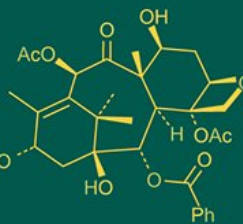
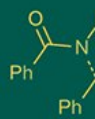


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Seasonal variation in the goat rumen microbiome: A comparative 16S RRNA gene sequencing analysis of cold and warm seasons

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Abstract

The rumen microbiome plays a fundamental role in feed digestion, nutrient utilization, host health and environmental adaptation in ruminants. Seasonal variation in climatic conditions and feed resources may influence the diversity and composition of rumen microbial communities. The present study investigated seasonal variation in the goat rumen microbiome using publicly available 16S rRNA gene sequencing data. Six rumen microbiome samples were retrieved from the National Center for Biotechnology Information Sequence Read Archive under BioProject PRJNA1050866, comprising three cold-season samples (SRR27169185-SRR27169187) and three warm-season samples (SRR27169218-SRR27169220). Raw paired-end sequence data were processed using the QIIME 2 bioinformatics pipeline, including quality filtering, denoising, chimera removal and amplicon sequence variant inference. Phylogenetic relationships among representative sequences were reconstructed for phylogeny-based diversity analyses. Alpha diversity was assessed using the Shannon index and Faith's phylogenetic diversity, whereas beta diversity was evaluated using Bray-Curtis dissimilarity and unweighted UniFrac distances. Seasonal differences in alpha diversity were assessed using the Kruskal-Wallis test, while differences in overall microbial community structure were evaluated using permutational multivariate analysis of variance. Significant seasonal differences were observed in alpha-diversity measures ($P < 0.05$), with contrasting patterns in taxonomic and phylogenetic diversity between seasons. However, beta-diversity analyses did not demonstrate statistically significant differences in overall microbial community composition between cold and warm seasons (PERMANOVA, $p > 0.05$). Krona-based taxonomic visualization nevertheless indicated descriptive differences in the relative representation of microbial taxa between seasons. Overall, the findings suggest that seasonal conditions may influence within-sample microbial diversity while the broader rumen microbial community remains relatively stable. This apparent stability may reflect resilience of the core goat rumen microbiome to seasonal environmental variation and could provide useful insights for microbiome-informed nutritional, management and breeding strategies aimed at improving goat adaptation and productivity.

Keywords: Goat; rumen microbiome; Seasonal variation; 16S rRNA gene sequencing; Alpha diversity; Beta diversity; Faith's phylogenetic diversity; UniFrac; QIIME 2; Microbial adaptation

Introduction

Ruminant animals possess a unique digestive system that enables them to efficiently utilize fibrous and low-quality plant materials that cannot be directly digested by the host. This ability is primarily dependent on the diverse microbial community inhabiting the rumen, which converts complex plant polysaccharides into fermentation products that can be utilized as major energy sources by the host. Consequently, the rumen microbiome represents a fundamental biological interface between the animal, its diet and the surrounding environment and plays an important role in ruminant productivity, health and adaptation (Agrawal *et al.*, 2014) [1].

The rumen is often regarded as a highly efficient natural bioreactor because of its capacity to support extensive microbial fermentation and degradation of complex plant carbohydrates (Codron and Clauss, 2010) [5]. It harbours a diverse community of microorganisms, including bacteria, archaea, protozoa and anaerobic fungi, which interact with one another and with the host to maintain rumen function. Among these microbial groups, bacteria are generally the

most abundant, followed by archaea, protozoa and anaerobic fungi. These microbial populations differ considerably in abundance and biomass contribution but collectively perform complementary roles in the degradation and fermentation of dietary substrates (Wang *et al.*, 2017) [13]. Through these interactions, rumen microorganisms contribute to the breakdown of cellulose, hemicellulose and other complex dietary components, while also participating in hydrogen metabolism and methane production. Therefore, changes in the composition and activity of the rumen microbiome can influence nutrient utilization, fermentation efficiency, animal performance and environmental outputs (Tapio *et al.*, 2017; Ebeid *et al.*, 2020) [12, 6].

The rumen microbial ecosystem is dynamic rather than static. Although a core microbial community is maintained within the rumen, its composition and relative abundance may respond to several host and environmental factors, including diet, age, physiological status, geographical location and management conditions. Seasonal variation represents another potentially important source of microbial variation because changes in ambient temperature are frequently accompanied by differences in forage availability, dietary composition and feeding management. Such changes may alter the substrates available for microbial fermentation and consequently influence microbial diversity and community structure. Understanding the response of the rumen microbiome to seasonal environmental variation is therefore relevant for improving our understanding of ruminant adaptation to changing climatic and nutritional conditions.

Historically, knowledge of the rumen microbial ecosystem was largely derived from culture-based investigations. Early studies described the establishment and composition of ruminal microorganisms and provided important information regarding the colonization of the rumen by bacteria and other microbial groups (Bryant *et al.*, 1958; Fonty *et al.*, 1987; Minato *et al.*, 1992) [4, 7, 10]. However, culture-based approaches provide only a partial representation of rumen microbial diversity because a substantial proportion of rumen microorganisms are difficult to cultivate under laboratory conditions. The development of molecular approaches based on microbial marker genes substantially improved the characterization of complex rumen microbial communities.

