United States), and run paired-end 250 nucleotides on a v2, 500-cycle flow cell of an Illumina MiSeq sequencer at the University of Tennessee Genomics Core.

TABLE 1 Incision scores of steers two weeks after the trocar cannulation surgery to access the rumen wall.

Score	Count
0	18
1	24
2	2
3	1

A score of 0 represents no swelling to a 20 × 20 mm swollen site, 1 represents a greater than 20 × 20 mm to 40 × 40 mm site, a 2 represents a greater than 40 × 40 mm to 60 × 60 mm site, and a 3 represents a greater than 60 × 60 mm site.

2.7 Processing of 16S rRNA gene sequences

Resultant fastq sequences were analyzed in the RStudio environment (R version 3.6.2). Quality was assessed using package 'fastqcr' v0.1.2 (Alboukadel, 2019) and sequences were filtered and trimmed using the function *filterAndTrim* from the package 'phyloseq' v1.40.0 (McMurdie and Holmes, 2013). Parameters for *filterAndTrim* included a truncLen of 240

nucleotides for forward and reverse reads. The trimLeft parameter was used to remove the first 20 nucleotides of reverse reads. Maximum expected errors were 2 for forward reads and 2 for reverse reads. Filtered reads were inspected for an average quality score (Q) of 30 or greater. Reads with less than Q30 were removed. Error rates were learned using the *learnErrors* function in the 'dada2' package v.1.24.0 (Callahan et al., 2016; Prodan et al., 2020). Sequences were then denoised using the *dada* function, which produced amplicon sequence variants (ASVs) for each sample. Denoised forward and reverse reads were merged using the *mergePairs* function in 'dada2', with a minimum of 12 bp overlap. A sequence table was then created from the merged reads and sequence lengths were checked. Chimeras were removed from data using *removeBimeraDenovo* with the consensus method. Taxa were assigned to the SILVA 138.1 database with a minimum bootstrap confidence of 80 using *assignTaxonomy* (Quast et al., 2012; Yilmaz et al., 2013; Glöckner et al., 2017; Henderson et al., 2019). The ASV table, assigned taxa, and metadata were merged into a phyloseq object for downstream analysis. Taxa that were identified as unclassified at any rank were reassigned to their last known taxonomic rank and stored in a separate phyloseq object. Alpha diversity was measured using the observed ASVs, Chao1 metric, and Shannon Diversity Index using the *estimate_richness* function from the 'phyloseq' package. Next, sample counts were transformed and ordinated using Bray-Curtis Dissimilarity to calculate distances for a principal coordinates analysis (PCoA) to assess beta diversity using 'vegan' v2.6.2 (Oksanen et al., 2022). The *core_members* function in the 'microbiome' package (v1.18.0) (Lahti et al., 2020) was used to determine within-study core epimural bacteria based on ASVs with greater than 1% detection and 90% prevalence across all epimural samples.

2.8 Statistical analyses

Scores from the incision sites were evaluated in RStudio (R version 3.6.2). A histogram was created to visually assess the distribution of scores.

The alpha diversity measurements of observed ASVs, Chao1, and Shannon Diversity Index were visually assessed for normality and then tested using a Shapiro-Wilks test (W), with normality determined at $W > 0.90$ and $P > 0.05$. *A* Kruskal-Wallis H test was then used to test differences between sampling sites. Next, sample counts were transformed from total counts and distances were calculated by the Bray-Curtis Dissimilarity Matrix to calculate beta diversity measurements. Following, dispersion estimates were calculated and then distances were tested by a permutational multivariate analysis of variance (PERMANOVA) with 999 permutations using the *adonis* function in the package 'vegan' (Oksanen et al., 2007). Significance was determined at $P < 0.05$ for all analyses.

To test for differential abundances of bacterial communities between the rumen wall and the ruminal liquid fraction, the 'ANCOMBC' package v1.6.2 (Lin and Peddada, 2020) was used, as recommended by Nearing and others. (Nearing et al., 2022). For ANCOM-BC, the phyloseq object was used with the formula of sample type for the *ancombc* function, with the fixed effect of sampling site. Further, ASVs with prevalence less than 10% across samples were removed from the analysis. Multiple testing was addressed using Benjamini-Hochberg corrections (Benjamini and Hochberg, 1995). Due to the nature of the samples, there were structural zeroes in the data, where taxa were classified as structural zeroes based on their asymptotic lower bounds. A conservative variance estimator was used for the test statistic based on the assumption that there were large differentially abundant taxa. Significance was determined at $Q < 0.05$.

