Distinguishing the Rumen Microbiome of Montana Ruminants

by

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Characterizing the rumen microbiome of native Montana ruminants

ABSTRACT

Ruminants are hoofed herbivorous mammals characterized by their habit of chewing cud (rumination). Ruminants can eat a diverse range of plants, and the microbiome (microbial community) found in the rumen is important in breaking down those plants. The rumen allows for the digestion and fermentation of food via microbes before entering the true stomach. Ruminants are not able to produce the enzymes to break down complex plant saccharides thus, they rely on microbes for the breakdown. When wild ruminants are considered, several species' rumen microbiome have been characterized. This is not the case with the rumen microbiome of Montana ruminants, many of which are keystone species. Thus, in this study I aimed to characterize the rumen microbiome of eight native Montana ruminant species utilizing samples collected around Montana and sequenced and sent into a bioinformatic pipeline for use in statistical analysis. The results from the analysis indicated that the phyla of Bacteroidetes and Firmicutes were the two most abundant microbial phyla found in all the ruminant samples. The results revealed that the rumen microbiomes of all species grouped together in accordance with their dominant feeding strategy of grazer, browser, or intermediate.

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ACKNOWLDGEMENTS

This study has been a collaborative effort made over the past half a decade originating from Savannah Grace, a master's student at Montana State University. Savannah Grace recruited and trained volunteer hunters on sample collection; she also performed the DNA extractions for 16S rRNA sequencing. It is to be acknowledged that PhD student Avia Simmons has also put effort into this collaboration after Savannah Grace's time at Montana State University. Avia Simmons trained me in bioinformatics including the use of DADA2, filtering, and taxonomic classification of 16S rRNA gene reads. Further, she helped with coding and interpretation of the multivariate and univariate methods. For my honors project, Avia continued to analyze the larger dataset, which forms the basis of a manuscript in preparation for ISME. I focused on a smaller subset of the data for my honors project.

INTRODUCTION

Ruminants are amongst the most successful group of herbivorous mammals on the planet, with about 200 ruminant species represented by 75 million wild ruminants and 3.5 billion domesticated ruminants (Henderson et al., 2015). Ruminants belong to the suborder Ruminantia (order Artiodactyla or Cetartiodactyla), which include taxa such as pronghorn antelope (family Antilocapridae), deer (Cervidae), and the bovids: cattle, Old World antelope, sheep, and goats (*Bovidae*). These mammals are distinct from others as they have a four-chambered "stomach" (the pre-absorptive digestive tract) and an even number of toe digits (Lotha, 2024). Ruminants have played a significant role in the development of many human cultures as a result of their unique rumen gut microbiome. There is a symbiotic relationship between ruminants and the rumen microbiome, with microbes facilitating the conversion of plant material that is indigestible for many mammals (e.g., cellulose). Thus, the digestive physiology of ruminants has benefitted humans through the conversion of the indigestible plant material into readily accessible resources such as dairy products, fiber, and meat (Henderson et al., 2015).

Food digestion via microbes

On average, 35% – 40% of a cow's day is spent ruminating (Linn et al., 2021). Fine feed such as grain or ground rations takes less time to digest, while grazing feed such as long hay requires more time. The stomach of ruminants is composed of four compartments: the rumen, reticulum, omasum, and abomasum (Parisch & Karisch, 2023). It is often considered that the reticulum and the rumen are one organ as both are hosts to microbes and function as a fermentation vat, only separated by a small muscular fold of tissue. The difference is that the reticulum often has heavy or dense feed drop into this compartment (Linn et al., 2021). The reticulorumen is home to the microbes and is responsible for fermenting and breaking down of cell walls into carbohydrate

fractions and volatile fatty acids. These materials will be used for fat synthesis, glucose synthesis, protein synthesis, vitamin synthesis, and energy metabolism (Lotha, 2024).

