



COMPARING MICROBIAL COMMUNITIES ACROSS THE GASTROINTESTINAL TRACT OF MOOSE

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ABSTRACT: Coevolution of microbial communities and their hosts has allowed herbivores to extract energy from recalcitrant complex carbohydrates in plants, and benefit from synthesized protein and vitamins. Ruminants have further evolved 3 locations within their gastrointestinal (GI) tract that house and that facilitate fermentation of plant fiber, the rumen/reticulum, cecum, and colon, but little is known about how the microbiome varies across these organs, especially in free-ranging browsing herbivores like moose (*Alces alces*). Therefore, we took advantage of a unique opportunity to sample each of these organs from vehicle-struck and hunter-harvested moose in south-central Alaska and eastern Washington/northwestern Idaho, USA. Using culture-free microbial techniques to extract, identify, and quantify microbes in the samples, we found few differences in microbial composition, richness, and diversity among fermentation organs, but large differences between geographic regions. Our results suggest that differences in the macroenvironment and diets across regions play a greater role in microbial communities than different microenvironmental conditions within organs of the GI tract. Furthermore, our findings support microbiome measurements from fresh fecal samples as a surrogate for microbial communities in the rumen and cecum. Understanding the function and composition of microbial communities within the fermentation organs is increasingly important for quantifying how ruminants such as moose will respond to changes in diet and environment, and how these changes may influence their interactions with plant resources.

ALCES VOL. 60: 123–145 (2024)

Key Words: Alaska, *Alces alces*, cecum, colon, Idaho, microbial communities, Washington

Ruminants have evolved a complex, anoxic foregut portion of their gastrointestinal (GI) tract that houses a large and diverse microbial community (i.e., 10^{10} to 10^{11} organisms/ml, (Newbold and Ramos-Morales 2020). This adaptation allows them to convert cellulose (the most abundant biomolecule on Earth), other recalcitrant structural carbohydrates, and elemental nutrients of plants to microbial cell protein and short chain fatty acids that fuel metabolism and build body tissue (Hofmann 1989, Solden et al. 2018). Approximately 80% of the energy derived by the ruminant host is produced from fermentation by the rumen microbiota (Van Soest 1994). Through

anaerobic fermentation, microbes in the ruminant foregut not only extract energy and nutrients from plants ingested by their host for their own use, but also synthesize vitamins, and aid in detoxifying plant secondary metabolites (Kartzinel et al. 2015), which provides critical benefits to their host in the ultimate symbiotic relationship.

The evolutionary benefits of microbial fermentation are enhanced when herbivores have multiple fermentation chambers that can increase digestion and absorption of nutrients from the plants they consume and improve detoxification efficiency. Ruminants (Bovidae, Cervidae, and Antilocapridae)

have 3 sites in their GI tract that house most of the microbes important to nutrition: the rumen/reticulum in the foregut, and the cecum and colon that are collectively referred to as the hindgut. The rumen is the largest fermentation organ, accounting for approximately 16% of a ruminant's total weight and 84% of the total GI volume (Van Soest 1994, Niehaus et al. 2019). The rumen is the primary location for fermentation and has large papillae and many folds to increase surface area for absorption of energy-rich byproducts of microbial fermentation. The papillae develop as the animal ages, stimulated by the presence of ingested food available for fermentation (Robbins 1994). Papillary structure and density are partly influenced by the animal's diet but are generally larger and denser in the ventral regions of the rumen where nutrient absorption is most pronounced (Van Soest 1994). Fine food particles wash out of the rumen into the reticulum, which is tasked with sorting and handling these particles, aided by its unique honeycomb texture. Like the rumen, the reticulum also hosts fermenting microbes, but its main role is to ensure larger particles stay in the rumen to be further diminished by rumination and fermentation while finer particles and microbial cells move on to the next chamber (Van Soest 1994)(Van Soest 1994).

Further along the GI tract beyond the gastric stomach (i.e., abomasum), fermentation also occurs in the cecum and colon. The cecum and colon are often understudied in ruminants because they are smaller than the foregut and are more difficult to access for sampling in live animals. The ruminant cecum, situated between the small intestine and colon, composes approximately 2% of the GI volume (Hoover 1978, Russell 2002). The cecum is a secondary site of fermentation in ruminants, containing a diverse microbial community. The rate and extent of

cecal fermentation depends on the amount and type of fermentable digesta that escapes foregut fermentation and gastric catabolism. During periods of heightened food intake (e.g., summer, lactation), passage rate through the GI tract is increased to avoid overfilling the rumen by reducing rumination time and drinking more water (Asano et al. 2007). Under these conditions, larger particles of food are more likely to escape the rumen before they can be fermented fully, at which point the cecum can provide a second chance to capture energy and nutrients from hemicellulose and cellulose (Allen et al. 1986, Van Soest 1994).

The most distal fermentation site of ruminants, the colon, composes approximately 12.5% of the total GI volume (Hoover 1978), and is the primary location of water, sodium, and copper absorption. However, the colon also houses microbial communities capable of fermentation. As in the cecum, colon communities specialize on the fermentable substances that by-pass the foregut or get washed out before being fully fermented (Hoover 1978, Van Soest 1994). The colon also plays a major role in the recovery and transfer of nitrogen from the microbes to the blood, where it can then be returned to the rumen and used for microbial protein synthesis (Hoover 1978).

In most mammals, the GI microbiome is dominated by bacterial phyla: Firmicutes, Bacteroidota, Actinobacteria, and Proteobacteria (Ley et al. 2008, Manichanh et al. 2010). Because Firmicutes and Bacteroidota are the most abundant phyla in the gut of mammals, including ruminants (Magne et al. 2020, Pinnell et al. 2022, Bensch et al. 2023), and they have different functions, the ratio of the 2 has often been used to evaluate proper gut functioning, such as identifying various health conditions, hormone shifts, and seasonal changes in the diet (Springer et al. 2017). Although Firmicutes

and Bacteroidetes are both important for carbohydrate metabolism, Firmicutes primarily ferment nonfibrous carbohydrates and proteins whereas Bacteroidetes digest and uses cellulose and exogenous peptides as a food source (Hayes and Bleakley 2018, Wu et al. 2022, Fu et al. 2024). The ruminant's microbiome also includes bacteria species from Acidobacteria, the Chloroflexi phyla, and some fungal and protozoan species (Ishaq and Wright 2014, Ishaq et al. 2015, Solden et al. 2017). Three bacteria species, *Fibrobacter succinogenes* within the Fibrobacterota phylum and *Ruminococcus albus* and *Ruminococcus flavefaciens* within the Bacillota phylum, have been considered the predominant agents of cellulolysis in the rumen, but the full suite of microbial taxa that are key to cellulose degradation in the rumen has yet to be identified (Weimer 2022).

