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Original Article

Research on the intestinal microbial diversity of Wild Rock Sheep on the Qinghai-Tibet plateau

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Abstract

The Qinghai-Tibet Plateau, China's largest ecologically fragile and sensitive region, is critical for understanding wildlife-environment interactions. To clarify the gut microbiota characteristics of Wild Rock Sheep (*Pseudois nayaur*) in this region and explore their potential ecological implications, we performed 16S rDNA sequencing on field-collected fecal samples. A total of 386,588 high-quality sequences were obtained, with an average of 111,382 valid combined sequences per sample. At the phylum level, Firmicutes dominate over Bacteroidetes, with notable inter-individual variations in their relative abundances. At the genus level, UCG-005 and Rikenellaceae-RC9-gut-group were the key differential taxa. with distinct abundances across samples. α-diversity indices (Chao1, ACE, Simpson, and Shannon) indicated high species richness, diversity, and evenness in the microbial communities, reflecting a stable gut ecosystem. Functional prediction revealed that the gut microbiota was primarily involved in core metabolic processes, including carbohydrate, amino acid, cofactor and vitamin metabolic processes, as well as terpenoid/polyketide, nucleotide, energy, and lipid metabolism.
Compared to prior studies on Rock Sheep gut microbiota, our work uniquely focuses on the ecologically fragile Qinghai-Tibet Plateau, linking microbial traits to potential adaptations to high-altitude environments. These findings enhance understanding of wildlife-microbiota-environment interactions and provide baseline data for conservation and ecological management in the region.

Keywords: Wild Rock Sheep, 16S rRNA sequencing, Gut microorganism, Microbial diversity

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Introduction

The Wild Rock sheep (also known as bharal, cliff sheep, or stone sheep), a Class II protected animal in China, belongs to the order Artiodactyla, family Bovidae, and subfamily Caprinae. It is primarily distributed on the Qinghai-Tibet Plateau and adjacent regions, with populations also found in Nepal, Pakistan, India, and Kashmir (Schaller, 1997). In China, Wild Rock Sheep inhabit Tibet, Sichuan, Gansu, Qinghai, Ningxia, Xinjiang, and Inner Mongolia (Wang et al., 2021), though existing studies on their ecology and conservation have focused mainly on Tibet, Qinghai, and Ningxia (Schaller et al., 1998). As herbivores feeding primarily on grasses, shrubs, and sedges (Liu et al., 2007), they inhabit high-altitude areas (2500-5500 meters), including Plateaus, hills, and alpine bare rock zones, and typically live in herds with an estimated total population of over 40,000 individuals (Kusuda et al., 2006). To date, research on Wild Rock Sheep has focused largely on habitat selection, population ecology, behavioral ecology, and gut microbiota of captive populations (Chi et al., 2019). For example, Sun et al compared gut microbiota differences between captive and wild individuals but did not explore the Qinghai-Tibet Plateau, an ecologically fragile region critical for their survival (Sun et al., 2019). Zhu et al highlighted seasonal and gender effects on their gut microbiota but neglected links to high-altitude adaptation mechanisms. These gaps leave a critical knowledge void: how does the gut microbiota of Wild Rock Sheep in the Qinghai-Tibet Plateau support their survival in extreme environments (Zhu et al., 2020).

Gut microbiota, as key regulators of host health and environment adaptation, mediate core physiological processes such as nutrient absorption, energy metabolism, and immune function (Zeb et al., 2020; Li et al., 2023). Their dysbiosis can disrupt host health (Czajkowska et al., 2020; Meng et al., 2020), Additionally, while stability is significantly shaped by environmental factors like altitude making microbiota a promising marker for wildlife adaptation (Rinninella et al., 2018). In mammals, gut microbiota is dominated by Firmicutes and Bacteroidetes, which form symbiotic relationships with hosts to maintain ecological balance (Marcobal et al., 2011; Hillman et al., 2017), underscoring the value of microbiota studies for conservation.

This study addresses the aforementioned knowledge gap by investigating gut microbiota of wild rock sheep in the Qinghai-Tibet Plateau, hypothesizing their microbial composition is uniquely adapted to high altitudes (differing from captive or other regional conspecifics) and specific taxa correlate with health and extreme environment adaptation. Using fecal samples, we sequenced the 16S rRNA V3-V4 region via Illumina MiSeq to analyze their gut microbiota, aiming to characterize core composition, assess health via microbial profiles, and support gastrointestinal disease prevention and conservation. Novel focusing on understudied microbiota of this species in the Qinghai-Tibet Plateau, it advances Wild Rock Sheep conservation by linking microbiota to health and adaptation.