In particular, sequencing of the 16S rRNA gene has become an important approach for investigating bacterial and archaeal diversity in the rumen. The 16S rRNA gene contains conserved and variable regions that permit the taxonomic identification and characterization of microbial populations. Molecular studies have demonstrated that the rumen contains a complex and diverse bacterial community, often revealing microbial populations that were underestimated or not detected using conventional culture-based methods (Stevenson and Weimer, 2007; Jami *et al.*, 2013) [11, 9]. High-throughput 16S rRNA gene sequencing has further expanded the capacity to characterize microbial communities by enabling simultaneous assessment of thousands of sequence variants across multiple samples. For example, 16S rRNA gene sequencing has been successfully applied to characterize bacterial diversity in the goat rumen and to investigate variation in microbial composition among animals and developmental stages (Han *et al.*, 2015) [8]. Bioinformatic approaches have become equally important for interpreting high-throughput microbial sequencing data.

Platforms such as QIIME 2 enable quality control, sequence denoising, inference of amplicon sequence variants and calculation of microbial diversity indices. Within-sample diversity can be assessed using alpha-diversity metrics, such as the Shannon index, which incorporates both richness and evenness and Faith's phylogenetic diversity, which incorporates the evolutionary relationships among microbial taxa. Between-sample variation can be examined using beta-diversity measures. Bray-Curtis dissimilarity, originally developed as a measure of ecological community dissimilarity by Bray and Curtis (1957) [3], provides an abundance-based assessment of differences in community composition. In contrast, unweighted UniFrac incorporates phylogenetic relationships and evaluates differences in the presence or absence of microbial lineages. The combined application of taxonomic and phylogenetic diversity measures can therefore provide a broader understanding of microbial community variation.

Although numerous studies have investigated the influence of diet and host-related factors on the rumen microbiome, comparatively less attention has been given to the extent to which seasonal environmental variation influences rumen microbial diversity in goats. This question is particularly relevant because goats are widely recognized for their ability to adapt to diverse and fluctuating production environments. Seasonal changes may impose simultaneous variation in climatic conditions and feed resources, potentially creating selective pressures on rumen microbial communities. However, whether such seasonal variation results in substantial restructuring of the overall microbial community or is accommodated through changes in within-community diversity remains incompletely understood.

Therefore, the present study investigated seasonal variation in the goat rumen microbiome through a secondary analysis of publicly available 16S rRNA gene sequencing data. Rumen microbial communities from cold- and warm-season samples were compared using complementary alpha- and beta-diversity approaches. Shannon diversity and Faith's phylogenetic diversity were used to assess within-sample taxonomic and phylogenetic diversity, respectively, whereas Bray-Curtis and unweighted UniFrac metrics were used to evaluate differences in community composition among samples. Principal coordinates analysis and permutation-based statistical tests were further used to examine patterns of seasonal variation. In addition, Krona-based taxonomic visualization was used to provide a descriptive comparison of microbial composition between seasons. The study was designed to determine whether seasonal conditions are associated with changes in rumen microbial diversity and overall community structure. By examining publicly available rumen microbiome data through a standardized bioinformatic workflow, this study provides preliminary insights into the potential resilience and adaptability of the goat rumen microbiome under contrasting seasonal conditions. Such information may contribute to future investigations aimed at developing microbiome-informed nutritional, management and breeding strategies to improve the environmental adaptation and productivity of goats.

2. Materials and Methods

2.1. Study design and data source

The present study was conducted as a secondary bioinformatic analysis of publicly available goat rumen 16S rRNA gene sequencing data. Raw sequencing datasets were

retrieved from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under BioProject accession PRJNA1050866. The dataset was derived from a goat rumen microbiome study associated with Zhejiang University. A total of six rumen microbiome samples representing two seasonal conditions were included in the present analysis. Three samples represented the cold season (SRR27169185, SRR27169186 and SRR27169187), whereas three samples represented the warm season (SRR27169218, SRR27169219 and SRR27169220). Samples were grouped according to their reported seasonal classification and compared to evaluate potential seasonal variation in rumen microbial diversity and community structure. As the present investigation involved secondary analysis of publicly available sequencing data, no animals were directly handled or sampled for this study.

2.2. Sequence data processing and ASV inference

Raw paired-end 16S rRNA gene sequencing reads were processed using the QIIME 2 bioinformatics platform. Demultiplexed sequence reads were imported into QIIME 2 and examined for sequence quality prior to downstream processing. Quality filtering, denoising, paired-end read merging and chimera removal were performed using the DADA2 plugin implemented in QIIME 2. Forward and reverse reads were truncated at 280 bp and 220 bp, respectively, based on the observed sequence quality profiles. Reads containing poor-quality regions or sequencing artefacts were removed during the denoising process. DADA2 was used to infer amplicon sequence variants (ASVs), which represent unique biological sequence variants after correction for sequencing errors. Chimeric sequences were identified and removed as part of the denoising workflow. The resulting ASV feature table and representative sequence dataset were used for subsequent taxonomic and diversity analyses.