Microbes within the same GI organ spatially segregate, depending on their roles or preferred food sources (Van Soest 1994). Microorganisms that ferment cellulose in plant fiber will often etch pits into the available surfaces of the fibrous material to anchor themselves to the fiber and remain in the rumen longer, but microbes in the rumen liquid are typically non-cellulolytic (Kong et al. 2010). Some bacteria attach to the rumen wall, and they are often ureolytic and aid in urea and oxygen transfer. Bacteria that are not attached to a substrate as well as free floating protozoans and fungi tend to wash out of the rumen at the liquid passage rate, and are later digested by the animal in the abomasum (gastric stomach, Van Soest 1994, Kong et al. 2010, Weimer 2022). Describing and comparing the microbial communities spatially separated in the digestive tract is in its early stages, and has focused solely on hindgut fermenters or ruminant livestock that consume more grasses (Kong et al. 2010,

Mao et al. 2015, Holman and Gzyl 2019). Therefore, little is known about these communities for browsing ruminants that consume plants with high levels of insoluble lignin and often toxic plant secondary metabolites (PSMs, Godoy-Vitorino et al. 2012, Svartström et al. 2017).

Not only is the microbial community expected to vary across the GI tract based on the morphology and function of the fermentation organ, but also with the composition of diets consumed, and thus the habitats in which herbivores forage. Because of their circumpolar distribution, moose reside in areas that vary greatly in available forage resources. Moose are large, near-obligate dietary specialists on boreal woody plants, primarily deciduous leaves during the growing season and stems and evergreens during the dormant period from mid-autumn through mid-spring. Their diets usually include willows (*Salix* spp.) and birch (*Betula* spp.) in Alaska and the Rocky Mountains, and conifers in Maine and Sweden (Shipley 2010). Dormant deciduous and evergreen shrubs not only contain high amounts of cellulose, but also high amounts of lignin and cutin (e.g., 15% - 20%) that cannot be fermented by GI microbes to extract energy (Spaeth et al. 2002, Spalinger et al. 2010). In addition, leaves of both deciduous and evergreen shrubs are chemically defended by PSMs such as condensed tannins and monoterpenes (Palo 1984, McArt et al. 2009, Tremblay et al. 2019). Therefore, moose rely on adaptations to help consume and digest these diets, including specific mouth morphology (e.g., prehensile tongue and lips, a wide mouth, large salivary glands (Hofmann and Nygren 1992, Van Soest 1996, Clifford and Witmer 2004), proline-rich saliva that binds tannins (Hagerman and Robbins 1993), and, most importantly, their GI microbiota (Ishaq and Wright 2012, Ishaq et al. 2015, Solden et al. 2017).

Our objectives were to describe and compare the microbial communities in the rumen, cecum, and colon of free-ranging moose, and compare microbial communities of moose in 2 different study areas in the United States, southcentral Alaska (Alaska, hereafter) and eastern Washington and northwestern Idaho (Inland Northwest, hereafter). We hypothesized that the diversity of microbial communities would decrease along the GI tract, thus the highest diversity in the rumen and the lowest in the colon. This hypothesis is supported by research with domestic cattle consuming traditional livestock rations that found lower microbial diversity in the hindgut (Holman and Gzyl 2019), likely because the gastric stomach removes much of the dietary substrates that provide food for microbiota in the hindgut. In addition, the cecum and colon have shorter retention times and less spatial separation than the rumen, so the microbes also have less time to fully establish into unique subcommunities (Dehority 2002). Because the rumen is the first major fermentation organ chamber in the GI tract, we also hypothesized that its microbial community would be dominated by organisms best able to ferment and transform cellulose and detoxify PSMs that reduce the animal's burden for detoxification later in the GI tract. Therefore, we predicted that the rumen would have a higher abundance of cellulolytic bacteria whereas the cecum would have a higher relative abundance of hemicellulose digestors (Kohl et al. 2014, Cholewińska et al. 2021, Pinnell et al. 2022). Finally, we hypothesized that the ratio of Firmicutes to Bacteroidetes would be lowest in the rumen and increase down the GI tract from the cecum to colon, because Bacteroidota would be most beneficial in fermenting complex carbohydrates, especially cellulose, and Firmicutes most beneficial in fermenting starches and proteins that escape the rumen.

METHODS

Study area

We opportunistically collected samples of the GI microbiome of moose in 2 study areas. The samples from Alaska came from 5 moose struck by vehicles in Game Management Units (GMU) 14A and 14C, which included the cities of Anchorage (61°13' N 149°54' W), Wasilla (61°34' N 149°27' W), and Palmer (61°36' N 149°07' W, Table A1). This area houses approximately 40% of the human residents of Alaska, however, most of the area (66% or 19,900 ha) is woodland (mixed, deciduous, coniferous, and shrub). The woodlands consisted of paper birch (*Betula papyrifera*), white spruce (*Picea glauca*), black spruce (*Picea mariana*), quaking aspen (*Populus tremuloides*), balsam poplar (*Populus balsamifera*), and black cottonwood (*Populus trichocarpa*). Shrubs were primarily willow, alder (*Alnus* spp.), and high-bush cranberry (*Viburnum edule*). Vehicle collisions were a significant source of mortality for the local moose population, accounting for 200 to 300 moose killed annually depending on snowfall (Peltier 2015). In 2022, this area accumulated approximately 178 cm of snowfall (Alaska Climate Research Center 2022), had an average minimum temperature of -13 °C (U.S. Climate Normals 2021). Vehicle-struck moose provided easily accessible and less invasive samples than wild-caught or captive animals.

In the Inland NW study area, we collected microbiome samples from both vehicle-struck and hunter-harvested animals, including 2 from northwestern Idaho, GMUs 8 and 11, near the cities of Moscow (46°44' N 117°00' W) and Troy (46°44' N 116°46' W), and 5 from eastern Washington, GMU 124, near the city of Spokane (47°40' N 117°25' W, Table A1). This area is characterized by rolling hills and an open landscape

that supports plant communities dominated by big sagebrush (*Artemisia tridentata*), Idaho fescue (*Festuca idahoensis*), and bluebunch wheatgrass (*Pseudoroegneria spicata*; Looney and Eigenbrode 2012). Snowberry (*Symphoricarpos albus*), quaking aspen (*Populus tremuloides*), and willow (*Salix* spp.) grow along riparian areas, and scattered ponderosa pines (*Pinus ponderosa*) are distributed along the foothills. There is also extensive farming in the area, producing primarily wheat and legumes. The mean minimum temperature in winter across all years was -4.3°C , and mean annual precipitation in this area between 2018–2022 was 52 cm, falling mostly as snow in the winter (U.S. Climate Normals 2021). To maintain sample quality, we only collected samples from animals that could be accessed within 3 hours of death as reported by the motorist or hunter. This ensured minimal microbial degradation as the animal tissues begin to break down.