Further, the 'MaAsLin2' package v1.10.0 (Mallick et al., 2021) was used to test for differential abundances of bacterial communities between the rumen wall and the ruminal liquid fraction. Using the *Maaslin2* function, the ASV counts were normalized using the cumulative sum scaling (CSS) method (Paulson et al., 2013). Counts were log-transformed and the linear model was used, as recommended by package developers (Mallick et al., 2021). The statistical analyses used are further supported by Nearing et al. (Nearing et al., 2022). To maintain consistency and quality control, ASVs with a less than 10% prevalence across samples were removed from the analysis. The minimum abundance for each feature was set to 0.0001. Fixed effects included sample type, which was either epimural or liquid samples. Multiple testing was addressed using Benjamini-Hochberg corrections (Benjamini and Hochberg, 1995), with α set to 0.05.

The significantly different features between the ruminal liquid and epimural samples across both ANCOM-BC and MaAsLin2 were used for reporting differential abundance data.

3 Results

3.1 Surgery results

Visual assessment scores of the surgical site two weeks following surgery revealed a right-skewed distribution, with the majority of animals within the 0-1 range (Table 1). Within score ranges, 18 steers had a score of 0, 24 had a score of 1, two had a score of 2, and one had a score of 3. In addition, slight discharge around the incision site was present in four animals, irrespective of incision site score.

3.2 Sequencing results

Libraries were sequenced on an Illumina MiSeq and yielded an average of 157,741 read pairs per sample, with a minimum of 35,498 reads and a maximum of 319,966 reads. After taxonomic assignment, 15,405 taxa were identified out of the 90 samples. Within the rumen wall bacterial communities, 23 phyla and 212 genera were identified. When analyzing the ruminal liquid fraction, 26 phyla and 272 genera were assigned.

3.3 Alpha and beta diversity of bacterial communities

Analysis of the alpha diversity indices of observed ASVs, Chao1, and Shannon Diversity Index did not reveal differences in richness and evenness of ASVs between the rumen wall and rumen liquid bacterial communities ($P > 0.05$) (Figure 2). However, when assessing distances measured by Bray-Curtis dissimilarity, there were significant differences based on sampling site ($P < 0.05$) (Figure 3).

3.4 Abundances of epimural bacterial communities

The top phyla present in the epimural bacterial communities were Bacteroidota (46.94%), Firmicutes (39.62%), and Proteobacteria (6.07%), representing greater than 90% of total communities. Verrucomicrobiota (2.43%) and Spirochaetota (1.66%) followed in abundance. Other phyla present represented less than 1% of the relative abundance.

The top ten bacterial genera within the epimural bacterial communities were *Prevotella* (21.03%), *Succiniclasticum* (6.99%), *Rikenellaceae RC9 gut group* (6.03%), *Christensenellaceae R-7 group* (3.85%), *Ruminobacter* (3.20%), *Ruminococcus* (2.71%), *Ruminococcaceae NK4A214 group* (2.27%), *Prevotellaceae UCG-003* (2.09%), *Treponema* (1.37%), and *Prevotellaceae UCG-001* (1.19%). There were 197 genera present at less than 1% of the relative abundance. Unassigned reads to the genus level represented 28.49% of the relative abundance. All bacterial communities identified are present in Supplementary Table 1.

3.5 Abundances of liquid-associated bacterial communities

The top phyla in the ruminal liquid bacterial communities, representing greater than 90% of the total relative abundance, were Bacteroidota (52.17%), Firmicutes (34.96%), and Proteobacteria (4.65%). Other phyla present at greater than 1% abundance were Verrucomicrobiota (3.05%), Spirochaetota (1.47%), and Patescibacteria (1.12%).

The top ten bacterial genera within the planktonic communities were *Prevotella* (26.26%), *Rikenellaceae RC9 gut group* (5.54%), *Succiniclasticum* (4.49%), *Christensenellaceae R-7 group* (2.62%), *Ruminobacter* (2.47%), *Prevotellaceae UCG-003* (2.10%), *Ruminococcus* (1.78%), *Prevotellaceae UCG-001* (1.46%), *Ruminococcaceae NK4A214 group* (1.36%), and *Treponema* (1.17%). There were 259 genera present at less than 1% of the relative abundance. Unassigned reads to the genus level represented 32.02% of the relative abundance. All bacterial communities identified are present in Supplementary Table 1.