Characterizing ruminants

It is presumed that the morphological, physiological, and behavioral characteristics of evolved feeding strategies are responsible for the microbial community composition of ruminants (Henderson et al., 2015). Feeding strategies can be grouped into browsers ("concentrate-fed"), grazers ("forage-fed"), and intermediate feeders (Parisch & Karisch, 2023). Grazers such as domestic cattle are the least selective and most versatile in their diet. Feeding on a diet of grasses, shrubs, forbs, and the lower leaves of deciduous trees, they have the largest rumens and slowest fermentation times. Intermediate feeders such as domestic goats have a seasonally dependent diet of grasses, sedges, forbs, shrubs, & leaves. They have a smaller rumen size and moderate fermentation times. Browsers such as roe deer feed on tree leaves, forbs & shrubs (Lindquist, 2020). They have the smallest rumens and the highest fermentation rates, metabolic rates, and frequency of feeding.

Rumen microbiome

Microbes are tiny living organisms that are too small to be seen by the naked eye and include bacteria, archaea, viruses, fungi, and protozoa (Gupta et al., 2017). A microbiome is a community of microbes that are found living together in any given environment, including the body or a part of the body. Most microbiomes that are found on or in an animal's body tend to be symbiotic with the host, often providing benefits such as epidermal protection or processing of food. A smaller portion of all microbiomes are pathogenic and could promote disease (Ursell, 2022). The rumen microbiome is a collection of archaea, protozoa, and bacteria that function to

degrade food and metabolize it into major nutrient sources for the host animal (Henderson et al., 2015).

Ruminants do not produce enzymes needed to break down complex plant saccharides found in their food and thus rely on microbes to aid in the breakdown (Henderson et al., 2015). Microbes digest 30 – 50% of the fiber units, cellulose and hemicellulose in the rumen. Sixty percent or more of the starch is degraded, depending on the amount and the speed that ingested materials move within the rumen. Most sugars are digested completely within the rumen (Wexler, 2007). The rumen microbiome is well-defined for many different ruminant species, but Montana ruminants were largely uncharacterized.

16S rRNA genes

The 16S rRNA gene is consistently used for characterizing microbial communities inhabiting host organisms via sequencing. By characterizing the diversity and composition of microbial communities, researchers can gain the identity of microbes that live within a microbiome (Jeong et al., 2021). The entirety of the 16S rRNA gene is ~1500 base pairs and within that sequence, it can provide the taxonomic resolution for the microbial species and strain categories (Johnson et al., 2019). Within this study, the 16S rRNA gene V4 region was utilized to characterize the rumen microbiome.

Problem statement

The field of ruminant rumen microbiology is understudied. Furthermore, the phylogeny of bacteria and their functions are relatively unknown, with only 14% of all bacteria falling under a named species, 16% within a phylum, and 70% not within a formally recognized genus (Henderson et al., 2015). Characterization of the rumen microbiome allows agricultural and

veterinary knowledge to be furthered. For example, larkspur (*Delphinium occidentale*), a native North American flower, is responsible for 10% of annual domestic cattle death within North America. Wild ruminants certainly encounter larkspur and yet their microbiome is able to degrade this toxic plant. A potential husbandry solution might be microbial transplant in which microbes are incorporated into feed. Hypothetically, this would mean that a microbe that can degrade larkspur could be added to feed, enabling domestic cattle to degrade larkspur toxins (Pfister et al. 1996). By characterizing the rumen microbiome, we are one step closer to this idea.

Thesis

Although domesticated ruminants and a few wild ruminant species have been extensively studied, this is not the case with the ruminant's native to Montana. For this study, the microbial community was analyzed from rumen samples of 159 individuals belonging to eight wild Montana ruminant species. The aim of this study was: (1) To characterize the rumen microbiome of wild Montana ruminant species, and (2) assess whether the microbiome is associated with their species-specific dietary habits.