Sampling methods

We used sterile instruments to open the animal's abdomen, rumen, cecum, and fecal samples from the colon (hereafter referred to as colon samples), collecting a mixture of fluid and solid digesta in equal proportion from each organ. We homogenized the solid and fluid contents to minimize potential bias caused by the animal's body position that could cause the pooling of fluids. Samples were placed on ice immediately after collection, and frozen at -20°C until extraction, which was accomplished no later than 6 months after collection. We analyzed each sample from the rumen, cecum, and colon for microbial composition and diversity using a 16S rRNA approach. This gene sequencing method is commonly used for identifying and quantifying microbes found in samples that could have thousands of different bacterial species. This method targets

the 16S rRNA genes, a highly conserved region of the transcriptional machinery found in all DNA-based life forms, making it an ideal target for sequencing DNA (Combrink et al. 2023). We extracted samples using the Qiagen RNEasy PowerMicrobiome Kit with manufacturer recommendations. The amount of DNA in each sample was measured using a Qubit (Invitrogen, Inc., Carlsbad, CA), and 5 μl of each DNA suspension was amplified by polymerase chain reaction (PCR) using a MasterCycler thermocycler (Eppendorf, Westbury, NY). Samples were multiplexed and sequenced using Illumina MiSeq Platform using the 300 cycle MiSeq Reagent kit V2 at the Advanced Instrumentation for Microbiome Studies (AIMS) core facility (Brauner and Briggs 2023). We sequenced samples using a 600-cycle MiSeq Reagent Kit v3 (Illumina®) in the presence of 25 % PhiX DNA. Following sequencing, samples were processed using the software Quantitative Insights Into Microbial Ecology (QIIME2), beginning with demultiplexing and removing samples that did not amplify, removing sequences that had less than 300 bp. We then denoised and dereplicated samples using the Divisive Amplicon Denoising Algorithm (DADA2) module within QIIME2 and removed sequences that had less than 300 bp. Unique amplicon sequence variants (ASVs) were generated using DADA2 and assigned a taxonomy using the SILVA 138 ribosomal RNA database. ASVs that could not be assigned to phylum were removed. Downstream analysis was done in R v 4.0.735. We imported QIIME2 readable files (*.qza) into R using the package qiime2R v0.99 (Bisanz 2024). We then used the phyloseq package (McMurdie and Holmes 2013) to remove non-bacterial, mitochondrial, and chloroplast ASVs, we then removed the negative controls (i.e., DNA-free water).

Statistical analysis

We examined the effects of GI organ (rumen, cecum, colon) and geographic region (Alaska, Inland NW) on taxonomic alpha diversity, beta diversity, and relative abundance of the most abundant microbial taxa. Alpha diversity quantified diversity within individual samples, allowing comparison across sample groups using 2 metrics—overall species richness and the Shannon-Weiner Diversity Index. We characterized richness as the number of unique microbial sequences per sample. We calculated the Shannon Index, which is the number of unique reads and their proportional abundance in the sequence, using the phyloseq package (McMurdie and Holmes 2013). We examined the effects of GI organ and geographic region starting with a linear mixed effects model with the individual moose as the grouping variables using the lme4 and lmerTest packages (Bates et al. 2017, Kuznetsova et al. 2013). We calculated estimated marginal means (emmeans) and lower and upper limits of the 95% confidence interval using the emmeans package (Lenth et al. 2024). Lastly, we performed a post-hoc Levene's test to compare variance in alpha diversity among GI organ and study locations (Derrick et al. 2018).

We calculated beta diversity to compare the dissimilarity between samples (moose location and organ), creating a distance matrix between all pairs of samples. To quantify the beta diversity of the microbiome, we first filtered and clustered sequences into amplicon sequence variants (ASVs). ASVs were used to reconstruct phylogenies, allowing us to calculate microbial beta diversity and relative abundances (Combrink et al. 2023). We then transformed the read counts of our non-rarefied data to relative abundance by dividing the number of reads for each taxon within a sample by the total

number of reads for that sample. We performed a Permutational Multivariate Analysis of Variance (PERMANOVA) to compare Bray-Curtis distances among GI organs and geographic regions, using the adonis2 function in the vegan package v2.6-4 (Oksanen et al. 2024). To visualize differences among independent variables, we used a Principal Coordinates Analysis with the Bray-Curtis distance that considers presence/absence of different ASVs and their abundance and an Analysis of Similarity (ANOSIM). We compared the response of the relative abundance of selected microbial families or phyla, including differences in the abundances of Firmicutes and Bacteroidota phylum, to GI organs and geographic regions using ANOVAs, and means were separated using the Tukey test. For our analyses, we set the statistical significance at $\alpha = 0.10$. We used this α (higher than the conventional ($\alpha = 0.05$)) to better align with our exploratory research objectives (McDonald 2009).

RESULTS

Of the 30 samples collected from 3 GI organs of 5 moose in Alaska and 7 moose in the Inland NW, we removed 4 samples because they did not amplify during PCR. Two of the Alaska moose samples had with organs that had burst and therefore we were unable to collect a sample. In our linear models that contained both geographic region and GI organ, microbial richness did not vary with region (Alaska: emmeans = 551, SE = 119, Inland NW: emmeans = 481, SE = 96, $P = 0.66$) or GI organ (all $P > 0.1$). However, the Shannon Index was lower in Alaska (emmeans = 3.08, SE = 0.56) than the Inland NW (emmeans = 4.09, SE = 0.46, $F = 6.30$, $P = 0.03$) but did not differ among organs (all $P > 0.1$, Fig. 1). The variance of microbial richness was greater in the Alaska