Material and Methods

Sample collection

The samples collected in this study were from the feces of Tibetan Wild Rock Sheep living in the wild near cliffs in Yumai Township, Lounze County, Shannan City, Tibet. Sampling was conducted in December 2024 (winter). A total of 3 sets of fecal samples were collected (labeled as YY1, YY2, YY3, respectively). All samples were collected immediately after natural defecation. Soil-contaminated and externally exposed fecal samples were discarded and only the interior feces were used for sampling. Collected samples were transferred to pre-sterilized 2 mL centrifuge tubes, labeled, and immediately preservation in liquid nitrogen. After transportation to the laboratory, samples were stored at -80°C until subsequent 16S rRNA gene analysis. All procedures involving Wild Rock Sheep were approved by the Animal Ethics Review Committee of the Tibet Agricultural and Animal Husbandry College (Approval No.: XZA-2025-015).

DNA extraction from feces and 16S rRNA amplicon sequencing

Preserved samples were sent to Kedio Company (Guangdong, China) for DNA extraction and sequencing. Genomic DNA was extracted using a DNA extraction kit (Beijing TianGen Biotech Co., Ltd.) following the manufacturer’s instructions. After extracting, DNA concentration was measured, and its integrity was verified via 1% agarose gel electrophoresis. Extracted DNA was stored at -20°C until further use. Using the extracted total DNA as a template, the V3-V4 hypervariable region of the 16S rRNA gene was amplified with barcode-tagged specific primers: Forward primer (341F): 5'-CCTACGGGNGGCWGCAG-3'; Reverse primer (806R): 5'-GGACTACHVGGGTATCTAAT-3'. The PCR amplification program was as follows: initial denaturation at 95°C for 3 minutes; 25 cycles of denaturation at 95°C for 45 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds; final extension at 72°C for 5 minutes. PCR products were purified via gel extraction using the AxyPrep DNA Gel Recovery Kit (AXYGEN) according to the manufacturer's protocol. Quantification of the recovered DNA was performed using the QuantiFluor^{TM}-ST Blue Fluorescence Quantitation System (Promega). Sequencing libraries were constructed, and paired-end sequencing (2×250 bp) was conducted on the Illumina MiSeq platform.

Bioinformatics analysis process

The raw data was generated by removing adapters and barcodes from the sequencing output. Sequences were then filtered to retain only those longer than 250 bp without ambiguous bases (N). Paired-end reads were merged using FLASH v1.2.7. The merged sequences underwent further quality control using QIIME2 v2021.2 (https://qiime2.org). Chimeric sequences were eliminated using mothur v.1.45.3 Operational Taxonomic Units (OTUs) were generated at 100% sequence similarity using the DADA2 pipeline within QIIME2. Taxonomic classification of OTUs was performed against the SILVA reference database (v138). The resulting feature tables, taxonomic assignments results, phylogenetic trees, and representative sequences were uploaded to Microbiome Analyst (https://www.microbiomeanalyst.ca) for downstream analysis. Alpha diversity indices, including ACE, Chao1, PD-tree, Shannon, Simpson, and Good's Coverage, were calculated. Rarefaction curves, relative abundance histograms, and community heatmaps were generated in R (v3.3.1). For bata diversity analysis, hierarchical clustering, Principal Coordinates Analysis (PCoA), and Non-metric Multidimensional Scaling (NMDS) were performed. Differences in species abundance among groups were statistically evaluated using the Kruskal-Wallis test, with a significantly threshold set at $P < 0.05$. Finally, PICRUSt v1.0.0 was utilized to predict bacterial functional profiles based on high-quality sequences.

Results

Sequence analysis

A total of 386,588 high-quality sequences were obtained from the three fecal samples, with an average of 111,382 effective sequences per sample. The length of the amplified region ranged from 203 to 478 bp, providing sufficient coverage for subsequent microbiota analysis.

Analysis of the structure and composition of microbiota at different taxonomic levels in the gut microbiota in Wild Rock Sheep.

The gut microbiota composition of Wild Rock Sheep (YY1, YY2, YY3) was analyzed at the kingdom, phylum, class, order, family, and genus levels, with a focus on dominant taxa and inter-sample patterns. At the kingdom level: over 99% of the microbiota belonged to the bacteria kingdom across all samples (YY1: 99.28%, YY2: 99.72%, YY3: 99.68%), indicating a highly conserved bacterial dominance (Figure 1A). At the phylum level: Firmicutes was the most dominant phylum, with a gradual increase from YY1(69.39%) to YY3 (77.05%). In contrast, Bacteroidetes showed a decreasing trend (YY1: 21.53%; YY3: 11.32%). Verrucomicrobiotea was the third dominant phylum, with relative abundances of 5.23% (YY1), 7.82% (YY2), and 5.78% (YY3) (Figure 1B). These trends suggest a potential shift in the Firmicutes/Bacteroidetes ratio, which is often associated with energy metabolism in ruminants. At the class level: Clostridia (a class within Firmicutes) was the dominant class, with relative abundances increasing from YY 1 (67.35%) to YY3 (67.35%) (Figure 1C). This aligns with the increasing trend of Firmicutes at the phylum level, reflecting a consistent taxonomic hierarchy. At the order level, Oscillospirales showed the highest relative abundance in YY3 (37.31%), followed by YY2 (34.30%) and YY1 (31.68%). In contrast, Lachnospirales was most abundant in YY2 (21.72%), while Bacteroidales (linked to Bacteroidetes) was most abundant in YY1 (21.44%) and decreased in YY2 (15.02%) and YY3 (11.17%) from (Figure 1D). These patterns mirror the phylum level trends, reinforcing the stability of taxonomic shifts across hierarchical levels. At the family level, Oscillospiraceae (associated with Oscillospirales) increased from YY1 (18.68%) to YY3 (24.86%), consistent with the order level trend. Lachnospiraceae (linked to Lachnospirales) was most abundant in YY2 (21.64%), while Rikenellaceae and Akkermansiaceae (a key family in Verrucomicrobia) showed variable but low abundances across samples (Figure 1E). At the genus level, the top 10 genera were dominated by UCG-005 (highest in YY2: 19.76%) Rikenellaceae-RC9-gut-group (highest in 8.18%), and Akkermansia (highest in YY2: 7.66%) (Figure 1F). These genera are often associated with fiber degradation and gut homeostasis, suggesting their potential role in the Wild Rock Sheep's digestive physiology.