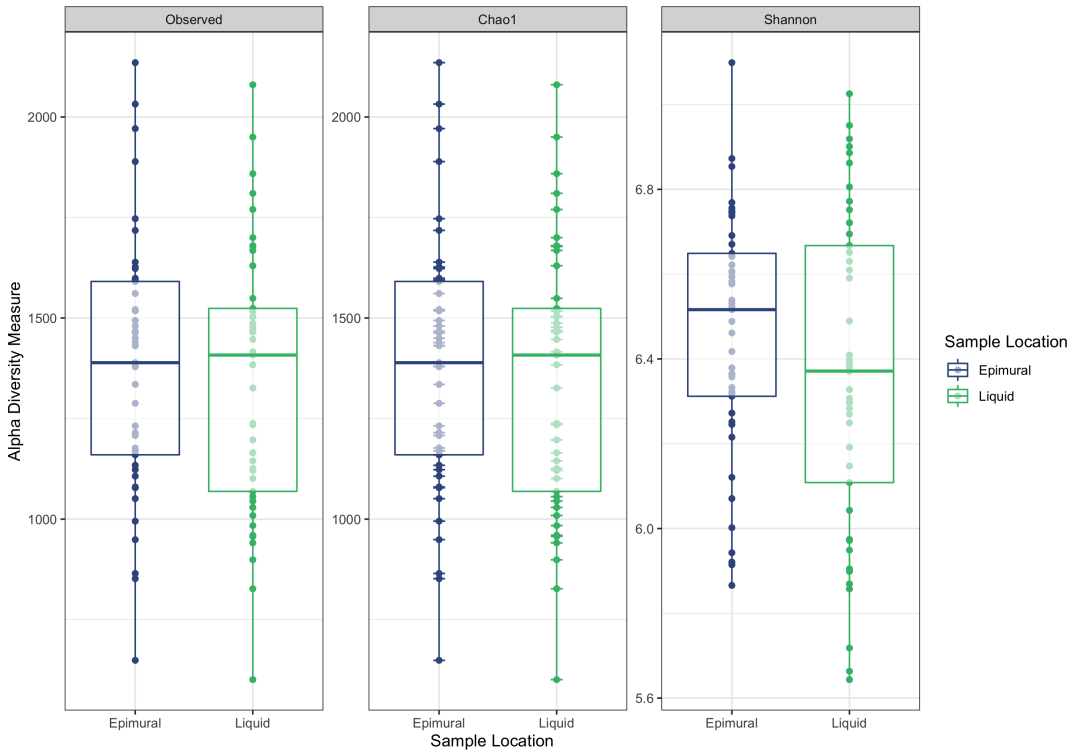

FIGURE 2
Alpha-diversity measurements between epimural and liquid bacterial communities. Measurements include the observed ASVs, Chao1, and Shannon Diversity Index. Epimural communities are represented in blue and liquid communities are represented in green.