METHODS

Obtaining rumen samples

Foregut rumen samples were collected from eight different wild ruminant species by volunteer hunters under legal methods during the designated Montana hunting season (September – December) in the fall and winter of 2019 and 2020. Hunters drew the license for specific species under normal Montana government law. Montana State University collaborated with Fish, Wildlife, and Parks to recruit volunteer hunters holding licenses for the following ruminants: bighorn sheep (*Odocoileus virginianus*), bison (*Bison bison*), elk (*Cervus canadensis*), moose

(*Alces alces*), mountain goat (*Oreamnos americanus*), mule deer (*Odocoileus hemionus*), pronghorn antelope (*Antilocapra americana*), and white-tailed deer (*Odocoileus virginianus*) (**Table 1**). The volunteer hunters were given verbal and written instructions on how to locate and sample the rumen.

Table 1*: Sample population characteristics*

Individual Characteristics	
	N=159
Species	
Bighorn sheep	14 (8.81%)
Bison	11 (6.92%)
Elk	11 (6.92%)
Moose	13 (8.18%)
Mountain Goat	19 (11.9%)
Mule Deer	53 (33.3%)
Pronghorn	19 (11.9%)
White-Tailed Deer	19 (11.9%)
Grazing Species	$N_G = 25$
Bighorn Sheep	14 (56%)
Bison	11 (44%)
Intermediate Species	$N_I = 49$
Elk	11 (18.6%)
Mountain Goat	19 (38.8%)
Pronghorn	19 (38.8%)
Browsing Species	$N_B = 85$
Moose	13 (15.3%)
Mule Deer	53 (62.3%)
White-Tailed Deer	19 (22.4%)

During harvest, rumen contents were sampled via an incision into the rumen wall and collection of a mixture of liquid and solid gut content in a 50 mL polypropylene conical tube. These

samples were placed in a freezer (-20 °C) and kept frozen until they were returned to the laboratory at Montana State University. The time between initial freezing and drop off at the laboratory was a maximum of four months. Any samples that were thawed, lacked ruminal fluid, or showed viability concerns were discarded from the sample pool.

16S rRNA sequencing

To obtain the microbial 16S rRNA, the DNA of the rumen samples was extracted and the 16S rRNA genes of each rumen sample were sequenced. Initially, the samples were amplified utilizing tagged PCR primers, which would introduce a barcode into each of the sequences thus purifying the samples when they were filtered. The PCR products were purified to remove excess primers and nucleotides. Purified samples underwent cycle sequencing, tagging each fragment with terminator bases with fluorescent dye for different lengths. The products were purified to remove unincorporated dye terminators and the lengths of each fragment were determined from gel electrophoresis. Fragments of DNA were sequenced separately.

Bioinformatic pipeline

Having obtained the 16S rRNA sequences, the next step was to send these sequences into a bioinformatic pipeline and ascribe a taxonomy based on a reference database. We used R Statistical Software (R Core Team, 2021) for the bioinformatic pipeline and statistical analysis. For the bioinformatic pipeline, the package Mothur (Schloss, 2020) was utilized to filter, parse, remove chimeras, merge, and assign taxonomy for the 16S rRNA sequence. Amplicon Sequence Variants (ASVs) were utilized for assigning taxonomy. Thus, 16S rRNA data went through a similarity threshold of 100% and were grouped up higher in the classification table. Singletons and doubletons were removed from the 16S rRNA data. Furthermore, taxa that had a maximum

relative abundance < 0.01 across all ruminant samples were removed. The taxonomy assigned was based on the Silva database (version 128) and there were a total of 1186 ASVs from the cumulative rumen microbiomes of 159 ruminants. The ASVs were sorted to the genus level. An additional dataset was created that sorts the ASVs to the phylum level. This dataset was created by grouping together all the ASVs in the same phylum and summing their relative abundance. This allows for the relative abundance of bacterial phyla to be determined.