2.3. Feature table processing and sequencing depth standardization

The ASV feature table was summarized to evaluate sequencing depth and the distribution of feature frequencies among samples. Summary statistics, including the number of samples, number of ASVs, total feature frequency and read frequency distribution, were generated using the QIIME 2 feature-table summary function. Because sequencing depth varied among samples, downstream diversity analyses were standardized to reduce the potential influence of unequal sampling effort. Samples were rarefied to a sequencing depth of 9,000 reads, corresponding to the lowest retained sequencing depth among the samples included in the analysis. The rarefied feature table was subsequently used for alpha- and beta-diversity analyses.

2.4. Taxonomic Classification

Representative ASV sequences were taxonomically classified using the QIIME 2 feature-classifier plugin. Taxonomic assignment was performed using the consensus BLAST+ approach against the SILVA reference database (version 138.2). Taxonomic classifications were assigned at a confidence threshold of 0.7. The resulting taxonomic assignments were used to characterize the microbial composition of the rumen samples and for visualization of seasonal taxonomic profiles.

2.5. Phylogenetic reconstruction

Representative ASV sequences were aligned using a multiple sequence alignment workflow implemented in QIIME 2. Phylogenetic reconstruction was subsequently performed to generate both unrooted and rooted phylogenetic trees. The rooted phylogenetic tree was used for phylogeny-based diversity analyses, including Faith's phylogenetic diversity and unweighted UniFrac distance calculations. The phylogenetic framework enabled the incorporation of evolutionary relationships among microbial lineages into the diversity analyses.

2.6. Alpha diversity analysis

Within-sample microbial diversity was evaluated using two complementary alpha-diversity metrics: the Shannon diversity index and Faith's phylogenetic diversity (Faith's PD). The Shannon diversity index was calculated to assess within-sample diversity by incorporating both the number of observed microbial taxa and their relative abundance distribution. Higher Shannon values indicate greater diversity arising from increased richness, evenness, or both. Faith's phylogenetic diversity was calculated using the rooted phylogenetic tree and represents the cumulative phylogenetic branch length encompassed by the microbial taxa present within each sample. This metric provides an estimate of diversity that incorporates evolutionary relationships among observed taxa.

Differences in alpha-diversity indices between cold- and warm-season samples were assessed using the Kruskal-Wallis test implemented through the QIIME 2 alpha-group-significance function. Where applicable, pairwise comparisons were adjusted for multiple testing using the Benjamini-Hochberg false discovery rate procedure. A *p*-value of less than 0.05 was considered statistically significant.

2.7. Beta diversity analysis

Between-sample variation in microbial community composition was assessed using both Bray-Curtis dissimilarity and unweighted UniFrac distance. Bray-Curtis dissimilarity was used as a non-phylogenetic, abundance-based metric to evaluate differences in the relative abundance structure of microbial communities among samples. Unweighted UniFrac distance was used as a phylogeny-based metric that evaluates differences in community composition according to the presence or absence of microbial lineages and their evolutionary relationships. Distance matrices generated from both metrics were used for downstream ordination and statistical analyses.

2.8. Principal Coordinates Analysis

Principal coordinates analysis (PCoA) was performed on the Bray-Curtis and unweighted UniFrac distance matrices to visualize patterns of similarity and dissimilarity among rumen microbial communities. The proportion of total variation explained by each principal coordinate axis was recorded. The relative distribution of cold- and warm-season samples in ordination space was examined to identify potential seasonal clustering or separation. PCoA was interpreted as an exploratory visualization of multivariate community structure. Apparent separation of samples in the ordination space was not considered evidence of statistical

significance without confirmation through permutation-based statistical testing.

2.9. Statistical analysis of beta diversity

2.9.1. Permutational multivariate analysis of variance

PERMANOVA was performed to test whether overall rumen microbial community composition differed between cold- and warm-season samples. The analysis was conducted separately for Bray-Curtis and unweighted UniFrac distance matrices using the QIIME 2 beta-group-significance function. The seasonal grouping variable was used as the explanatory factor and statistical significance was assessed using 999 permutations. A p-value less than 0.05 was considered statistically significant.

2.9.2. Analysis of similarities

Analysis of similarities (ANOSIM) was performed using the unweighted UniFrac distance matrix to further assess the degree of separation between seasonal groups. The analysis was conducted with 999 permutations. The ANOSIM R statistic was used to quantify the relative separation between groups. Values approaching +1 indicate greater separation between groups relative to within-group variation, whereas values close to zero indicate limited separation. As the present study included only three samples per season, the results of PERMANOVA and ANOSIM were interpreted cautiously, with particular emphasis on effect patterns and p-values rather than treating visual ordination patterns alone as evidence of significant seasonal differences.

2.10. Krona visualization of taxonomic composition

Krona diagrams were generated to provide a hierarchical visualization of the taxonomic composition of the rumen microbiome during the cold and warm seasons. Taxonomic abundance information was combined with the ASV feature table to generate multi-layered interactive Krona visualizations using the q2-krona workflow. The hierarchical diagrams display taxonomic relationships from higher taxonomic ranks towards lower taxonomic levels, with segment size corresponding to the relative abundance of individual taxonomic groups. Separate Krona visualizations were generated for the cold- and warm-season groups to facilitate descriptive comparison of their microbial taxonomic composition. Taxa contributing at least 0.1% relative abundance were retained for labeling in the final visualization. The Krona plots were used for descriptive comparison and were not interpreted as statistical evidence of differential abundance between seasons.