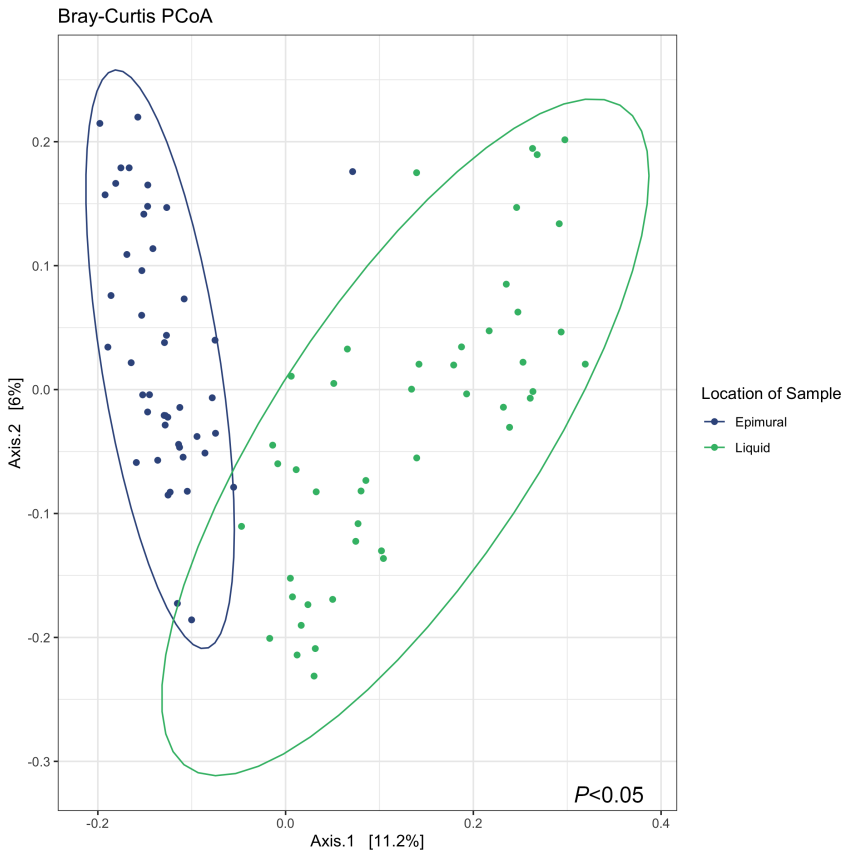

FIGURE 3
Bray-Curtis principle coordinates analysis (PCoA) describing the dissimilarity between sample locations, where the epimural samples are represented in blue, and the liquid samples are represented in green. Testing differences with a PERMANOVA with 999 permutations revealed differences in between sample diversity ($P < 0.05$).

3.6 Prevalent epimural bacteria

To describe the most prevalent epimural bacterial communities, the communities were based on ASVs with a greater than 1% detection and 90% prevalence among epimural samples. The 99 ASVs filtered based on these criteria were assigned to 23 unique genera (Table 2).

A full table representing the last known taxonomic assignment of unassigned bacteria at the genus level is located in Supplementary Table 2.

3.7 Differential abundances between epimural and liquid bacteria

After filtering MaAsLin2 and ANCOM-BC results for the overlapping bacterial communities, 451 ASVs significantly differed between the epimural and liquid fractions ($Q < 0.05$). These ASVs represented 91 taxa, where 62 were identified at the genus level and the remaining 29 were associated with their last-known taxonomic rank. Log_2 fold changes were calculated for all significantly different ASVs, shown in Figure 4. Log_2 fold changes for all significantly different ASVs, including unclassified genera that were reclassified to their last known taxonomic rank, are present in Supplementary Figure 1. The returned MaAsLin2 coefficients and ANCOM-BC log_2 values are available in Supplementary Table 3.

The top 10 ASVs in greater abundance in the epimural samples are found in Table 3 ($Q < 0.05$). Their respective genus-level assignments belonged to *Prevotella*, *Succinivibrio*, and *Rikenellaceae RC9 gut group*. Unassigned ASVs at the genus level were mapped back to their last-known taxonomic rank, belonging to family-level *Eubacterium* and *Prevotella*.

The top 10 ASVs that were in greater abundance in the planktonic, or liquid-associated, samples are found in Table 4 ($Q < 0.05$). Their respective genus-level assignments belonged to *Prevotella* and *Weissella*. Unassigned ASVs at the genus level were mapped back to their last-known taxonomic rank, belonging to family-level *Bacteroidales RF16 group* and *F082*.

4 Discussion

Most current approaches for sampling these ruminal epimural communities rely on two main methods: 1) harvesting the animal and collecting rumen papillae that are washed to collect epimural communities or, 2) cannulating the animal to sample papillae or directly swab the rumen wall. Few previous studies have suggested

10.3389/fanim.2023.1270550

TABLE 2 The most prevalent bacterial communities present based on a criteria of amplicon sequence variants (ASVs) with a greater than 1% detection and prevalence across all epimural samples.

Genus	ASV	Genus-level relative abundance (%)
[Eubacterium] ruminantium group	ASV227	1.05
Candidatus Saccharimonas	ASV127	0.04
Christensenellaceae R-7 group	ASV22, ASV39, ASV46, ASV75	3.85
Methanobrevibacter	ASV174, ASV285	0.55
Mitsuokella	ASV156	0.24
Unassigned	ASV9, ASV15, ASV19, ASV25, ASV33, ASV44, ASV51, ASV59, ASV89, ASV104, ASV105, ASV113, ASV124, ASV148, ASV150, ASV177, ASV196, ASV204	28.49
Ruminococcaceae NK4A214 group	ASV123, ASV169, ASV243	2.27
p-1088-a5 gut group	ASV165	0.43
Prevotella	ASV13, ASV16, ASV27, ASV29, ASV34, ASV36, ASV45, ASV47, ASV49, ASV57, ASV61, ASV62, ASV63, ASV71, ASV81, ASV92, ASV99, ASV101, ASV115, ASV141, ASV186, ASV252	21.03
Prevotellaceae Ga6A1 group	ASV18, ASV35, ASV43	1.09
Prevotellaceae NK3B31 group	ASV109	0.97
Prevotellaceae UCG-003	ASV116	2.09
Prevotellaceae UCG-004	ASV66, ASV103	0.66
probable genus 10	ASV215	0.41
Pseudobutyrivibrio	ASV121	0.26
Pyramidobacter	ASV280	0.14
Rikenellaceae RC9 gut group	ASV4, ASV12, ASV17, ASV94, ASV110, ASV158, ASV322, ASV385	6.03
Ruminobacter	ASV3, ASV7, ASV11, ASV26	3.20
Ruminococcus	ASV166, ASV201	2.71
Saccharofermentans	ASV68, ASV98, ASV132, ASV153	1.16
Schwartzia	ASV96, ASV242	0.32
Succiniclasticum	ASV1, ASV5, ASV8, ASV14, ASV20, ASV28, ASV38, ASV48, ASV138, ASV139, ASV255, ASV319	6.99
Succinivibrionaceae UCG-002	ASV21, ASV37, ASV50	1.02
Veillonellaceae UCG-001	ASV205	0.22