Statistical analysis

Compiling all the ASVs into a .csv file, statistical analysis was conducted to characterize the composition of the microbiome and analyze microbial composition through the variables of feeding strategies and ruminant species. Non-metric multi-dimensional scaling (NMDS), principle coordinate analysis (PCoA), pie charts, and boxplots were created using R Statistical Software to visualize the rumen microbiomes. Multivariate and univariate statistical techniques were then utilized to look at the microbiome as a whole and at ASVs individually. These graphics enabled the characterization of the ruminant microbiomes and correlation of microbial abundance to feeding strategies. To test that datasets were significantly different from each other, a two-tailed, two-sample unequal variance t-test was conducted.

To analyze the variation among samples, Analysis of Similarity (ANOSIM) and Permutational Multivariate Analysis (PERMANOVA) were calculated using the Jaccard dissimilarity index. ANOSIM is a non-parametric test based on the rank distances among sample units. This test looks at grouping variables, thus the mean rank distance among sample units within a group will be smaller than the rank distance between sample units from different groups. PERMANOVA is

a non-parametric multivariate statistical permutation test used to compare variation between groups to the variation within groups.

Parametric tests assume that the data follows a Gaussian distribution in the underlying population, which is suitable for situations when groups have different amounts of variability. Non-parametric tests do not rely on assumptions about the distribution of the underlying population, which is suitable for when the data are not normally distributed. Additionally, non-parametric tests are robust against outliers and resistant to extreme values. For this study, the Gaussian distribution was not observed with the microbial abundance data. The data has many outliers and extreme values thus non-parametric tests were conducted.

RESULTS

The rumen microbiome varies by ruminant species & by feeding strategy:

The principle coordinate analysis (PCoA) of the entire ruminant microbiome (Fig. 1) revealed that grazers predominantly grouped to the right side of the figure, while the browsers clustered on the left side. Intermediates, with more flexible diets, covered areas from both grazers and browsers. The PERMANOVA R^2 indicated that both ruminant species and feeding strategy significantly explained the variation within the rumen microbiome, with species contributing 12.4% of the observed variation, and feeding strategy contributing 4.6%. ANOSIM analysis revealed greater similarity in microbial composition among ruminants of the same species ($R = 0.3451$) compared to those with similar feeding strategies ($R = 0.1942$).