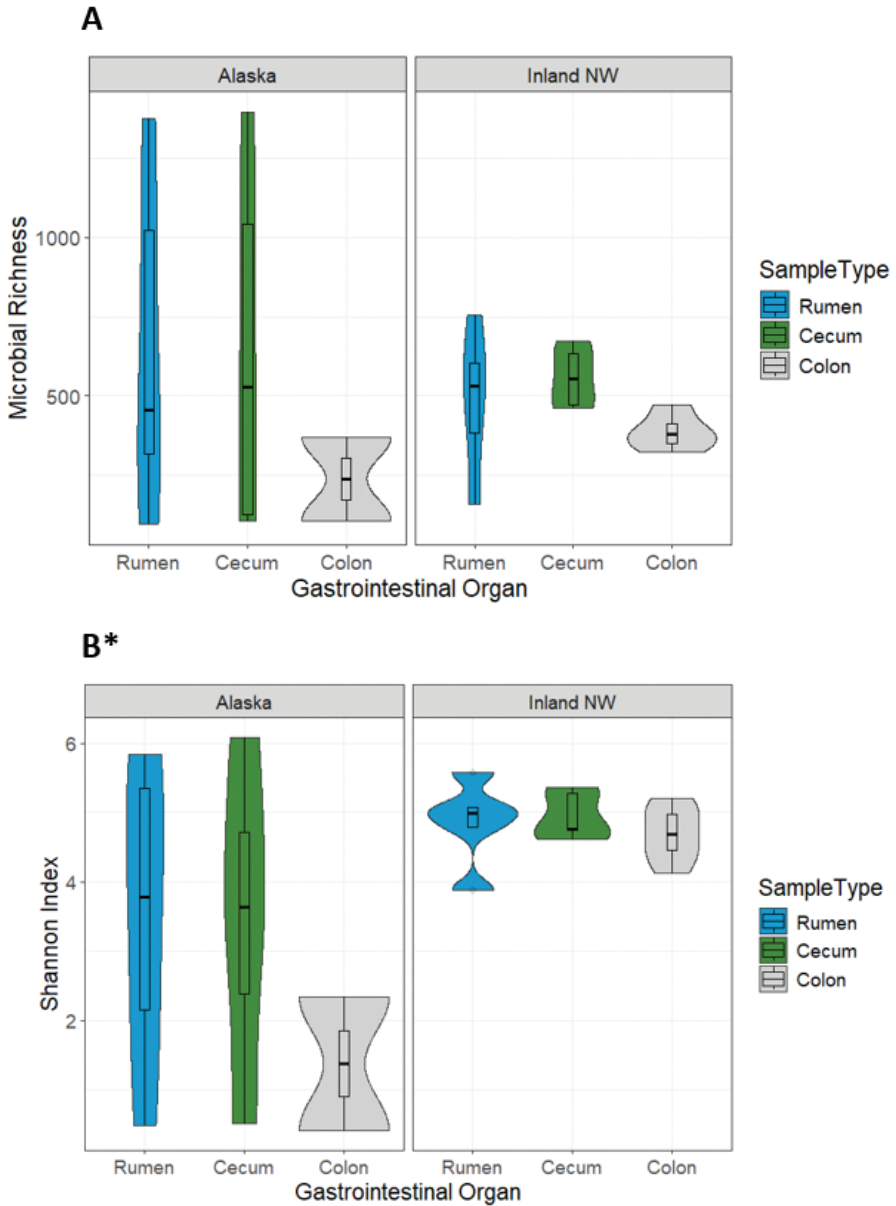


Fig. 1. Microbial richness (A) and Shannon Index (B) of the microbial community found in the rumen, cecum, and colon of moose in Alaska and the Inland NW (eastern Washington and northwestern Idaho), United States. Samples were collected from vehicle collisions and hunter harvests during the fall and winter seasons, 2022–2024. Asterisk denotes a difference between regions for the Shannon Index Values.

samples (var = 261,746) than the Inland NW samples (var = 22,640, $F = 9.29$, $P = 0.005$), and for both areas combined higher in the

rumen (var = 155,147) and cecum (var = 154,995) than the colon (var = 11,613, $F = 2.52$, $P = 0.1$; Fig. 1). The variance of the

Shannon Index was also greater in the Alaska samples ($var = 4.55$) than the Inland NW samples ($var = 0.20$, $F = 26.32$, $P < 0.0001$). However, the variances did not differ across GI organs ($F = 0.034$, $P = 0.96$; Fig. 1). Much like alpha diversity, we found that geographic region ($F = 7.14$, $R^2 = 0.21$, $P = 0.001$), influenced beta diversity but GI organ did not ($F = 0.63$, $P = 0.96$; Fig. 2).

The ANOSIM also showed that regions differed significantly ($F = 7.14$, $R^2 = 0.24$, $P = 0.001$) but there were no differences among GI organs. The first two axes of the PCoA using the Bray-Curtis distance for ordinations accounted for 30% of the variation in beta diversity for moose in both regions, clustering by geographic region but not GI organ (Fig. 2).

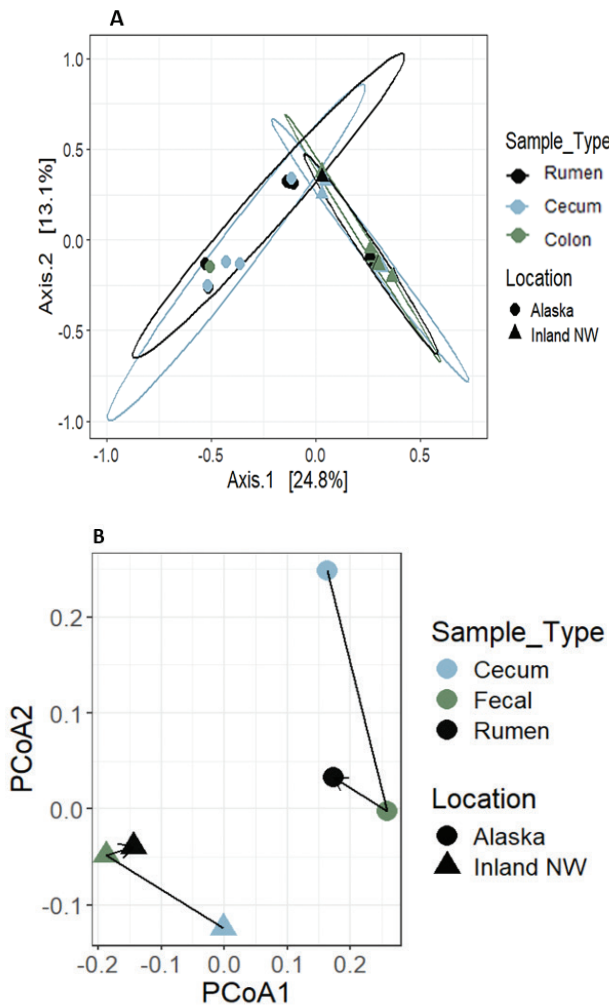


Fig. 2. Ordinations of microbial beta diversity obtained from the rumen, cecum, and colon of moose in Alaska and Inland NW (northwestern Idaho and eastern Washington), United States. Samples were collected from vehicle collisions and hunter harvests in the fall and winter seasons, 2022–2024. Beta diversity ordinations based on a PCoA with the Bray-Curtis distance metric (A), ordinations were then replotted using just the centroids with the arrows indicating the direction of change (B).