Cluster heatmaps (Figure 2A, 2B) revealed that 11 phyla and 20 genera clustered closely across samples, indicating high taxonomic consistency among individuals. The similarity in bacterial composition (Figure 2C) suggests a stable core microbiota in Wild Rock Sheep, with minor variations driving the observed trends.

Table-1. Statistics of the number of OTU and Tags.

Sample Name	Raw Reads	Clean Reads	Raw Tags	Clean Tags	Chimera	Effective Tags	Effective Ratio (%)
YY1	131253	131104	128005	127266	12496	114770	87.44
YY2	131300	131136	127872	126918	13886	113032	86.09
YY3	124005	123853	120657	119564	13219	106345	85.76

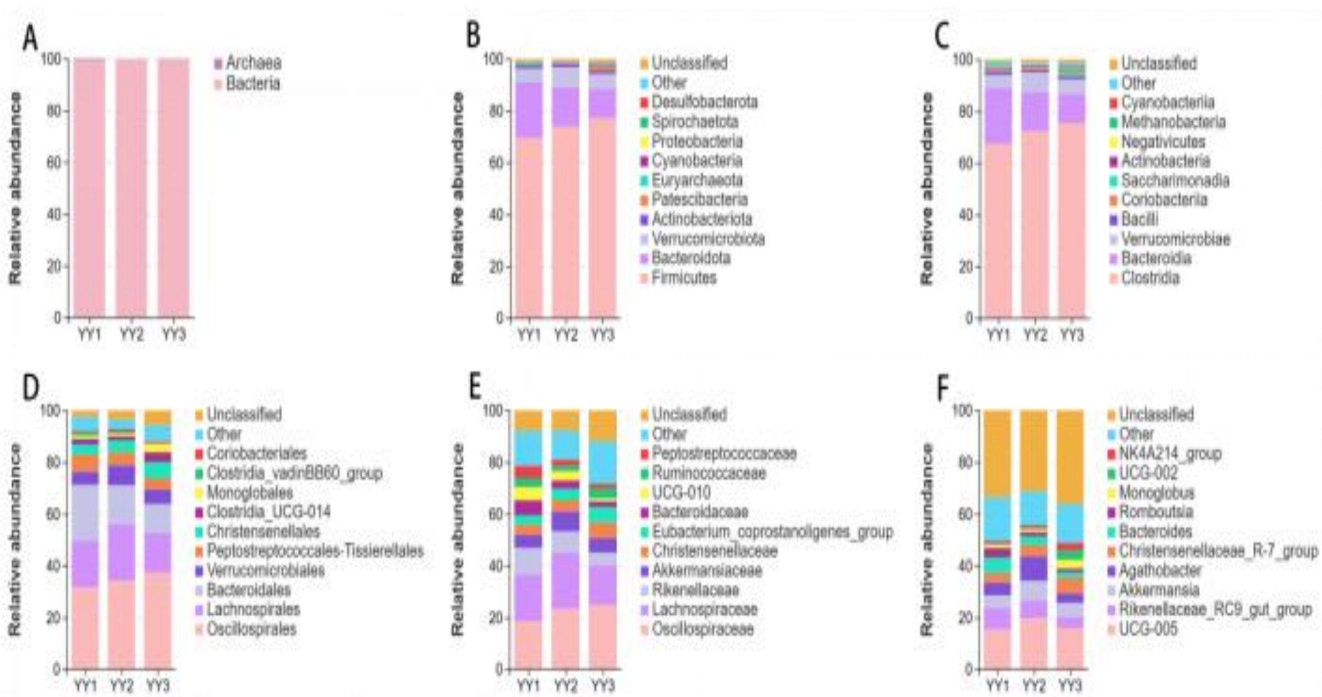

Figure-1. Relative abundance levels of TOP10 abundance in wild rock sheep feces. (A) kingdom, (B) phylum, (C) class, (D) order, (E) family, (F)genus.