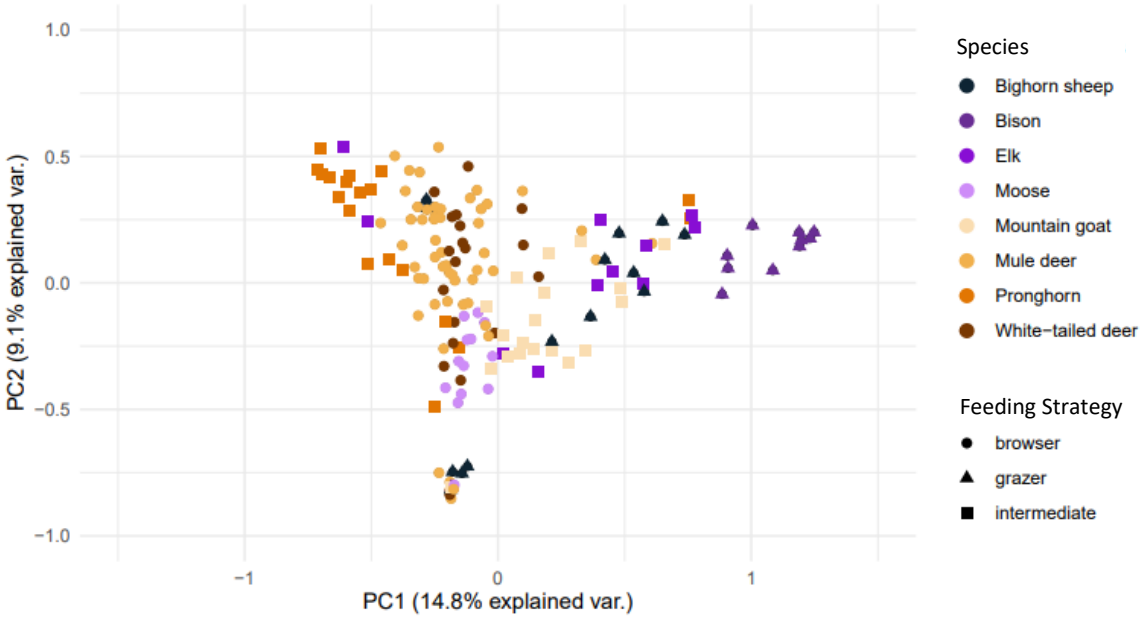

Figure 1. Each data point on this principle coordinate analysis (PCoA), represents the entire ruminant microbiome from a rumen sample. Within this figure, there are 159 ruminant samples and each data point represents an entire microbiome from a ruminant. These points are characterized by color for ruminant species and by shapes for feeding strategies. This graph is indicative of similarity between variables. Thus, the closer two points are, the more similar the corresponding samples are to each other. This indicates that points in close proximity have similar microbiomes and points distant from each other have widely different microbiomes.

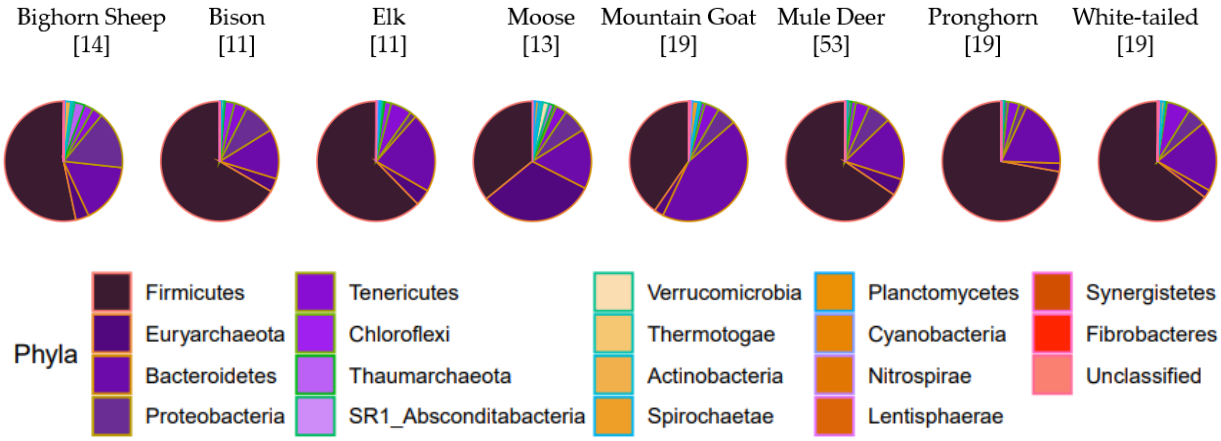

Figure 2. The legend below the pie charts represents bacterial and archaeal phyla obtained during ascribing taxonomy. The most abundant bacteria and archaea from all ruminants, start at the top and moves counterclockwise of the pie chart from most to least abundant.

The relative abundance of bacterial phyla in the microbiomes are illustrated in Figures 2-4. The phyla Firmicutes and Bacteroidetes (Fig. 2) were the most abundant across all ruminant species, albeit varying in distribution among species. A two-tailed, two-sample unequal variance t-test indicates a significant difference between Firmicutes and Bacteroidetes with a value of p = $2.53\ x\ 10^{-45}$. Boxplots reveal dominant families within each phylum, such as *Lachnospiraceae*, *Christensenellaceae*, and *Ruminococcaceae* in Firmicutes, and *Prevotellaceae* and *Rikenellaceae* in Bacteroidetes.