3. Results and Discussion

3.1. Sequencing quality and data processing

Six goat rumen microbiome samples, comprising three cold-season samples (SRR27169185, SRR27169186 and SRR27169187) and three warm-season samples (SRR27169218, SRR27169219 and SRR27169220), were processed using the QIIME 2 workflow for quality filtering, denoising, paired-end merging, chimera removal and downstream microbial diversity analysis. The sequencing quality statistics are summarized in Table 1.

Table 1: Quality filtering, denoising, merging and chimera removal statistics for goat rumen microbiome samples.

Sample ID	Season	Input reads	Filtered reads	% input passed filter	Denoised reads	Merged reads	% input merged	Non-chimeric reads	% input non-chimeric
SRR27169185	Cold	83,884	67,604	80.59	64,305	49,068	58.50	30,076	35.85
SRR27169186	Cold	85,239	66,247	77.72	62,412	34,898	40.94	15,450	18.13
SRR27169187	Cold	82,769	71,279	86.12	68,341	46,077	55.67	21,576	26.07
SRR27169218	Warm	112,545	104,049	92.45	97,488	22,511	20.00	9,000	8.00
SRR27169219	Warm	112,985	105,286	93.19	99,253	22,312	19.75	9,626	8.52
SRR27169220	Warm	118,495	109,156	92.12	101,524	22,195	18.73	12,774	10.78

Raw sequencing depth ranged from 82,769 to 118,495 reads per sample. The proportion of reads passing the initial quality-filtering step ranged from 77.72% to 93.19%. Although warm-season samples showed higher retention during the initial filtering step, a greater proportion of cold-season reads was retained after paired-end merging and chimera removal. The proportion of merged reads ranged from 40.94% to 58.50% in cold-season samples and from 18.73% to 20.00% in warm-season samples. Consequently, the final number of non-chimeric reads was higher in cold-season samples (15,450-30,076) than in warm-season samples (9,000-12,774).

These differences in sequencing retention should not, however, be interpreted as biological evidence of seasonal differences in microbial diversity. Variation in read retention and paired-end merging may arise from differences in sequencing quality, amplicon length variation, overlap between forward and reverse reads, library preparation or sequencing runs. Therefore, downstream diversity analyses should be standardized to minimize the potential influence of unequal sequencing depth. All diversity analyses were consequently interpreted using a standardized sampling depth based on the lowest retained sample depth. This normalization is important because differences in sequencing depth can influence the detection

of rare taxa and may bias alpha- and beta-diversity estimates.

3.2. Feature Table Characteristics and Microbial Community Complexity

The final feature table contained 3,443 amplicon sequence variants (ASVs) across the six rumen samples, with a total frequency of 98,502 reads. The feature distribution indicated a complex microbial community characterized by a relatively large number of low-abundance ASVs together with a smaller number of highly represented features.

Table 2: Summary statistics of the feature table generated from the goat rumen microbiome samples.

Summary statistic	Value
Number of samples	6
Number of features	3,443
Total frequency	98,502
Minimum frequency per sample	9,000
Maximum frequency per sample	30,076
Mean frequency per sample	16,417
Minimum frequency per feature	1
Median frequency per feature	17
Maximum frequency per feature	888
Mean frequency per feature	28.6

The median feature frequency was substantially lower than the maximum feature frequency, indicating an uneven abundance distribution across ASVs. Such distributions are characteristic of complex microbial communities, in which a limited number of highly represented taxa coexist with numerous low-abundance taxa.

However, because only six samples were included in the present analysis, the detection of rare ASVs should be interpreted cautiously. Low-frequency features may represent biologically rare taxa but can also be sensitive to sequencing depth and bioinformatic filtering thresholds. Therefore, the observed ASV richness provides an overview of community complexity rather than definitive evidence for a stable “rare biosphere.”

3.3. Seasonal variation in alpha diversity

Alpha diversity was evaluated using the Shannon diversity index and Faith's phylogenetic diversity (Faith's PD), representing complementary aspects of microbial community diversity. The Shannon index reflects both richness and relative abundance distribution, whereas Faith's PD incorporates the evolutionary relationships among taxa.

Table 3: Alpha diversity indices of goat rumen microbiome samples during cold and warm seasons.

Sample ID	Season	Faith's PD	Shannon index
SRR27169185	Cold	40.27	8.58
SRR27169186	Cold	34.19	8.54
SRR27169187	Cold	47.61	8.58
SRR27169218	Warm	70.40	8.08
SRR27169219	Warm	55.36	8.08
SRR27169220	Warm	54.37	8.08

Cold-season samples exhibited Shannon index values ranging from 8.54 to 8.58, whereas warm-season samples

showed consistently lower values of 8.08. Thus, the cold-season samples displayed greater Shannon diversity, suggesting a higher combined contribution of taxonomic richness and evenness. In contrast, Faith's PD was lower in the cold season (34.19-47.61) than in the warm season (54.37-70.40). This indicates that the warm-season rumen microbiome encompassed a greater phylogenetic breadth, with taxa distributed across a larger amount of evolutionary branch length. As illustrated in Figure 2, the distribution of Faith's PD differed between the two seasonal groups, indicating the contrasting pattern between the two diversity metrics is biologically informative. Higher Shannon diversity during the cold season indicates a comparatively more diverse or even taxonomic community, whereas higher Faith's PD during the warm season indicates representation of more phylogenetically distinct microbial lineages.