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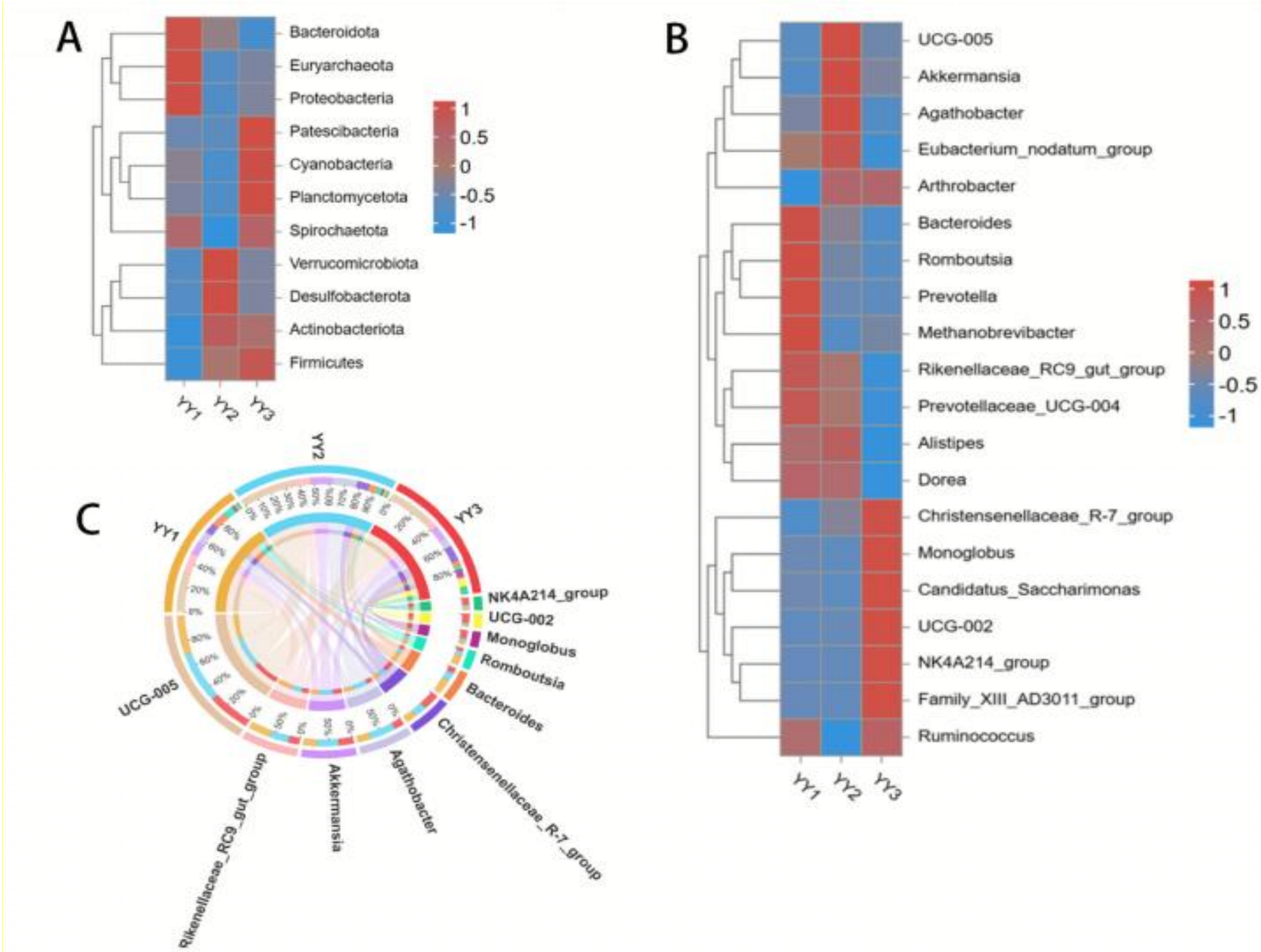

Figure-2. Heat map of species abundance clustering. (A, B) The horizontal axis is the sample name, and the vertical axis is the species name. The variation in abundance of different species in each sample is shown using a color gradient that can reflect the similarity and differences of the sample at different taxonomic levels. The values corresponding to the color gradient are shown to the right of the plot. (A) 11 common phylum in different colonies; (B) 20 common genera in different colonies; (C) The microbial composition situation between the genus level species.