A total 99 identified ASVs were assigned to 23 unique genera.

using a trocar for collecting ruminal liquid. However, those methods are not directed toward sampling epimural microbiota and not widely used (Follis and Spillett, 1972; Wilson et al., 1977). Minimizing animal sacrifice and pain remains a key concern for animal welfare in research, providing the impetus for developing a live animal, minimally-invasive method to sample the microbial communities present on the rumen epithelium important. Following surgery, animals were monitored daily for changes in feed intake and health conditions. During the immediate postoperative period, animals showed no decreased appetite or mobility. To determine the procedure's invasiveness, incision sites were scored based on inflammation and swelling two weeks following the surgery. Few steers had discharge and swelling around the incision site and all steers retained normal mobility; therefore, the method was considered minimally invasive. Due to the limited techniques to sample from the rumen wall, many studies use small animal populations that impact the ability to determine differences within microbial communities. The surgery method allows for a non-invasive, quick technique to sample the rumen wall via swabbing through a trocar while capturing a comprehensive view of the microbial communities present.

While the surgical method allows for a swab to reach the rumen wall of the ventral sac, the swab must be pushed through the fiber mat and the rumen liquid fraction to reach the rumen wall. The cotton swab absorbing some of the liquid content from the rumen results in contamination from the liquid-associated bacterial communities. However, the sampling method did not aim to sample exclusively epimural bacterial communities but rather provide a minimally-invasive method to detect differences between the liquid and the epimural bacteria. The method is best used in a study that will examine the epimural communities in addition to the liquid and/or solid-associated microbial communities to aid in distinguishing the origin. Ultimately, there may be many microbial communities that overlap between epimural and liquid-associated samples using this sampling technique.

Limited research is available regarding the mechanisms of attachment of bacteria to the rumen epithelia. The associated bacterial communities have been previously proposed to adhere via fibrous carbohydrate coats (McCowan et al., 1978; Cheng and Costerton, 1980) or pili (Cheng and Costerton, 1980). Due to the mechanisms of attachment and the sloughing of the rumen epithelia, there is a likelihood that luminal microbiota may also be associated with or nearby epimural communities. As the proximity of these microbial communities may indicate their ability to interact with one another, distinguishing truly epithelial-associated versus planktonic microbes may be difficult. Some studies investigating the epimural microbial communities will rinse the excised rumen papillae with saline (Petri et al., 2013; Wetzels et al., 2017), while others will scrape the rumen epithelium as well (Chen et al., 2011; De Mulder et al., 2016). The swabbing method proposed in the current study may differentially separate adhered microbes from papillae compared to rinsing methods.

The composition and structure of the rumen microbiome has been demonstrated to alter in cases of major changes, such as alteration of diet, management practices, and environment. Steers underwent surgery on day 35 of the study and liquid samples were

FIGURE 4
Significantly different $\log_2$ fold changes in the abundance of amplicon sequence variants (ASVs) when using epimural as a reference level ($P < 0.05$; $Q < 0.05$). A negative $\log_2$ fold change represents ASVs more abundant within the epimural microbiome and a positive $\log_2$ fold change represents ASVs more abundant within the liquid microbiome. Colors represent different phyla, and rows label the ASVs to the genus level.