In addition to differences in microbial alpha and beta diversity, the 2 regions had distinct microbial community compositions (Fig. 3, Table A2). We identified 7 families that were present only in the Alaska samples and 8 present only in the Inland NW. Furthermore, families that were identified in both regions differed in relative abundance. The top family identified in the Alaska samples, *Enterobacteriaceae*, had a higher abundance in the Alaska samples ($\bar{x} = 28.49$, SE = 31.39) than the Inland NW ($\bar{x} = 3.39$, SE = 3.32, $F = 15.08$, $P = 0.002$, Fig. 3). The top 3 identified families for Inland NW moose, *Oscillospiraceae* (NW $\bar{x} = 20.95$, SE = 2.03 vs. AK $\bar{x} = 15.49$, SE = 8.57, $F = 17.17$, $P = 0.01$), *Lachnospiraceae* (NW $\bar{x} = 17.06$, SE = 3.85 vs. AK $\bar{x} = 10.88$, SE = 7.46, $F = 38.60$, $P = 0.002$), and *Christensenellaceae* (NW $\bar{x} = 13.12$, SE = 2.66 vs. AK $\bar{x} = 8.31$, SE = 5.73, $F = 43.51$, $P = 0.002$) all had greater relative abundances than in Alaska (Fig. 3, Table A2). However, across GI organs within regions, we found that each organ had the same top 5 families except *Oscillospiraceae* in the Alaska samples ($F = 3.40$, $P = 0.08$), where relative abundance for *Oscillospiraceae* was highest in the cecum ($\bar{x} = 20.83$, SE = 7.01), followed by the rumen ($\bar{x} = 2.57$, SE = 3.96) and finally the colon ($\bar{x} = 6.93$, SE = 1.01, Fig. 3). In addition, the relative abundance of Firmicutes was lower in the colon ($\bar{x} = 1.76$, SE = 0.99) than in the rumen ($\bar{x} = 5.38$, SE = 2.08, $P = 0.09$) and cecum for Alaskan moose ($\bar{x} = 4.93$, SE = 1.66, $P = 0.1$, Table 1). We find no differences between the 2 regions or within the Inland NW samples for either Firmicutes, Bacteroidota, or the F:B ratio between geographic location or among GI organs (all $P > 0.1$, Fig. 4).

DISCUSSION

Although we expected to find differences in microbial diversity and composition across

the 3 fermentation organs of moose GI tracts (i.e., rumen, cecum, and colon), these differences were minimal in both geographic regions we sampled. On the other hand, we found differences in microbial diversity and composition between the 2 geographic regions. Taken together, our results suggest that regional environmental characteristics, such as diet composition and seasonal availability of forages, are more important drivers of microbial community diversity and composition than the internal microenvironment caused by physiological and functional differences along the GI tract. Because of the minimal differences in the microbiome from the rumen to the colon, our findings support sampling the microbiome from fresh fecal samples as a reasonable surrogate for microbial communities in the rumen and cecum.

We expected that microbial diversity would decrease along the GI tract because of the decrease in the amount of substrate for the microbes and the faster passage rate after the gastric stomach (Van Soest 1996, Stevens and Hume 1998, Clauss and Hummel 2005). However, microbial diversity and richness did not vary among the GI fermentation organs of moose in either region. These similar levels of microbial diversity may reflect the need for functional redundancy in GI microbial community structure that addresses the most important task of breaking down the complex fibers in their diet and adds resilience as diet changes across seasons and locations (Ribas et al. 2023). On the other hand, diets consumed by moose during the non-growing season (e.g., deciduous twigs and conifers) are relatively less diverse than in the growing season, which might have resulted in a relatively low diversity overall (Solden et al. 2017). For example, the mean value of the Shannon index across the GI tract of moose in our study was lower (3.08 in Alaska and 4.09 in Inland

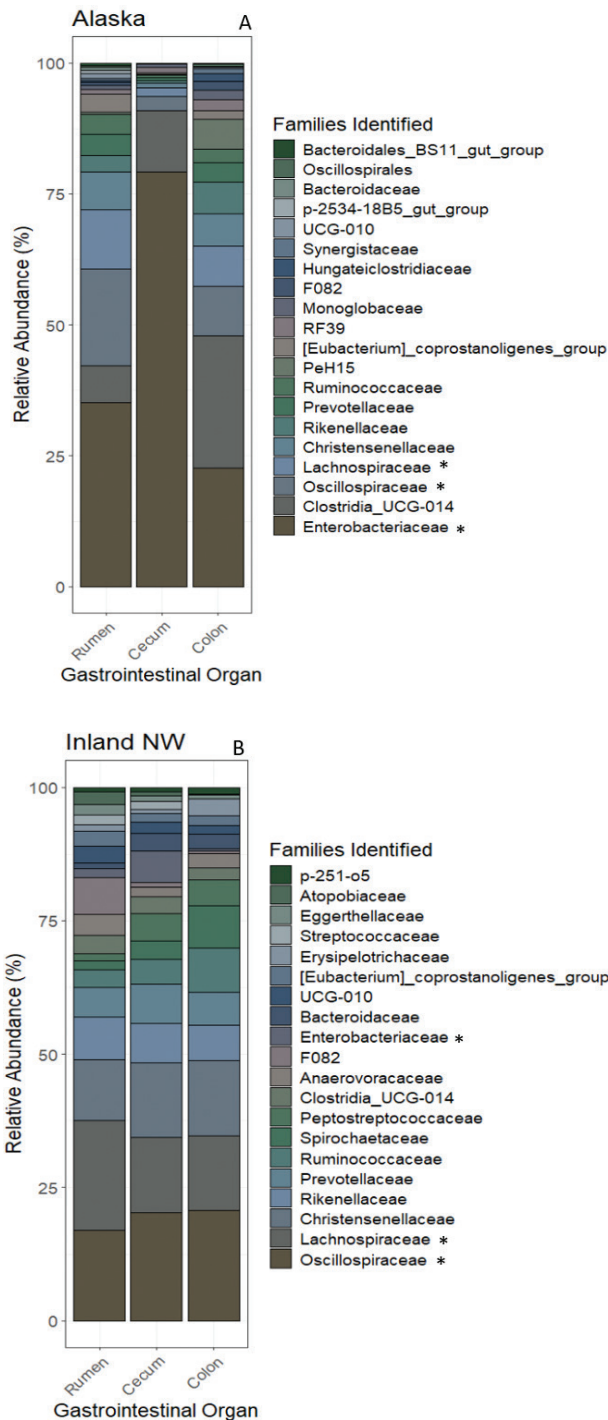


Fig. 3. Top 20 bacterial families identified in the rumen, cecum, and colon of moose in Alaska (A) and Inland NW (B, northwestern Idaho and eastern Washington), United States. Samples were collected from vehicle collisions and hunter harvests in the fall and winter seasons between 2022 and 2024. Families are ordered from most to least abundant from bottom to top. Asterisks denote significant differences between the two geographic regions, $\alpha = 0.10$.