Analysis of intestinal microbial diversity in Wild Rock Sheep

α diversity indexes (Chao1, ACE, PD-tree, Shannon, Simpson) and coverage (Good's Coverage) were used to assess microbial richness and diversity (Figure 3). Good's Coverage exceeded 99% for all samples, confirming sufficiently sequencing depth to capture the core microbiota. Overall, YY3 showed the highest microbial richness and diversity: Chao1 (2579.94) ACE (2744.97) indexes (richness) were higher in YY3 than in YY1 and YY2, while the Shannon index (7.81) (diversity) was also highest in YY3. The simpson index (a measure of evenness) was higher in YY1 (0.98) and YY3 (0.98) than in YY2 (0.97), indicating more balanced species distribution in YY1 and YY3. Rarefaction curves (Figure 4A) plateaued at >40,000 sequences, confirming sufficient sequencing depth. Rank-abundance curves (Figure 4B) showed a gentle slope, indicating high Species evenness. Together, these results suggest that YY3 has the most diverse and rich gut microbiota, while YY2 has the lowest diversity.

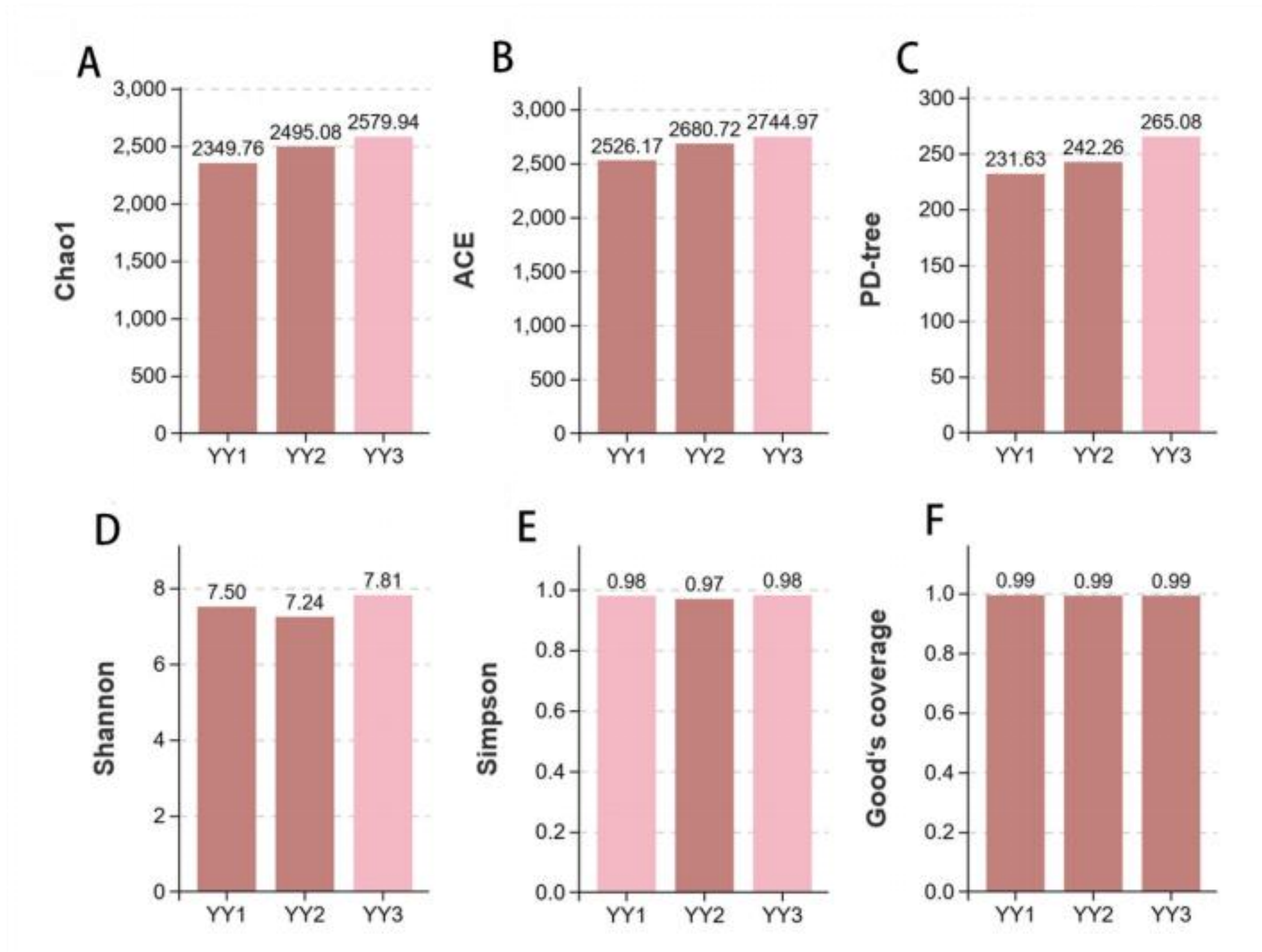

Figure-3. The α diversity index of the gut microbial communities among different individuals. (A) (B) Chao1 and ACE index mainly reflect the species richness of the sample. (C) The PD-tree index evaluates the degree of diversity, namely lineage diversity, based on the phylogenetic features of the OTU sequence evolutionary tree. (D) (E) Shannon and Simpson comprehensively reflect the richness and evenness of species, and the level of the index is also affected by the evenness. The more uniform the distribution of species in the sample, the higher the diversity. (F) Good's Coverage reflects the sequencing saturation of the sample.