collected on day 70. At this time, the microbiome should already have reached stability, or near stability, which should result in minimal fluctuations between sampling dates. The time between sampling dates allowed for animals to be closely monitored for changes in health conditions before tubing for rumen fluid. Several studies have investigated the stability of ruminal bacterial communities within the rumen over time, concluding that after adjustment to diet, the most abundant microbial communities remain relatively stable (Clemmons et al., 2019; Snelling et al., 2019). Within the last decade, host genetics have been suggested to play an increasingly important role on the composition of the rumen microbiome (Sasson et al., 2017; Difford et al., 2018; Li et al., 2019a; Wallace et al., 2019; Martínez-Álvaro et al., 2022) and are likely contributing to the stability of the rumen microbiome during this time period. Further, recent studies have identified that host-influenced microbial genes do not have major shifts over the course of a 56-day feeding trial, supporting previous literature findings that the rumen microbiome remains stable over time (Lima et al., 2023). As the diet, management, and environment for the steers enrolled in the current study were maintained consistently, no major perturbations in the rumen microbiome would occur during this time frame. Therefore, differences identified in the rumen bacterial communities within the current study are more likely due to true differences in sample type and not due to temporal changes in the community structure.

In a previous study examining four rumen-cannulated Holstein-Friesian cows, the alpha diversity indices for the epimural bacterial communities indicated an increased variance compared to the liquid and solid-associated bacteria (De Mulder et al., 2016), which was likely due to individual animal differences.

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TABLE 3 The top 10 amplicon sequence variants (ASVs) based on log_2 fold changes associated with the epimural bacterial communities present in the rumen.

ASV	Genus	Log_2 Fold Change	FDR-adjusted *P*-value (*Q*)
ASV74	*Prevotella*	-4.00	1.29e-7
ASV157	Family: *Eubacterium*	-3.77	1.04e-10
ASV59	Family: *Succinivibrio*	-3.65	5.64e-15
ASV52	*Prevotella*	-3.64	1.27e-9
ASV128	Family: *Eubacterium*	-3.49	2.06e-12
ASV80	Family: *Eubacterium*	-3.41	1.24e-13
ASV88	*Succinivibrio*	-2.43	6.26e-7
ASV385	*Rikenellaceae RC9 gut group*	-2.36	5.64e-15
ASV87	*Prevotella*	-2.32	1.19e-7
ASV93	Family: *Prevotella*	-2.19	2.33e-5

Unclassified genus-level bacterial communities were renamed to their last known taxonomic rank, denoted as the ranking name followed by a colon and the last known name (ex: an unassigned bacterial community at the genus level whose last known ranking was the family level and assigned to Eubacterium would be denoted as Family: Eubacterium).

TABLE 4 The top 10 genera based on log_2 fold changes associated with the planktonic bacterial communities present in the rumen.

ASV	Genus	Log_2 Fold Change	FDR-adjusted *P*-value (*Q*)
ASV24	Family: *Bacteroidales RF16 group*	6.84	2.09e-23
ASV54	Family: *Bacteroidales RF16 group*	4.42	1.49e-20
ASV2	*Prevotella*	4.22	6.42e-9
ASV10	*Prevotella*	4.11	3.50e-9
ASV41	Family: *Bacteroidales RF16 group*	4.05	1.61e-18
ASV6	*Prevotella*	4.05	1.50e-9
ASV23	*Prevotella*	3.72	4.37e-9
ASV32	Family: *F082*	3.49	9.13e-8
ASV1103	*Weissella*	3.21	8.93e-8
ASV67	Family: *F082*	2.83	5.63e-7

Unclassified genus-level bacterial communities were renamed to their last known taxonomic rank, denoted as the ranking name followed by a colon and the last known name (ex: an unassigned bacterial community at the genus level whose last known ranking was the family level and assigned to Bacteroidales RF16 group would be denoted as Family: Bacteroidales RF16 group).

Other studies examining the alpha diversity of the epimural samples compared to rumen content samples have found increased diversity in the ruminal epimural bacteria (Abbas et al., 2020). However, this is inconsistent across studies, with others noting no significant differences in alpha diversity between epimural and liquid samples (Zhou et al., 2021). No differences in any alpha diversity metrics (observed ASVs, Chao1, and Shannon Diversity Index) existed between sampling locations within the current study, indicating that the within-sample richness and diversity were similar. There is a high likelihood that the within-sample richness and diversity of ASVs between the ruminal epimural and liquid bacterial communities would be very similar, as they could be distinctly different but equally diverse.