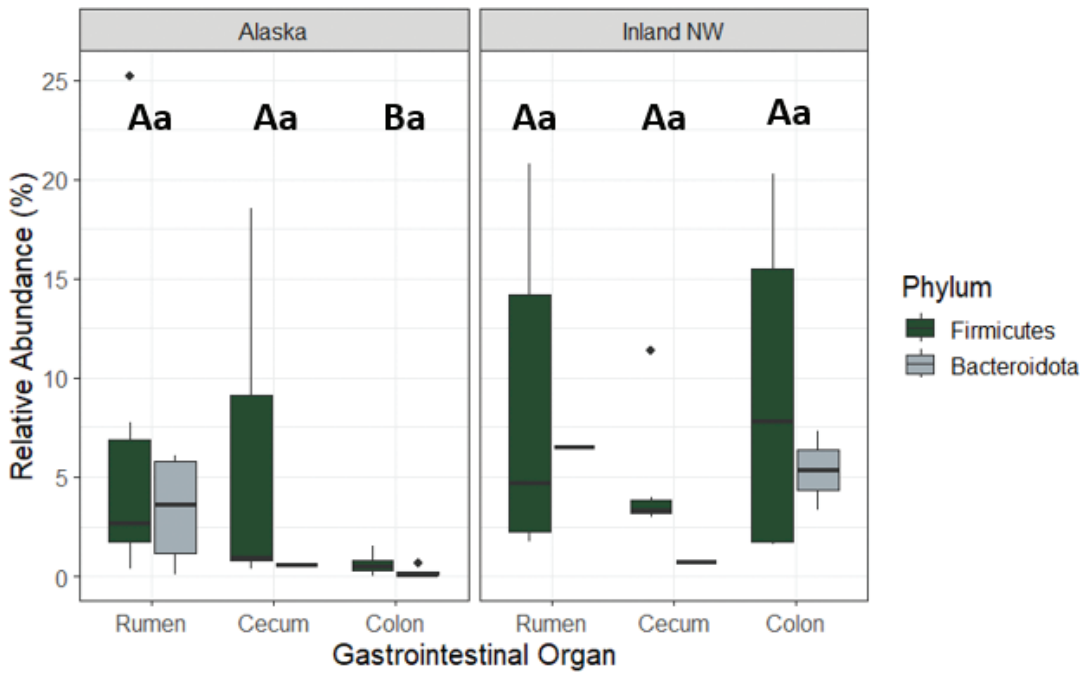


Fig. 4. Relative Abundances of the Firmicutes and Bacteroidetes phyla identified in the rumen, cecum, and colon of moose in Alaska and Inland NW (northwestern Idaho and eastern Washington), United States. Samples were collected from vehicle collisions and hunter harvests in the fall and winter seasons, 2022–2024. Different capital letters indicate differences among means for Firmicutes from the Tukey means separation tests ($\alpha = 0.05$). The same lowercase letters indicate that the relative abundance of Bacteroidota did not differ among organs within regions.

Table 1. Mean relative abundance (SE) for the Firmicutes and Bacteroidota phyla for moose by geographic region (Alaska vs. Inland NW [eastern Washington and western Idaho], USA) and gastrointestinal (GI) organ during the fall and winter seasons of 2022–2024.

Region	GI organ	Firmicutes	Bacteroidota	Ratio
Alaska	Rumen	5.38 (2.08)	2.49 (0.93)	2.16
	Cecum	4.93 (1.66)	1.45 (0.53)	3.41
	Colon	1.76 (0.99)	0.19 (0.09)	9.08
Inland NW	Rumen	6.70 (1.94)	3.44 (1.12)	1.94
	Cecum	6.36 (1.95)	4.45 (1.36)	1.43
	Colon	6.28 (1.91)	3.94 (1.32)	1.59

Northwest) than the value (i.e., 6) recorded for the American bison (*Bison bison*) during the fall in Colorado (Bergmann 2017), but were similar to that of pronghorn (*Antilocapra americana*) fecal samples (i.e., 4.3) in fall and winter in Wyoming (Buchanan et al. 2024).

We also expected the rumen to have the highest diversity of microbes and contain primarily cellulolytic bacteria compared to the colon because of the change in available substrates along the GI tract. Contrary to our expectations, however, microbial taxa and the Firmicutes: Bacteroidetes ratio were

generally similar among the fermentation organs, but we did note 1 microbial phylum and 1 family that differed in Alaskan moose. Firmicutes were more abundant in the rumen than the cecum and colon, whereas *Oscillospiraceae*, an epithelial group, were more abundant in the cecum than the rumen, and more abundant in the rumen than the colon. The higher abundance of Firmicutes in the rumen may be due to the energetic requirements of the season when the animals were sampled. Firmicutes can act as an efficient energy source, leading to more calorie absorption which is important during winter when food is scarce (Söllinger et al. 2018). Likewise, domestic cattle (*Bos taurus*) fed a high forage diets had higher abundances of Firmicutes, but when fed a high concentrate diet, they had higher abundances of Bacteroidota (Clemmons et al. 2019). The higher abundance of *Oscillospiraceae* in the hindgut is unsurprising based on the function of the cecum in foregut fermenters, which serves as a site for absorption of water and nutrients from bacterial fermentation. As an epithelial group, *Oscillospiraceae* would have the ability to absorb the microbial proteins, the nutrients that were partially digested in the acid stomach, and the metabolic products from other microbes that can then be used as a substrate by other microbes (Grond et al. 2021). Microbes engage in cross-feeding, whereby they produce SCFAs that are not only useful to the host and other microbes, but can also digest those microbes themselves (Solden et al. 2018). Cecal fermentation can be important to a ruminant because both the microbes and the animal can take advantage of nutrients that require prior breakdown, either through digestion or fermentation in the rumen and small intestine (Hoover 1978).

Although none of our 3 hypotheses were strongly supported, our results align with some studies with other species. For

example, research with ruminant livestock (Mao et al. 2015), captive-raised bison (Bergmann 2017), and moose in Vermont (Ishaq and Wright 2012) found differences in the relative abundances of different bacterial orders among GI organs, but did not find a consistent decrease in the abundances along the GI tract. Like Ishaq and Wright (2014), our samples also contained a relatively high abundance of the *Lachnospiraceae* family. *Lachnospiraceae* have been found in the human large intestine (Flint et al. 2012) and are fiber degraders often found in the solid digesta fractions (Deusch et al. 2017). Because of its ability to produce butyrate, this family can help reduce the side effects of gastrointestinal inflammation and can help stimulate growth of papillae (Ishaq and Wright 2014). One potential reason for the amount of overlap in the microbial species between the GI organs in moose is that the general purpose of each organ is essentially the same, fermenting cellulose, hemicellulose, and other nutrients to capture energy contained in SCFAs, break down food particles, and increase the digestive efficiency of the animal (Van Soest 1996, Kohl et al. 2014). This functional redundancy across the GI tract ensures that the animal can extract the most energy and nutrients from a complex diet, well-defended by insoluble fiber. In contrast to our moose, woodrats (*Neotoma* spp.), which have a small foregut fermentation portion of their bilocular stomach and a larger cecum, were found to have a foregut community that was better suited for detoxification of PSMs and a hindgut community that was better suited for SCFA production (Kohl et al. 2014).