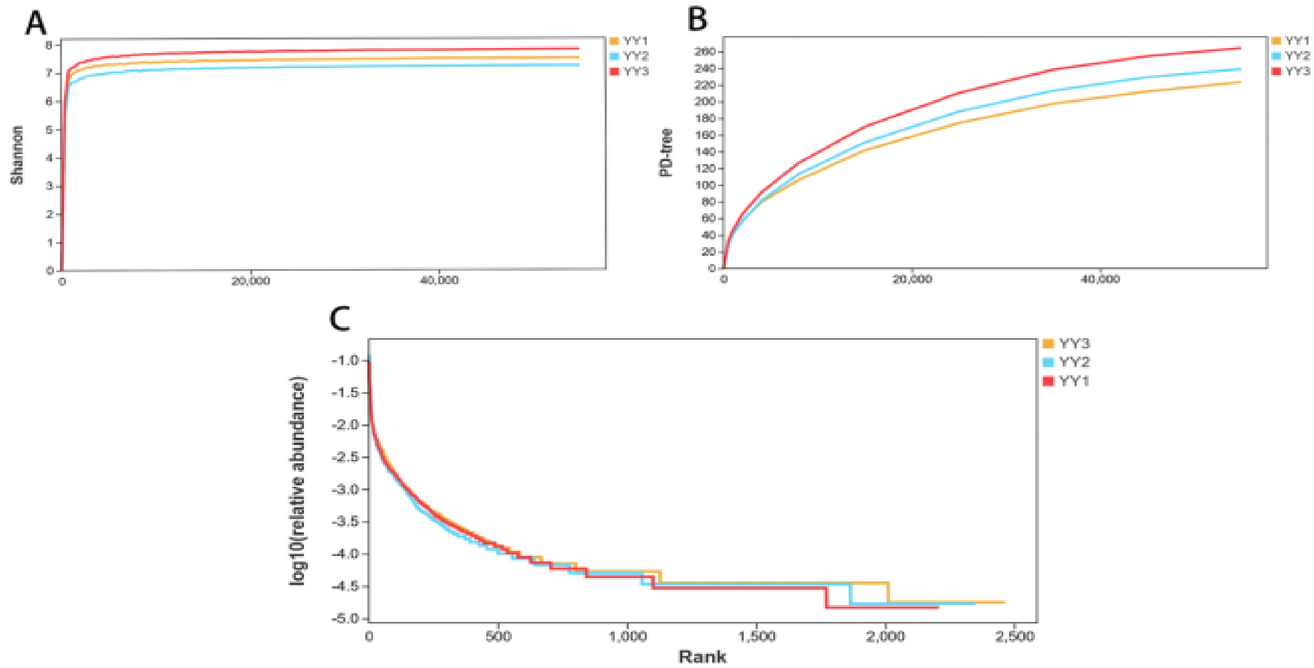

Figure-4. (A) The Simpson diversity index curve. (B) The PD diversity index curve. The dilution curve mainly reflects whether the sequencing depth (the amount of sequencing data) is reasonable, and also reflects the difference of species diversity of the samples. (C) The Rank abundance curve. Grade abundance curves assess the uniformity and abundance of the species contained in the samples, and each curve represents one sample.

Prediction of intestinal microbial ecological function in Wild Rock Sheep

Functional prediction (Figure 5) revealed that the gut microbiota primarily contributes to metabolism (e.g., carbohydrate, amino acid, and cofactor metabolism), followed by genetic information processing, cellular processes, and environmental information processing. The top 10 predicted functions were consistent across samples, with metabolism-related functions accounting for >50% of the total, reflecting the microbiota's role in energy acquisition and nutrient utilization in wild rock sheep (Table 2). Minor inter-sample variations in functional abundances aligned with taxonomic shifts (e.g., higher carbohydrate metabolism in YY3, potentially linked to its higher Firmicutes abundance).

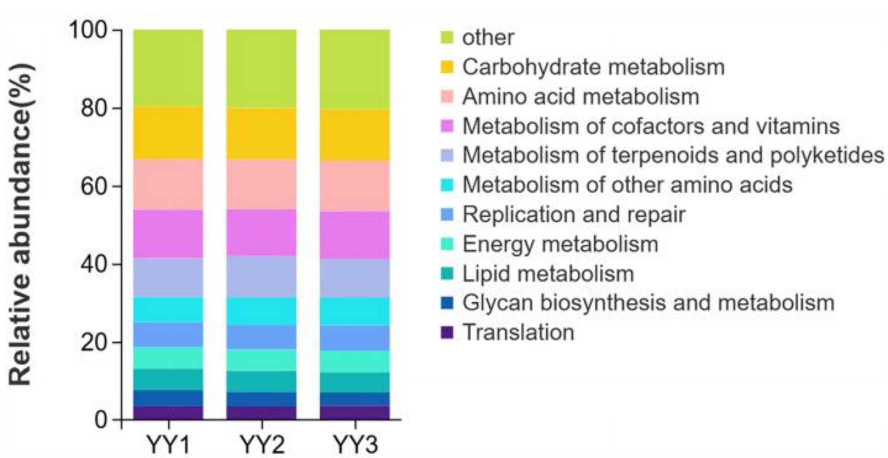

Figure-5. Ecological function prediction situation.

