High-Throughput Sequencing Analysis of Rumen Ciliate Protozoan Population Structure in Chuanzhong Black Goats (Postprint)

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Abstract

This study aimed to investigate the rumen ciliate population structure of Chuanzhong black goats using high-throughput sequencing technology. Three 4-month-old Chuanzhong black goats [body weight (15.53$±$0.21) kg] were selected; rumen fluid samples (A) were collected after normal feeding for 20 days, and rumen fluid samples (F) were collected again after an interval of 40 days. Total DNA was extracted from the samples, the V4 region of eukaryotic 18S rRNA was amplified, and the amplification products were sequenced using the Illumina MiSeq platform. The results showed that: 1) A total of 242,321 high-quality valid sequences were obtained, which clustered into 1,650 operational taxonomic units (OTUs). 2) There were no significant differences in alpha diversity Chao, ACE, Shannon, and Simpson indices between samples A and F ($P > 0.05$). 3) At the class level, the highest relative abundance in both time-point samples was Ciliophora, Litostomatea (46.0% in sample A; 44.7% in sample F), and the difference in relative abundance between the two samples was not significant ($P > 0.05$). 4) At the family level, the dominant family in sample A was Ophryoscolecidae (31.8%), followed by Isotrichidae (14.2%); the dominant family in sample F was Ophryoscolecidae (42.8%); moreover, the relative abundance of Ophryoscolecidae in sample F was significantly higher than that in sample A ($P < 0.05$), while the relative abundance of Isotrichidae in sample A was significantly higher than that in sample F ($P < 0.05$). 5) At the genus level, the ciliate genus with the highest relative abundance in both samples A and F was Polyplastron (20.9% in sample A; 25.4% in sample F), with no significant difference ($P > 0.05$); the ciliate genera with significant differences in relative abundance were Isotricha, Ophryoscolex, Diplodinium, and Eudiplodinium, among which the relative abundances of Isotricha (14.1% vs. 1.9%) and Diplodinium (2.8% vs. 1.5%) in sample A were significantly higher than those in sample F ($P < 0.05$), while the relative abundances

of Ophryoscolex (6.7% vs. 12.5%) and Eudiplodinium (0.3% vs. 2.5%) were significantly lower than those in sample F ($P < 0.05$). The results of this study indicate that Polyplastron was the most abundant ciliate genus in the rumen fluid of young Chuanzhong black goats, and there were still many unclassified and unidentified eukaryotes with relatively high abundance in the rumen, which require further investigation.

Full Text

Analysis of Rumen Ciliate Community Structure in Chuanzhong Black Goats Using High-Throughput Sequencing Technology

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Abstract: This study investigated the ruminal ciliate community structure in Chuanzhong black goats using high-throughput sequencing technology. Three healthy 4-month-old Chuanzhong black goats with an average body weight of (15.53$±$0.21) kg were selected and fed a normal diet for 20 days before rumen fluid collection (sample A). After a 40-day interval, a second rumen fluid sample was collected (sample F). Total DNA was extracted from each sample, and the V4 region of eukaryotic 18S rRNA was amplified and sequenced using the Illumina MiSeq platform. The results showed that: 1) A total of 242,321 high-quality valid sequences were obtained, clustering into 1,650 operational taxonomic units (OTUs). 2) No significant differences were observed in alpha diversity indices (Chao, ACE, Shannon, and Simpson) between samples A and F ($P>0.05$). 3) At the class level, Ciliophora, Litostomatea was the dominant taxon in both samples (46.0% in sample A; 44.7% in sample F), with no significant difference between them ($P>0.05$). 4) At the family level, Ophryoscolecidae was the dominant family in sample A (31.8%), followed by Isotrichidae (14.2%); Ophryoscolecidae was also dominant in sample F (42.8%). The relative abundance of Ophryoscolecidae was significantly higher in sample F than in sample A ($P<0.05$), while Isotrichidae was significantly more abundant in sample A ($P<0.05$). 5) At the genus level, *Polyplastron* was the most abundant ciliate genus in both samples (20.9% in sample A; 25.4% in sample F), with no significant difference between them ($P>0.05$). Significant differences between samples were observed for *Isotricha*, *Ophryoscolex*, *Diploplastron*, and *Enoploplastron*. Specifically, the relative abundances of *Isotricha* (14.1% vs. 1.9%) and *Diploplastron* (2.8% vs. 1.5%) were significantly higher in sample A ($P<0.05$), whereas *Ophryoscolex* (6.7% vs. 12.5%) and *Enoploplastron* (0.3% vs. 2.5%) were significantly more abundant in sample F ($P<0.05$). These

findings indicate that *Polyplastron* is the dominant genus in the rumen ciliate community of young Chuanzhong black goats, and that many highly abundant eukaryotic microorganisms in the rumen remain unclassified and require further investigation.

Keywords: Chuanzhong black goat; ruminal ciliate; community structure; diversity; MiSeq sequencing

Introduction

The rumen of ruminants harbors a vast and complex microbial ecosystem comprising bacteria, protozoa, fungi, and archaea. Among these, protozoa are predominantly ciliates, with population densities reaching 1$×10^{4}$ to 1$×10^{6}$ cells/mL. Although ciliates are less numerous than bacteria, their large biomass accounts for 30–80% of total rumen microbial biomass, conferring significant biological functions. These include stabilizing the rumen environment (pH, fermentation patterns, methanogenesis, and ammonia concentration), degrading cellulose and protein, and preventing host toxicity from nitrates, nitrites, and acids. Ciliate community structure is influenced by factors such as host age, dietary composition, and geographical region. Previous studies have demonstrated that newborn ruminants lack rumen ciliates, with community structure undergoing dramatic changes during early development. As research on ruminant nutrition and disease advances, characterization of rumen ciliate communities, their population dynamics, and complex relationships with the host has become a focal point in rumen metabolic studies.