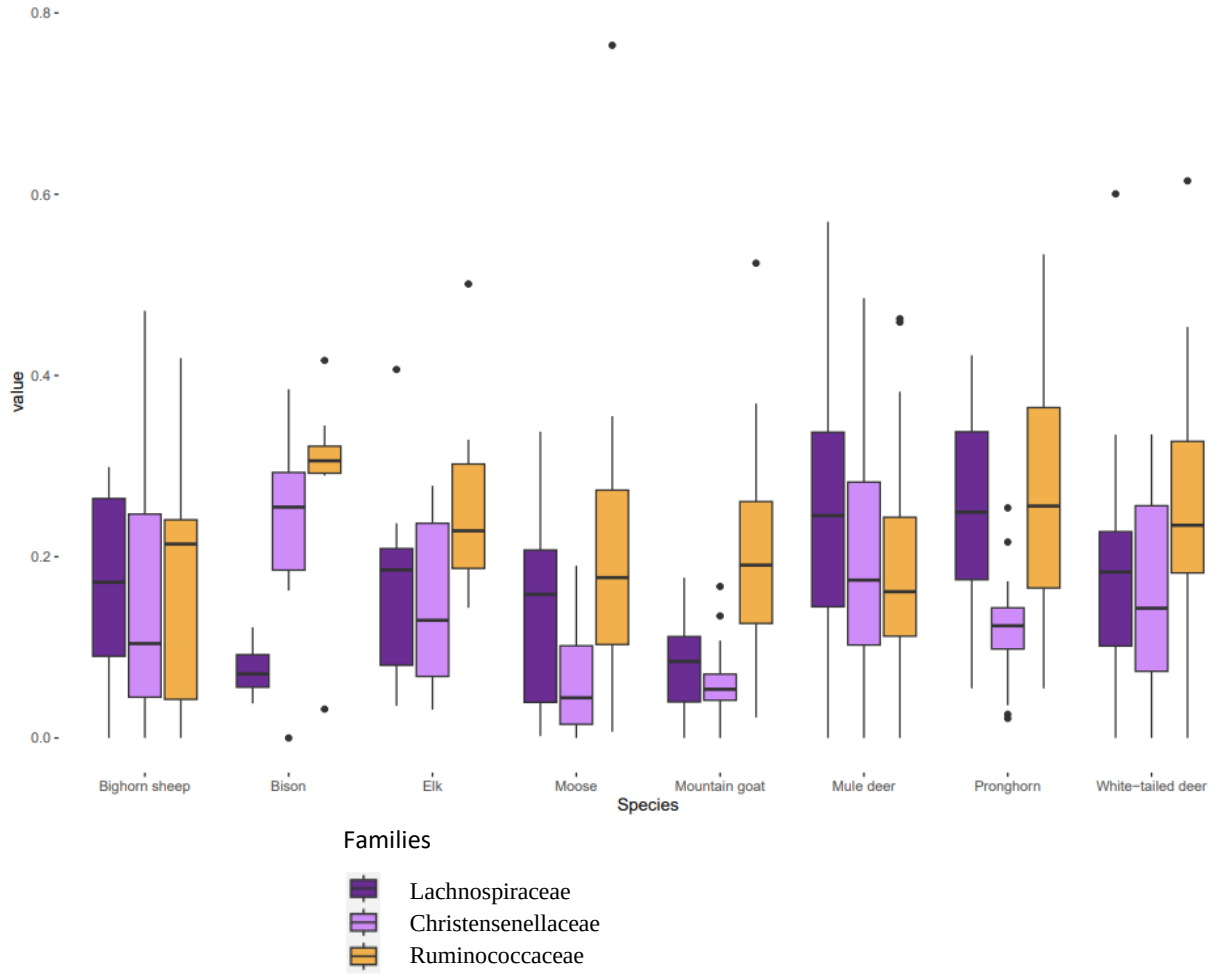

Figure 3. The legend indicates the three most abundant families were from the Firmicutes phylum. The boxplots indicate the median via the middle line within the box, and the box from bottom to top indicates the 2nd and 3rd quarter and are separated by the median line. The lines below and above the box are whiskers that indicate the 1st and 4th quarters. Dots are outlier samples.