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Table-2. Functional prediction among individuals in Wild Rock Sheep.

Level 1	Level 2	YY1	YY2	YY3
Metabolism	Carbohydrate metabolism	384087.2705	320739.6123	290932.5087
Metabolism	Amino acid metabolism	365901.4835	308909.8241	285979.9366
Metabolism	Metabolism of cofactors and vitamins	352383.587	291322.7151	268974.0106
Metabolism	Metabolism of terpenoids and polyketides	282166.285	255366.8184	219624.7572
Metabolism	Metabolism of other amino acids	182904.4704	170360.1805	157473.215
Metabolism	Energy metabolism	157973.7888	132839.5121	122166.5913
Metabolism	Lipid metabolism	155543.829	130372.7305	114682.6594
Metabolism	Glycan biosynthesis and metabolism	115592.3784	88490.3306	74356.3762
Metabolism	Xenobiotics biodegradation and metabolism	86392.664	69265.491	70754.2435
Metabolism	Biosynthesis of other secondary metabolites	58507.1199	56635.5895	42788.1847
Metabolism	Nucleotide metabolism	57901.1829	48321.8233	45199.0356
Genetic Information Processing	Replication and repair	179994.6684	152361.0886	143329.0668
Genetic Information Processing	Translation	99410.3126	84681.8311	80048.5016
Genetic Information Processing	Folding, sorting and degradation	94723.0945	80362.9278	75744.036
Genetic Information Processing	Transcription	38150.3472	32360.6197	31075.6072
Cellular Processes	Cell motility	92308.103	88642.7157	82212.7752
Cellular Processes	Cell growth and death	42031.4776	34747.1374	32970.9262
Cellular Processes	Cellular community - prokaryotes	5302.4419	4671.3481	4448.5433
Cellular Processes	Transport and catabolism	5350.8529	3994.4031	4523.134
Environmental Information Processing	Membrane transport	46602.836	40296.8926	37170.84
Environmental Information Processing	Signal transduction	11044.8505	10045.7088	9300.3771
Environmental Information Processing	Signaling molecules and interaction	2.6071	0	2.5357
Organismal Systems	Environmental adaptation	7080.6711	6409.382	5644.6243
Organismal Systems	Endocrine system	2943.4746	2564.829	2355.6249
Organismal Systems	Immune system	2498.2715	2100.9854	1901.1458
Organismal Systems	Digestive system	1048.9432	630.7776	425.42
Organismal Systems	Development	0	0	0.25
Organismal Systems	Excretory system	0.0235	0.1176	0.0294
Human Diseases	Infectious diseases	5520.4252	4482.4365	4115.624
Human Diseases	Cardiovascular diseases	0	1.6974	2.0724
Human Diseases	Immune diseases	0.7826	0.5652	0.5217

Discussion

The Wild Rock Sheep (*Pseudois nayaur*), a national second-class protected species endemic to high-altitude regions (2500~5500 m), has evolved unique adaptations to extreme environments, including hypoxia tolerance, cold resistance, and efficient digestion (Dolker et al., 2023). These animals exhibit remarkable adaptations to their environment, including efficient nutrient absorption and cold resistance, which are likely influenced by their gut microbiota (Schaller et al., 1998; Zhi et al., 2021).