The Shannon diversity index differed significantly between the two seasonal groups (Kruskal-Wallis test, $P < 0.05$), indicating variation in within-sample microbial diversity associated with seasonal conditions (Figure 1). Nevertheless, because the comparison involved only three samples per season, statistical significance should be interpreted together with the limited sample size. The results should therefore be considered preliminary evidence of seasonal variation requiring validation in a larger biological dataset. Importantly, Shannon diversity and Faith's PD should not be considered contradictory. Two microbial communities can have similar or even contrasting taxonomic diversity while differing in phylogenetic breadth. A community may contain a relatively even distribution of closely related taxa, resulting in high Shannon diversity but lower phylogenetic diversity, whereas another community may contain fewer or unevenly distributed taxa that span more distantly related evolutionary lineages.

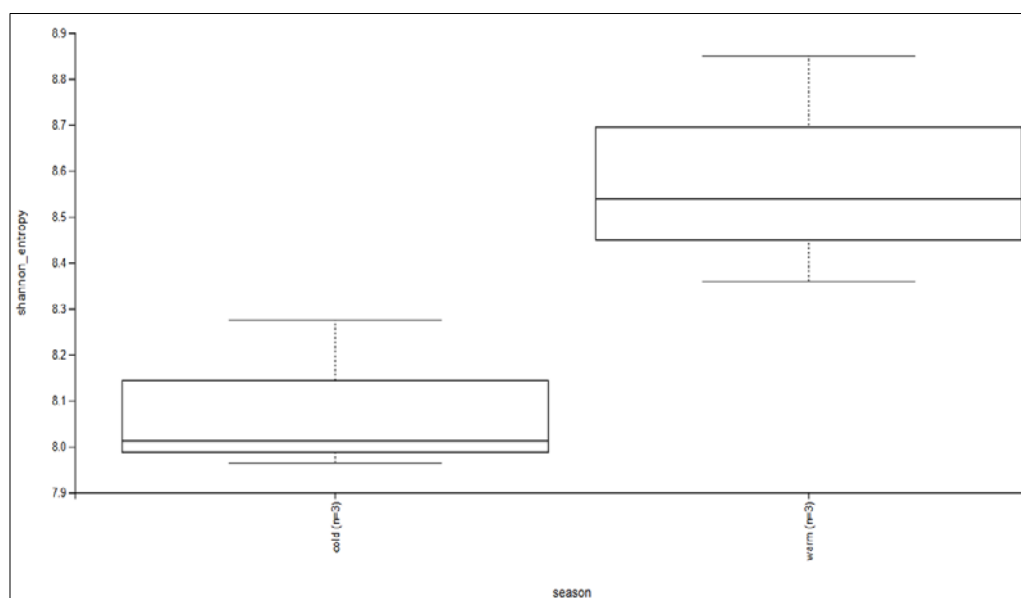


Fig 1: Alpha diversity comparison between cold and warm seasons.

Boxplots showing the distribution of Shannon diversity index values across cold ($n = 3$) and warm ($n = 3$) season samples. Horizontal lines indicate medians and whiskers

represent the minimum and maximum values. The asterisk indicates a significant difference between seasons (Kruskal-Wallis test, $P < 0.05$).