The abundance of Firmicutes families across ruminant species was correlated with their diets (Fig. 3). Mule deer and pronghorns expressed the highest abundance of *Lachnospiraceae*; *Christensenellaceae* was most abundant in bison; and *Ruminococcaceae* was the most abundant in elk, pronghorn, and white-tailed deer. The ruminant species that had the highest abundance of

families from Firmicutes include two of the three browsing species, namely the white-tailed deer and mule deer.

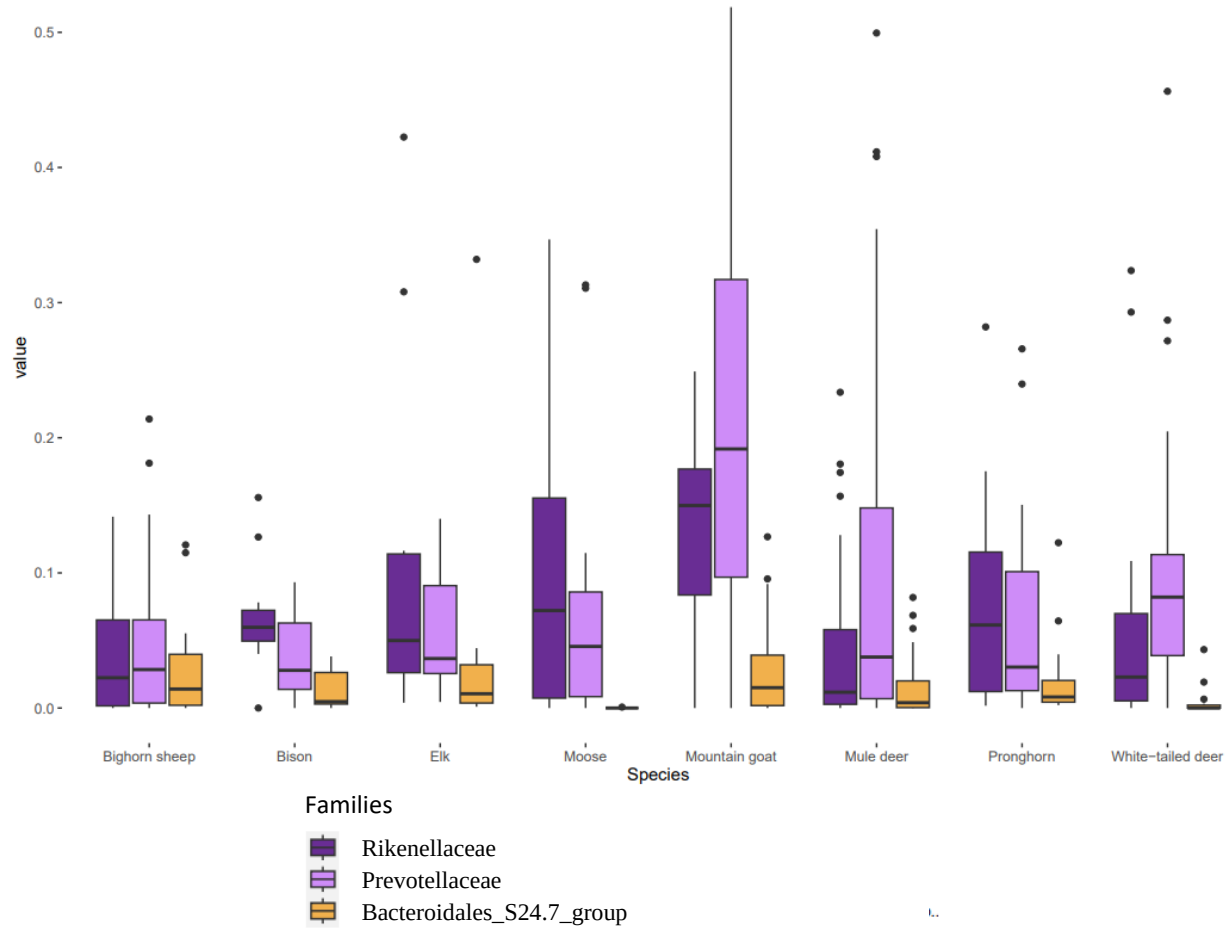

Figure 4 The legend indicates the three most abundant families from the Bacteroidetes phylum. The boxplots indicate the median via the middle line within the box and the box from bottom to top indicates the 2nd and 3rd quarter and are separated by the median line. The lines below and above the box are whiskers that indicate the 1st and 4th quarters. Dots are outlier samples.