Past studies have found that Firmicutes, Bacteroidetes, and Proteobacteria are the most abundant phyla in the epimural communities. However, these studies disagree on whether Firmicutes or Bacteroidetes are in the greatest abundance (Chen et al., 2011; Petri et al., 2013; De Mulder et al., 2016; Sbardellati et al., 2020; Zhou et al., 2021). In this study, Firmicutes was in greater abundance in the epimural bacteria compared to the liquid fraction. Overall, Bacteroidetes was the dominant phylum for both sampling locations. Due to diet influencing both the liquid and epimural microbiota (Petri et al., 2020), shifts between Bacteroidetes and Firmicutes are expected among different studies. Additionally, primers targeting different regions of the 16S rRNA gene differentially preference specific bacterial communities via amplification bias, resulting in some studies discovering greater levels of one microbe over another (Klindworth et al., 2012). Overall, this study captures the main phyla present in other epimural community studies.

Previous studies assessing the composition of the epimural bacterial or archaeal communities have recently been combined into a few meta-analyses. A meta-analysis aiming to identify core epimural microbial communities, defined as operational taxonomic units (OTUs) present in over 90% of samples, found 11 genera representing the epithelial bacterial communities (Pacífico et al., 2021). These 11 genera consisted of *Campylobacter*, *Ruminococcaceae NK4A214 group*, *Desulfobulbus*, *Christensenellaceae R-7 group*, *Comamonas*, uncultured *Neisseriaceae*, *Succiniclasticum*, *Ruminococcaceae UCG-014* and *UCG-010*, *Lachnospiraceae UCG-010*, and *Defluvitaleaceae UCG-011*. A separate meta-analysis assessing the core epimural bacterial communities across 17 16S rRNA gene datasets, defined as a community present in greater than or equal to 80% of samples, found 147 core OTUs from closed-reference clustering within cattle species across dairy and beef breeds (Anderson et al., 2021). When narrowing the criteria for a core microbiome to bacterial communities present in 90% of cattle samples, there were 63 OTUs associated with the epithelium (Anderson et al., 2021). The 63 OTUs corresponded to 24 genus-level assigned taxa, where at least 4 OTUs were assigned to *Butyrvibrio*, *Christensenellaceae R7 group*, *Desulfobulbus*, and *Fretibacterium*. Based on the above literature, the predominant bacterial communities present on the rumen epithelium have been well-described and therefore can be compared to the current study.

Based on existing literature regarding the core epimural bacterial communities, the swabbing method employed in the current study captures previously-identified core epimural bacterial communities. When the most prevalent, considered as "core" within the current study, ASVs were aggregated to the genus

level, many ASVs were assigned to *Succiniclasticum*, *Rikenellaceae RC9 gut group*, *Christensenellaceae R7 group*, *Ruminobacter*, *Ruminococcus*, *Ruminococcaceae NK4A214 group*, and *Prevotella*. There are several considerations to make when using a core microbiome analysis, such as sample size, prevalence criteria, and the difficulty in determining a true "core" microbiome across studies (Neu et al., 2021). While core bacterial communities are defined within the current study, the epimural bacteria identified are used strictly for comparison to validate that the microbiota present are reported in previous literature. Several genera that were in greater abundance in the epimural communities in comparison to the rumen fluid communities were also considered a core bacteria within the study. Several *Succiniclasticum*, *Ruminococcus*, and *Ruminococcaceae NK4A214 group* were in significantly greater abundance in the epimural samples.

When assigning ASVs to the genus level, the majority of genera belonged to either the epimural or rumen samples, with very few having genera represented in both locations. There were 20 distinct genera that were present in rumen samples; in comparison, 33 genera were identified exclusively in the epimural samples. While there were 9 genera that had ASVs in both sample locations, these possibly indicate different species or common genera between the two locations. As there were distinct genera associated with sample location, there is evidence that the sampling method is not solely contaminated by planktonic bacteria. The number of differentially abundant communities between sampling location indicates that the minimally-invasive swabbing method provides information on the epithelium-associated microbes independent from the liquid-associated bacteria. These findings help validate the sampling ability of the surgery method.

Although not typically considered a core bacteria on the rumen wall, *Prevotella* and several *Prevotellaceae* met this study's criteria as core bacterial communities at the genus and family level. The bacteria are well-characterized in literature for their presence in rumen fluid samples (Stevenson and Weimer, 2007; Myer et al., 2015), which may indicate the potential degree of contamination via the swabbing method. However, other studies that examine epimural bacteria have found *Prevotellaceae* in small to moderate abundances, suggesting that these luminal communities may be closely associated with the rumen wall and thus appear in epimural samples (Sbardellati et al., 2020). The ASVs assigned to *Prevotella* within our study separate into two groups depending on the sample location. When examining the $\log_2$ fold change between the epimural and rumen liquid samples, the observed *Prevotella* are presumably serving different functions based on locations. Some caution should be employed when using ASVs to represent different species, as there is the possibility that genomes are artificially split using strict ASV thresholds (Schloss, 2021); however, there is also evidence that ASVs can capture a higher resolution of variation within an environment (Callahan et al., 2016; Callahan et al., 2017). Therefore, these ASVs assigned to *Prevotella* are possibly different species present depending on the sample locations.