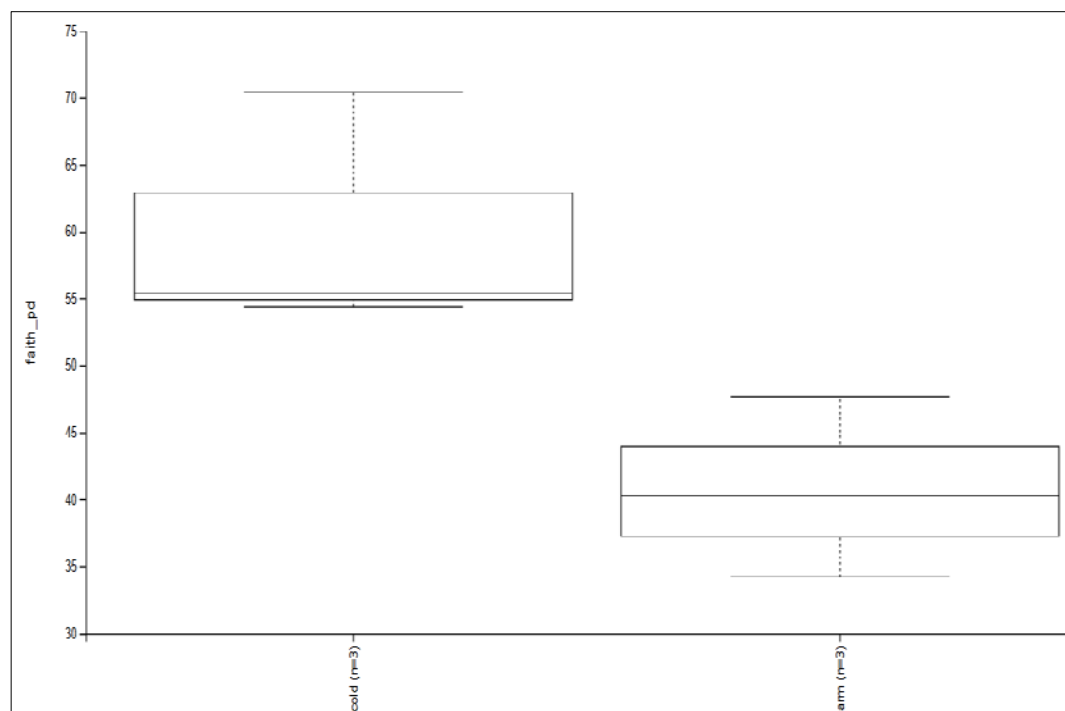


Fig 2: Phylogenetic diversity comparison between cold and warm seasons.

Boxplots showing the distribution of Faith's Phylogenetic Diversity (Faith's PD) values across cold (n=3, red) and warm (n=3, blue) season samples. Horizontal lines indicate medians; whiskers extend to minimum and maximum values. Asterisk denotes significant difference (Kruskal-Wallis test; * $p < 0.05$).

3.4. Beta diversity based on unweighted UniFrac

Unweighted UniFrac PCoA was used to evaluate differences in the phylogenetic composition of the rumen microbiome between seasons. Unlike abundance-based metrics, unweighted UniFrac primarily considers the presence or absence of microbial lineages in relation to their phylogenetic relationships.

Table 4: Principal coordinate analysis of unweighted UniFrac distances among cold- and warm-season goat rumen microbiome samples.

Sample ID	Season	PCoA1	PCoA2	PCoA3
SRR27169185	Cold	-0.272	-0.044	0.045
SRR27169186	Cold	-0.303	0.015	0.084
SRR27169187	Cold	-0.291	0.008	0.016
SRR27169218	Warm	0.409	0.165	0.342
SRR27169219	Warm	0.285	-0.426	-0.116
SRR27169220	Warm	0.173	0.283	-0.371

PCoA1, PCoA2 and PCoA3 explained 37.11%, 20.39% and 19.44% of the total variation, respectively. The first two axes together accounted for 57.50% of the variation. All cold-season samples had negative PCoA1 values, whereas all warm-season samples had positive PCoA1 values. This pattern indicates an apparent separation between seasons along the principal axis of phylogenetic variation.

The cold-season samples were relatively closely positioned along PCoA1 and PCoA2, whereas the warm-season samples showed greater dispersion along PCoA2. This pattern suggests that the warm-season samples exhibited greater within-group variability in phylogenetic community

composition. However, ordination plots are descriptive and do not independently establish statistical significance. Therefore, the visual separation observed along PCoA1 should be interpreted together with permutation-based statistical testing.

3.5. Beta diversity based on bray-curtis dissimilarity

Bray-Curtis PCoA was used to evaluate seasonal variation in abundance-based microbial community composition.

Table 5: Principal coordinate analysis of Bray-Curtis dissimilarities among cold- and warm-season goat rumen microbiome samples.

Sample ID	Season	PCoA1	PCoA2	PCoA3
SRR27169185	Cold	-0.285	0.000	0.575
SRR27169186	Cold	-0.285	-0.022	-0.332
SRR27169187	Cold	-0.285	0.090	-0.241
SRR27169218	Warm	0.202	-0.529	-0.016
SRR27169219	Warm	0.379	0.000	0.000
SRR27169220	Warm	0.275	0.460	0.014

The first three axes explained 20.22%, 20.03% and 20.03% of the total variation, respectively. PCoA1 accounted for 20.22% of the abundance-based variation and showed an apparent separation between cold- and warm-season samples. All cold-season samples had negative PCoA1 scores, whereas warm-season samples were positioned on the positive side of PCoA1. The cold samples also displayed relatively limited dispersion along PCoA2. In contrast, warm-season samples were more dispersed along PCoA2. The consistency of this pattern with the unweighted UniFrac analysis suggests that seasonal differences may influence both phylogenetic lineage composition and relative taxonomic abundance. Nevertheless, the strength and statistical significance of these patterns should be assessed using PERMANOVA rather than inferred solely from the visual arrangement of samples.

3.6. Statistical Evaluation of Seasonal Differences in Beta Diversity

The PERMANOVA and ANOSIM analyses indicated strong

descriptive separation between seasons but did not reach the conventional threshold for statistical significance.

Table 6: Statistical comparison of beta diversity between cold- and warm-season goat rumen microbiome samples.