The mountain goats' microbiome had the highest abundance of Bacteroidetes, particularly Prevotellaceae and Rikenellaceae.

DISCUSSION

The rumen microbiomes of various ruminant species distinctly reflect differences in their feeding ecology (Parisch & Karisch, 2023). We observed the microbiome of ruminants clustered by ruminant species and by feeding strategies. Species with the same feeding strategy exhibited more similar microbiomes than those with different feeding strategies. Firmicutes and Bacteroidetes emerged as the most abundant microbial phyla collectively accounting for over half of all rumen microbes across species. Firmicutes were the most abundant phyla in all species except for mountain goats, where Bacteroidetes were the most abundant phyla. This is consistent with Stojanov et al.(2020), who showed that these two phyla are the two most important in maintaining gut health.

The literature connects specific microbial families to dietary components. The phyla Firmicutes are responsible for carbohydrate, starch, lipid metabolization and fermentation, often playing a crucial role in health and homeostasis (Stojanov et al., 2020). The family *Lachnospiraceae* are associated with the degradation of plant cellulose and hemicellulose, characterized by their cellulose-decomposing activity (Wang et al., 2021). *Christensenellaceae* metabolizes amino acids with their relative abundance being inversely proportional to fat content (Jia et al., 2024). *Ruminococcaceae* are found in a diet of high fat, breaking down cellulose, and were negatively correlated to inflammation (Rinninella et al., 2019).

Connecting the literature to our results, it is likely that the ruminant samples from the bison species had less fat content in their diet than the other ruminant species due to the comparable abundance of *Christensenellaceae*. Furthermore, it is likely that elks, pronghorns, and white-tailed deer had a diet high in fat due to having a relatively higher abundance of *Ruminococcaceae*.

The phylum Bacteroidetes are the primary-starch degrading and cellulolytic bacteria in the rumen microbiome. They are often found in food that is high-grain and are negatively correlated with high fiber. Within high-grain food, there is more starchy material (Louis & Flint, 2016). The family of *Rikenellaceae* break down proteins and have a higher abundance in high-protein groups (Zhang et al., 2021). The family *Prevotellaceae* is central to carbohydrate and hydrogen metabolism, often breaking down a variety of polysaccharides and complex carbohydrates (Bentacur-Murillo et al., 2023) The abundance of Bacteroidetes correlates with the sampled mountain goats, having a high grain diet in comparison to a diet of high fiber. Additionally, the diet mountain goats consumed was rich in high-protein groups and varying complex carbohydrates and polysaccharides.

I also observed that while multivariate analysis indicates ruminant species group by feeding strategy. Specific microbial abundance did not strictly align with feeding strategies. For instance, microbial families' percentage were different between pronghorns and mountain goats, which are both intermediate feeders. These findings highlight the adaptability of ruminants to diverse nutritional ecological niches, facilitated by the microbial diversity in their rumen microbiomes. In particular, intermediate feeders demonstrate versatility in consuming various plant types, as evidence by their microbiome distributions resembling browsers or grazers. This suggests

potential adaptation to dietary shifts induced by climate change, although further research with larger sample sizes is warranted.

The rumen microbiome, reflects evolutionary adaptations to different feeding strategies, enabling ruminants to thrive in varied ecological niches. The abundance and diversity of microbes facilitate the consumption of diverse vegetation. Further studies could explore seasonal influences on rumen microbiome dynamics, enriching our understanding of ruminant physiology and ecology. Overall, characterizing rumen microbiome diversity enhances our understanding of interspecies differences among ruminants.

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