A second potential reason that microbial communities did not differ across the GI organs is our relatively small sample size and high variance among individuals in our study, even though we controlled for

individual variation in our models using a grouping variable. The variance for both microbial richness and Shannon Index was particularly high in the rumen and cecum of the Alaska samples. Experiments with captive ruminants in controlled conditions have shown substantial individual variation in diet selection, thus differences in microbial communities might be expected to be amplified in free-ranging animals in natural landscapes (Provenza and Balph 1987, Anderson 2020). Dietary differences might have the greatest influence on microbes in the rumen because the food entering the rumen has undergone only minimal transformation from saliva. The rumen provides a stable environment for microbes to establish larger communities, with perturbations that continually expose new substrates to the microbes (Troyer 1984, Weimer 2015).

Rumen microbial diversity also depends in part on the chemical complexity of the foods consumed, specialization of subcommunities for maximal biochemical efficiency, and the possibility that the best-suited microbes will displace others that are under-suited and create opportunities for new microbes (Russell 2002). Therefore, the rumen microbial community would be expected to vary the most among individuals based on the animal's early life experiences, health status, physiology, behavior, diet, and other members of the microbial community (e.g. cross feeding and the presence of protozoa [Troyer 1984, Ley et al. 2008, Rosenberg and Zilber-Rosenberg 2016, Clemmons et al. 2019, Fountain-Jones et al. 2020, Dearing and Weinstein 2022]). Variation in microbial communities in moose likely also reflects the substantial chemical diversity consumed in the form of PSMs. Deciduous and coniferous shrubs and trees are heavily defended by antimicrobial chemicals (terpenes and phenolic resins), complex

polyphenolics that are known to interfere with protein digestion (e.g., tannins), as well as a suite of other primarily small or intermediate-sized phenolics that can act as metabolic toxins to the animals (Richards et al. 2016). The microbial community within the GI tract can enhance the herbivore's innate detoxification system while also detoxifying PSMs themselves, lessening the overall burden on the host (Dearing et al. 2005).

Although microbial communities did not vary greatly among fermentation organs, microbial diversity and composition differed substantially between our study areas. These differences most likely reflect differences in diet and other macro-environmental conditions. Similarly, Ishaq and Wright (2014) found that the differences in region outweighed the differences in the GI organ sampled for moose in Vermont, Norway, and Alaska. In addition, microbial communities could in part reflect small differences in the GI tract of moose based on their phylogeny between our study regions (i.e., *A. a. gigas* in Alaska vs. *A. a. shirasi* in the continental U.S., Chong et al. 2018, Arshad et al. 2021). We sampled from 2 study regions to increase our sample size and to determine the universality of patterns in the GI microbiome but did not have a priori expectations about which geographic location would support the most diverse GI microbiome, in part because we lacked adequate information about their diets during fall and winter. Previous studies have demonstrated that moose in Alaska consume a large variety of willow species in both summer and winter, but the only published data on diets of Inland NW moose suggests that they might consume a greater variety of plant functional groups such as grass and forbs, at least in summer (Schrempp et al. 2019). Because our samples were collected in fall and winter, snow cover and reduced abundance of nutritious forages likely restricted diet

choices, especially for the Alaska moose (Hamilton et al. 1980, Hoy et al. 2022). For example, Cook et al. (2021) found that approximately 80% of the moose sampled in Northwestern Washington showed some level of starvation and starvation has also been the cause of winter mortality for many Alaskan moose (Testa 2004, Boertje et al. 2007, 2019). GI microbial communities respond to declining abundance and nutritional quality and diversity of diets from the summer to the winter (Solden et al. 2018, Zou et al. 2019). To gain insight into causes of regional differences in the GI microbiome of moose, future studies could directly link diet to microbiome by 1) collecting simultaneous rumen or fecal samples and identifying diet composition using molecular techniques similar to those of the microbiome (Kartzinel et al. 2015, Sonsthagen et al. 2020), 2) minimizing variation of sample collection dates or collect samples throughout each season, or 3) using controlled feeding studies with specific diet items of interest.

Not only did microbial diversity differ between Alaska and Inland NW, but the relative abundance of microbial families did also. In fact, almost half of the 20 families were not found in samples from the other region. The top family identified in the Alaska samples, *Enterobacteriaceae*, a member of the Proteobacteria phylum, is a facultative anaerobic family that ferments lactose and produces acid from glucose. This family is more abundant in hosts that live in high-elevation and/or cold environments, which would be consistent with the Alaskan region (Wu et al. 2022). The second most abundant was unclassified Clostridia_UCG-014, which is an order found in the Firmicutes phylum that could not be further refined to family or genus. This order has been correlated with tryptophan metabolism, helps maintain intestinal barrier function, and has

been found in the cecum of several species (e.g., chickens and geese, Jiang et al. 2023; Zhao et al. 2025). The third most abundant family was *Oscillospiraceae*, which was also the top identified family for the Inland NW samples. *Oscillospiraceae* has been positively associated with growth and lactation of ruminants, and can enhance growth and production of other microbes by mediating SCFA transport (Tong et al. 2018; Pinnell et al. 2022; Chen et al. 2024). The top three identified families for the Inland NW, *Oscillospiraceae*, *Lachnospiraceae*, and *Christensenellaceae*, are all families within the Firmicutes phylum. Using metagenomic analyses, future studies could further determine the function of various microbial taxa to better understand the relationship between microbial communities and the internal (GI tract) and external (regional) environments.