Distance metric	Statistical method	Test statistic	P-value
Bray-Curtis	PERMANOVA	Pseudo-F = 1.013	0.095
Unweighted UniFrac	PERMANOVA	Pseudo-F = 3.214	0.092
Unweighted UniFrac	ANOSIM	R = 0.852	0.096

The Bray-Curtis PERMANOVA analysis did not identify a statistically significant difference between seasons ($p = 0.095$). Similarly, unweighted UniFrac PERMANOVA did not reach statistical significance ($p = 0.092$). ANOSIM based on unweighted UniFrac produced a high R statistic ($R = 0.852$), indicating strong separation between within-season and between-season ranks of phylogenetic dissimilarity. However, the corresponding p-value was 0.096 and therefore did not meet the conventional significance threshold of $P < 0.05$. Accordingly, the beta-diversity results should not be reported as demonstrating statistically significant seasonal restructuring of the rumen microbiome. The appropriate interpretation is that the analyses revealed a consistent trend toward seasonal separation, particularly for phylogenetic

community composition, but the available sample size was insufficient to provide formal statistical confirmation. The combination of visual PCoA separation and relatively large ANOSIM R should therefore be considered hypothesis-generating rather than conclusive. The limited number of biological replicates is an important constraint and larger studies are required to confirm whether the observed seasonal pattern represents reproducible biological variation.

3.7. Seasonal Taxonomic Composition Based on Krona Visualization

The Krona diagrams provided a hierarchical visualization of the taxonomic composition of the rumen microbiome during the warm and cold seasons.

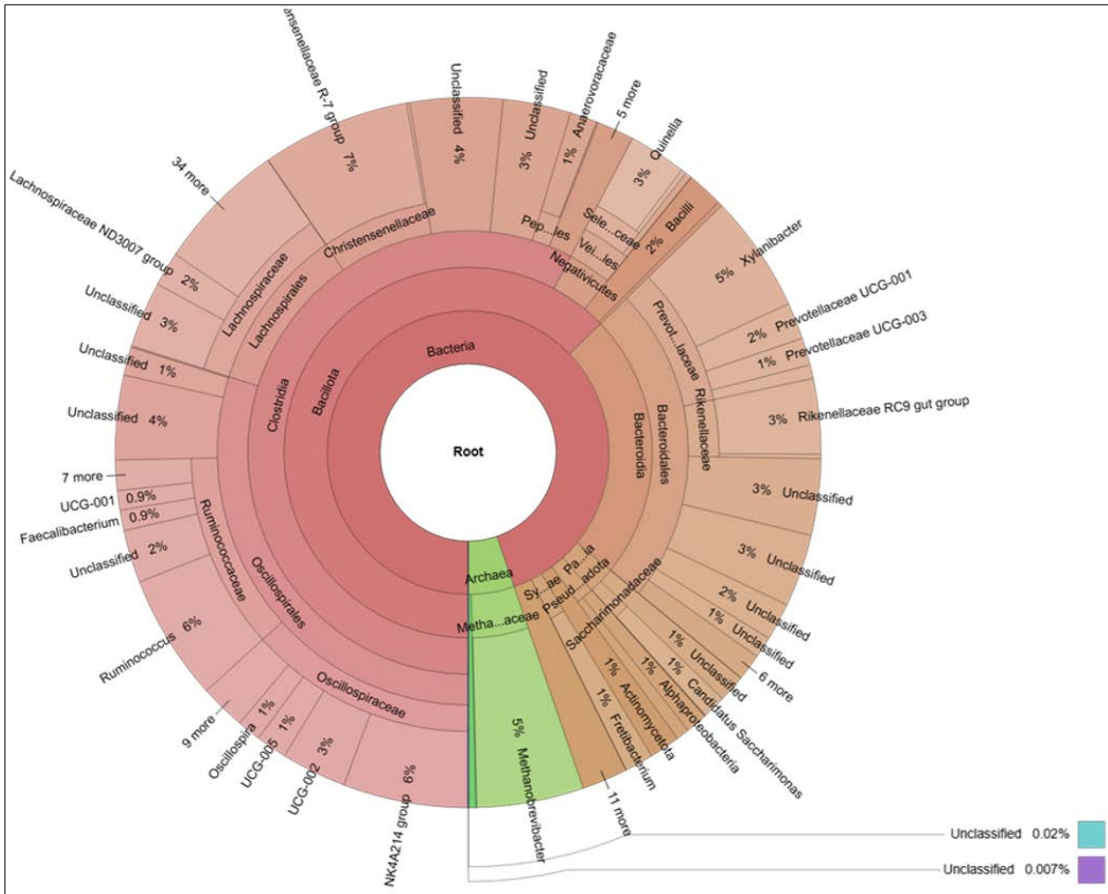


Fig 3: Krona visualization of the taxonomic composition of the goat rumen microbiome during the warm season.

The warm-season Krona diagram indicated a complex bacterial community composed of multiple taxonomic groups, with bacterial taxa forming the major component of the rumen microbiome. The hierarchical distribution of taxonomic branches indicated the presence of several lower-level bacterial lineages rather than complete dominance by a single lineage. The relatively higher Faith's PD values

observed during the warm season are consistent with the presence of phylogenetically diverse microbial lineages. However, the Krona visualization alone should be considered descriptive and cannot be used to statistically establish differences in the relative abundance of individual taxa.