Many ASVs identified as significantly different between the epimural and rumen fluid were unassigned at the genus level. Unassigned reads at the genus level are not uncommon in short-read amplicon studies (Henderson et al., 2019). Due to the difficulty in culturing ruminal bacteria (Creevey et al., 2014), many bacteria are unrepresented in databases, negatively impacting taxonomic resolution. One benefit of using ASVs is that they are not generally constrained to taxonomic classification. The unassigned ASVs could indicate bacterial communities that are not yet represented in databases. There are several attempts within the field of rumen microbiology to improve gaps within databases (Henderson et al., 2019), such as making rumen-specific databases (Seedorf et al., 2014; Seshadri et al., 2018) or improving culturing techniques (Kenters et al., 2011; Nyonyo et al., 2013). As these improve, more information will be available regarding unknown rumen species that are performing unique and important functions within the rumen.

Due to the proximity of the epimural microbiota to the rumen wall and their capacity to act as intermediary communicators to the ruminant host and rumen liquid fraction, there are opportunities to utilize the surgery method to elucidate functionality of the ruminal epimural communities. Studies have started to define the transcriptome of the microbial communities present at the rumen wall (Wang et al., 2017; Mann et al., 2018; Tan et al., 2021) and ruminal epithelial tissue (Kong et al., 2016; Wang et al., 2017; Elolimy et al., 2018). This has offered opportunities to better understand the functions that epimural bacterial and archaeal communities may perform. Studies examining the epimural microbiome have found that individual animal variation contributes to differences in abundance (Sbardellati et al., 2020), which may be explained by individual host control. These microbial communities could be modulating host-microbiome interactions. The non-invasive surgery method proposed within this work will allow for the investigation of mechanisms between the host and epimural microbial communities.

5 Conclusions

The non-invasive surgery method used within the current study captured epimural microbiota while also improving epimural sampling methods in the context of animal welfare in research. There is an opportunity within the field of rumen microbiology to use this minimally-invasive method to explore the microbiome on the rumen wall. In addition, this method can allow for examination of the cross-talk occurring between the rumen environment and the host, mediated by the epimural communities, through different -omics technologies.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA918692.

Ethics statement

The animal study was approved by University of Tennessee Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MH: Data curation, Formal Analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review and editing. TR: Investigation, Methodology, Supervision, Writing – original draft, Writing – review and editing. JB: Investigation, Methodology, Supervision, Writing – original draft, Writing – review and editing. PM: Methodology, Writing – review and editing. JS: Methodology, Writing – review and editing. BV: Investigation, Methodology, Writing – original draft, Writing – review and editing. JW: Investigation, Methodology, Writing – original draft, Writing – review and editing. LK: Investigation, Methodology, Writing – original draft, Writing – review and editing. PM: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fanim.2023.1270550/full#supplementary-material

SUPPLEMENTARY TABLE 1
Relative abundances and taxonomic assignments for all bacterial communities between the rumen liquid and epimural fractions.

SUPPLEMENTARY TABLE 2
The most prevalent epimural amplicon sequence variants based on a prevalence greater than 90% and detection greater than 1% across all epimural samples.

SUPPLEMENTARY TABLE 3
Results from the overlap of MaAsLin2 and ANCOMBC for differential abundance analyses, including coefficients, amplicon sequence variant numbers, taxonomic assignments, standard errors, p-values, and FDR-adjusted p-values.

SUPPLEMENTARY FIGURE 1
Significantly different $\log_2$ fold changes in the abundance of amplicon sequence variants (ASVs) when using epimural as a reference level ($P < 0.05$; FDR < 0.05). A negative $\log_2$ fold change represents ASVs more abundant within the epimural microbiome and a positive $\log_2$ fold change represents ASVs more abundant within the liquid microbiome. Colors represent different phyla, and rows label the ASVs to the genus level. The ASVs that were unassigned at the genus level are renamed to their last known taxonomic assignment, represented by the taxonomic rank followed by an underscore with the taxonomic assignment.

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