Elucidating the function and composition of microbial communities within fermentation organs is key to understanding how ruminants like moose respond to changes in diet and environment, and how these changes might influence their interactions with their plant resources. However, the logistics of sampling wild ruminants can often be a barrier to quantifying the complex interactions between the microbes and their hosts, especially considering the diversity of microbes within spatially segregated portions of the GI tract. Sampling recently killed vehicle-struck or hunter-harvested animals in our study allowed for relatively easy and precise sampling of potentially unique microbial communities (Kohl et al. 2014) within each fermentation chamber, without the ethical concerns related to surgical access to the GI in live animals or harvesting animals specifically to sample their microbiome. Although we were able to bypass some logistic limitations by opportunistically sampling vehicle-struck or

hunter-harvested animals, this approach cannot completely control for variation caused by postmortem physiological processes. Microbial communities change after the host dies as the stability of the environment and the substrates they rely on for food are no longer available. Although changes in the microbial communities after host death are used as a forensic technique on human cadavers (DeBruyn and Hauther 2017), to our knowledge there has not been a study on a ruminant that quantifies how the community changes postmortem.

Microbes capable of fermentation provide a quid pro quo relationship with their host. The microbes providing the host SCFAs, essential amino acids, vitamins, and microbial protein; whereas the herbivores provide a consistent anoxic environment and substrate (Troyer 1984, Söllinger et al. 2018). However, the characteristics of the GI microbiome have implications for more than just extracting nutrients from plants. Recent research suggests that the microbiome may have a role in mental and physical health, hormone balance, and immune response within humans, livestock, and wildlife (Delzenne et al. 2019, Anand and Mande 2022, Vos et al. 2022). Because climate change is expected to influence allocation of resources within plants, which in turn affects how ruminants and their symbiotic microbial communities can sustain themselves and interact with their environment, this research provides a baseline for future comparisons (Herms and Mattson 1992, Moore et al. 2015, Chen et al. 2022, Beale et al. 2023).

ACKNOWLEDGEMENTS

We would like to thank B. Briggs and the AIMS Core Lab at the University of Alaska Anchorage for running the 16S samples. We also appreciate the assistance

collecting moose samples provided by Alaska Department of Fish and Game, Washington Department of Fish and Wildlife, and Idaho Department of Fish and Game, especially M. DeVivo and I. Hull.

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APPENDIX

Table A1. Sample information moose sampled from the rumen, cecum, and colon in Alaska (*n* = 5) and the Inland NW (*n* = 7, eastern Washington and western Idaho), United States, from the fall and winter seasons of 2022–2024.

Geographic Region	Sample ID	Closest City	Date Collected	Sex	Age	GI Organ Sampled
Alaska	AK1	Anchorage	1/24/2022	Female	Adult	Rumen
Alaska	AK1	Anchorage	1/24/2022	Female	Adult	Cecum
Alaska	AK2	Palmer	1/14/2022	Female	Calf	Rumen
Alaska	AK2	Palmer	1/14/2022	Female	Calf	Cecum
Alaska	AK3	Wasilla	12/8/2022	Female	Calf	Rumen
Alaska	AK3	Wasilla	12/8/2022	Female	Calf	Cecum
Alaska	AK3	Wasilla	12/8/2022	Female	Calf	Fecal
Alaska	AK4	Wasilla	1/28/2022	Female	Adult	Rumen
Alaska	AK4	Wasilla	1/28/2022	Female	Adult	Cecum
Alaska	AK4	Wasilla	1/28/2022	Female	Adult	Fecal
Alaska	AK5	Palmer	2/19/2022	Female	Adult	Rumen
Inland NW	INW1	Palmer	2/19/2022	Female	Adult	Fecal
Inland NW	INW1	Moscow	9/23/2022	Female	Adult	Rumen
Inland NW	INW1	Moscow	9/23/2022	Female	Adult	Cecum
Inland NW	INW1	Moscow	9/23/2022	Female	Adult	Fecal
Inland NW	INW2	Spokane	10/4/2022	Male	Adult	Rumen
Inland NW	INW2	Spokane	10/4/2022	Male	Adult	Cecum
Inland NW	INW2	Spokane	10/4/2022	Male	Adult	Fecal
Inland NW	INW3	Spokane	10/12/2022	Male	Adult	Rumen
Inland NW	INW3	Spokane	10/12/2022	Male	Adult	Cecum
Inland NW	INW3	Spokane	10/12/2022	Male	Adult	Fecal
Inland NW	INW4	Spokane	11/28/2022	Female	Adult	Rumen
Inland NW	INW4	Spokane	11/28/2022	Female	Adult	Fecal
Inland NW	INW5	Mt. Spokane	3/20/2023	Female	Adult	Rumen
Inland NW	INW5	Mt. Spokane	3/20/2023	Female	Adult	Cecum
Inland NW	INW5	Mt. Spokane	3/20/2023	Female	Adult	Fecal
Inland NW	INW6	Moscow/Troy	10/25/2023	Female	Adult	Rumen
Inland NW	INW6	Moscow/Troy	10/25/2023	Female	Adult	Cecum
Inland NW	INW6	Moscow/Troy	10/25/2023	Female	Adult	Fecal
Inland NW	INW7	Spokane	11/12/2024	Male	Adult	Rumen
Inland NW	INW7	Spokane	11/12/2024	Male	Adult	Cecum
Inland NW	INW7	Spokane	11/12/2024	Male	Adult	Fecal

Table A2. Top 20 microbial families for moose sampled from the combined rumen, cecum, and colon in Alaska ($n = 5$) and the Inland NW ($n = 7$) eastern Washington and western Idaho), United States, from the fall and winter seasons, 2022–2024. Families are listed with their mean abundance and standard error. Asterisks denote significant differences between the two geographic regions, $\alpha = 0.10$.

Family	Alaska Mean Abundance	SE	Inland NW Mean Abundance	SE
Enterobacteriaceae*	28.49	31.39	3.39	3.32
Oscillospiraceae*	15.49	8.57	20.95	2.03
Lachnospiraceae*	10.88	7.46	17.06	3.85
Clostridia_UCG-014	9.04	10.42	3.08	1.10
Christensenellaceae	8.31	5.73	13.12	2.66
Peptostreptococcaceae	4.86	3.19	4.86	3.19
Rikenellaceae	4.1	2.58	5.99	1.78
Prevotellaceae	3.92	2.65	6.14	1.34
Spirochaetaceae	3.42	3.31	3.42	3.31
Ruminococcaceae	2.92	2.19	4.28	2.25
Anaerovoracaceae	2.64	1.11	2.64	1.11
[Eubacterium]_coprostanoligenes_group	1.81	1.06	2.13	0.68
UCG-010	0	0	1.97	0.60
Atopobiaceae	1.44	1.21	1.44	1.21
Erysipelotrichaceae	1.44	0.84	1.44	0.84
PeH15	1.41	1.54	0	0
F082	1.38	2.01	2.28	2.72
Bacteroidaceae	1.35	1.20	2.23	0.88
Eggerthellaceae	1.33	0.55	1.33	0.55
Streptococcaceae	1.30	0.96	1.30	0.96
RF39	1.17	0.70	0	0
p-251-o5	0	0	0.96	0.24