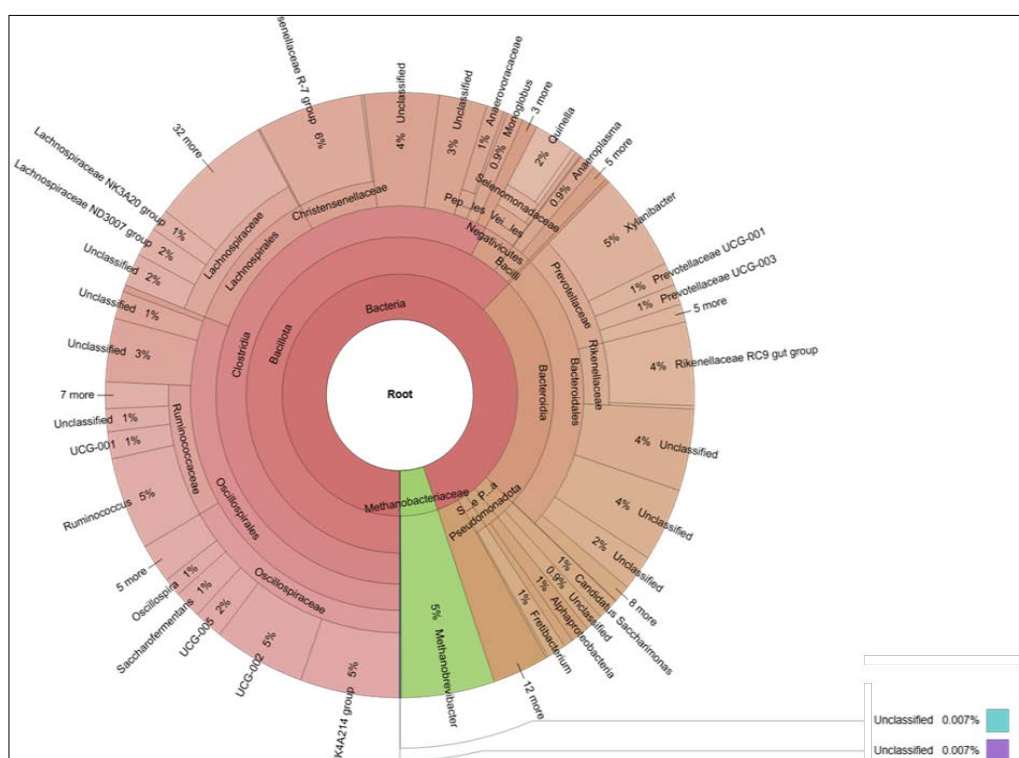


Fig 4: Krona visualization of the taxonomic composition of the goat rumen microbiome during the cold season.

The cold-season Krona diagram also demonstrated a complex microbial community dominated primarily by bacterial taxa, with additional representation of archaeal lineages. The hierarchical distribution showed the persistence of multiple taxonomic groups within the rumen ecosystem. Compared with the warm-season visualization, differences in the relative size and distribution of taxonomic sectors suggest variation in community composition between seasons. These observations are consistent with the alpha- and beta-diversity analyses, which indicated differences in within-sample diversity and apparent separation along the primary ordination axis. However, because the present study included only three samples per season and no differential-abundance analysis was performed, the Krona diagrams should not be used to claim statistically significant enrichment or depletion of specific taxa.

4. Conclusion

The combined alpha- and beta-diversity analyses indicate that season may influence the structure of the goat rumen microbiome. The two seasons differed significantly in alpha-diversity measures, with cold-season samples showing higher Shannon diversity and warm-season samples showing greater Faith's phylogenetic diversity. This pattern suggests that seasonal differences may affect not only the number and relative distribution of microbial taxa but also the evolutionary breadth of lineages present within the rumen community. The contrasting responses of Shannon diversity and Faith's PD emphasize the importance of evaluating microbial diversity using complementary taxonomic and phylogenetic metrics.

Both unweighted UniFrac and Bray-Curtis PCoA demonstrated a similar directional pattern along PCoA1, with cold-season samples occupying the negative side and warm-season samples occupying the positive side. The

consistency between abundance-based and phylogeny-based ordinations suggests an apparent seasonal structuring of the microbial community. Nevertheless, the PERMANOVA and ANOSIM results did not reach statistical significance at $P < 0.05$. Therefore, the observed separation cannot be interpreted as definitive evidence that season significantly altered overall rumen microbial community composition. Instead, the results suggest a potential seasonal trend, particularly in phylogenetic composition, that should be investigated using a larger number of independent biological replicates. The Krona visualizations further support the presence of seasonal variation in taxonomic structure, although these figures remain descriptive. Together, the results indicate that seasonal environmental conditions may be associated with changes in rumen microbial diversity and community organization. Confirmation of these findings will require larger sample sizes, detailed information on diet and management and quantitative differential-abundance analyses.

5. Data availability

The raw sequencing datasets analysed in the present study are publicly available through the National Center for Biotechnology Information Sequence Read Archive under BioProject accession PRJNA1050866, including the samples SRR27169185-SRR27169187 and SRR27169218-SRR27169220.

6. References

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