The gastrointestinal tract of mammals is one of the indispensable organs in the body, serving as the site for nutrient absorption and immune defense. When animals Encounter stress, bacterial invasion, or changes in their living environment, these adverse factors can impact the gastrointestinal tract (Hale et al., 2017; Liao et al., 2022). Gut microbiota composition is tightly inked to host ecology, particularly dietary habits and environmental conditions (Wang et al., 2019). Previous studies on Rock sheep have highlighted that seasonal shifts, altitude, and captivity status significantly shape their gut microbial communities (Sun et al., 2019; Zhu et al., 2020). Zhu et al reported marked seasonal and sex differences in Rock Sheep gut microbiota, with seasonal effects outweighing sex-related variations. Similarly, Sun et al demonstrated that altitude alters microbial structure, likely due to changes in edible plant composition across elevations. These findings underscore that Rock Sheep gut microbiota is a dynamic trait, finely tuned to environmental cues, a pattern consistent with our observations. At the phylum level, our results revealed that Firmicutes and Bacteroidetes dominated the gut microbiota of wild Rock Sheep, aligning with studies on other mammals (Duncan et al., 2008; Ley et al., 2008) and specific findings in Rock Sheep (Zhu et al., 2020). This consistency reflects the conserved role of these phyla in herbivorous digestion: Firmicutes specialize in degrading cellulose and complex carbohydrates (Patel et al., 2014), while Bacteroidetes excel at breaking down oligosaccharides (Wang et al., 2019). Notably, Firmicutes abundance was higher in YY3, followed by YY2 and YY1, whereas Bacteroidetes showed the reverse trend. This pattern mirrors Sun et al.'s observation that wild Rock Sheep have higher Firmicutes than captive individuals— a difference attributed to the wild diet's higher fiber content (e.g., alpine shrubs, sedges). Our results extend this finding by suggesting individual variation in Firmicutes/Bacteroidetes ratios may reflect fine-scale differences in foraging behavior: YY3's higher Firmicutes could indicate greater consumption of tough, fiber-rich plants, while YY2's higher Bacteroidetes might correspond to more oligosaccharide-rich food sources. At the genus level, differences in UCG-005 and Rikenellaceae-RC9-gut-group abundance between samples may reflect adaptations to specific dietary or climatic conditions. UCG-005 is associated with butyrate production (Salvi and Cowles, 2021). This short-chain fatty acid may enhance intestinal epithelial health and energy homeostasis, which are critical for surviving cold environments with elevated metabolic demands. Similarly, Rikenellaceae-RC9-gut-group may enhance nutrient absorption and pathogen resistance (Gu et al., 2022), aligning with its increased abundance in individuals exposed to harsher conditions. These findings echo studies on wild primates, where seasonal dietary shifts drive microbial community restructuring (Springer et al., 2017; Baniel et al., 2021), suggesting a conserved mechanism for environmental adaptation across species.

α diversity indices (Chao1, ACE, Shannon, Simpson) indicated YY3 had the highest microbial richness and evenness. High diversity in gut microbiota typically enhances functional redundancy (Peguero et al., 2017). a trait critical for Wild Rock Sheep, as it allows the community to maintain metabolic function when specific taxa are disrupted by environmental shifts (e.g., sudden cold, food scarcity). YY3's high diversity may thus reflect greater ecological resilience, enabling it to exploit a wider range of food sources or buffer against stressors. Consistent with Combrink et al and Chang et al, who linked high gut diversity to pathogen resistance in herbivores, YY3's profile suggests a microbiota better equipped to fend off pathogens an advantage in high-altitude ecosystems where immune challenges (e.g., cold-induced stress) are common (Combrink et al., 2023; Chang et al., 2022).

Functional prediction results showed the gut microbiota prioritizes carbohydrate, amino acid, and energy metabolism, functions aligning with Rock Sheep's high metabolic demands in extreme environments (Atzeni et al., 2020; Wu et al., 2024). YY1's higher predicted functional abundance may relate to individual metabolic traits, but with only three samples, this observation remains speculative.

Our findings highlight Wild Rock Sheep gut microbiota as a dynamic, adaptive trait: phylum-level ratios, genus-level functions, and α diversity collectively support efficient nutrient extraction, energy balance, and stress resilience— key for surviving high-altitude extremes. While limited by sample size and indirect functional data, this study provides a foundation for future research: larger cohorts, paired with dietary and environmental metadata, could clarify how microbiota mediates Rock Sheep's adaptation. Such insights may inform conservation strategies, emphasizing the need to preserve diverse foraging habitats to maintain gut microbial health in this endangered species.

Conclusions

This study reveals the compositional and structural characteristics of the gut microbiota in Wild Rock Sheep, which are mainly dominated by Firmicutes and Bacteroidetes, with significant differences in microbial composition among different individuals. Such microbial diversity and richness reflect the complexity and stability of the intestinal ecosystem of Rock Sheep, providing a scientific basis for assessing the health status of Wild Rock Sheep. The research results not only enhance our understanding of the intestinal microbial ecology of wild animals but also lay a theoretical foundation for wildlife conservation and disease prevention. Future studies can further explore the relationship between the microbiota and the health status of Rock Sheep, as well as its role in ecological adaptation and disease prevention, so as to provide more robust support for the formulation of wildlife management and conservation strategies.

Study Limitations

This study has several limitations. First, the small sample size (n=3) and lack of biological replicates limit statistical power and generalizability. Second, unrecorded variables such as dietary fluctuations, microhabitat differences, and individual health status may confound microbiota variations. Third, reliance on 16S rRNA sequencing precludes direct functional insights, necessitating complementary multi-omics approaches.

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Contribution of Authors

Ze LC & Duan MQ: Conceptualization, designed research methodology, data collection, analyses, interpretation and writing the original draft.
Wei MB, Wu GX, Nei HY, Ji JR & Tan ZK: Data curation and editing, investigations and literature review.
Chamba YZ & Shang P: Prepared plant samples, performed molecular analyses, data analyses, interpretation and wrote the manuscript.
All authors read and approved the final draft of the manuscript.

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