Most previous research on rumen microbiota in goats has focused on breeds such as Boer goats, with limited investigation of indigenous Chinese breeds. The Chuanzhong black goat, native to Jintang and Lezhi counties in Sichuan Province, is an excellent local breed characterized by large body size, rapid growth, superior meat quality, high reproductive rate, strong adaptability, and tolerance to roughage. Officially included in the *National Directory of Livestock and Poultry Genetic Resources* on January 15, 2010, this breed has received little attention regarding its rumen microbial ecology.

High-throughput sequencing technology has emerged as a powerful tool for microbial ecology, enabling comprehensive analysis of microbial community composition and structure. While this technology has been applied to study rumen bacteria in goats, research on rumen ciliates remains scarce. Therefore, this study employed high-throughput sequencing to characterize the rumen ciliate community structure in Chuanzhong black goats, aiming to provide a foundation for the conservation and utilization of this genetic resource and to support future research on microecological nutrition in this breed.

1.1 Experimental Animals and Diet

Three healthy 4-month-old male Chuanzhong black goats with an average body weight of (15.53$±$0.21) kg were selected for rumen fluid collection. The animals were housed individually and fed concentrate ad libitum with fresh grass, with free access to water. The concentrate composition and nutrient levels are presented in . Concentrate was provided at 2% of body weight, and roughage consisted of fresh grass fed daily at 08:00 and 17:00.

1.2 Sample Collection and Experimental Design

Following a 10-day adaptation period, the formal experiment commenced. Rumen fluid samples (sample A) were collected on day 21 before morning feeding after 20 days of normal feeding. A second collection (sample F) was performed 40 days later. Each sample was collected in triplicate using the stomach tube vacuum pump method described by Wang et al. [15].

1.3 18S rDNA Amplification and High-Throughput Sequencing

Total microbial DNA was extracted from rumen fluid samples using a Tiangen Biotech (Beijing) kit according to the manufacturer's instructions. DNA concentration was measured using a NanoDrop-ND1000 spectrophotometer, and quality was assessed by 1% agarose gel electrophoresis. Extracted DNA was stored at -20°C until use. Qualified DNA samples were sent to Shanghai Personal Biotechnology Co., Ltd. for amplification of the eukaryotic 18S rRNA V4 region (420 bp) using universal primers V547F (5 -CCAGCASCYGCGGTAATTCC-3) and V4R (5 -ACTTTCGTTCTTGATYRA-3). PCR products were identified by 2% agarose gel electrophoresis and purified using an Axygen gel extraction kit. After library quality control and quantification, qualified libraries were subjected to paired-end sequencing using the MiSeq Reagent Kit V3 (600 cycles).

1.4 Data Analysis

Raw sequencing data in FASTQ format were processed using Flash 1.2.7 software to filter sequences (based on primer and index information, assign them to corresponding samples, and remove chimeric sequences). High-quality sequences were obtained using QIIME 1.8.0 (requiring length $ $150 bp, no ambiguous bases other than N, $ $1 mismatched base at the 5 end of primers, and $ $8 consecutive identical bases). The Uclust alignment tool [16] was used to cluster high-quality sequences at 97% similarity and assign operational taxonomic units (OTUs) [17]. The most abundant sequence in each OTU was selected as the representative sequence, and an OTU abundance matrix was constructed based on sequence counts per sample. OTUs were compared against the NCBI database for taxonomic identification via GenBank. Alpha diversity indices (Chao, ACE, Shannon, and Simpson) were calculated using Mothur 1.30.1 software [18]. Significant differences in relative abundance between the two time points were analyzed using SPSS.

Results

2.1 Sample Sequencing Depth and Diversity Analysis

A total of 242,321 high-quality sequences were obtained from the six samples, with each sample containing (40,386$±$4,082) sequences. Clustering at >97% similarity yielded 1,650 OTUs. After removing rare OTUs, sample A contained 175 OTUs and sample F contained 197 OTUs, with no significant difference between samples (P>0.05). The two samples shared 143 OTUs ([Figure 1: see original paper]). The rarefaction curves for rumen fluid samples are shown in [Figure 2: see original paper]. Except for sample A1, all curves approached saturation, indicating that the sequencing depth adequately covered the majority of microorganisms in each sample.

Principal component analysis (PCA) results are presented in [Figure 3: see original paper]. The two samples clustered together, with principal component 1 (PC1) explaining 70.84% of variance and principal component 2 (PC2) explaining 23.80%. Alpha diversity indices reflecting community richness (Chao and ACE) and evenness/diversity (Shannon and Simpson) are shown in . Compared with sample A, sample F exhibited slightly lower richness indices (Chao1 and ACE) and slightly higher evenness and diversity indices (Shannon and Simpson), though none of these differences were significant (P>0.05).

2.2 Rumen Protozoal Ciliate Community Structure

The distribution of ciliates at class, family, and genus levels is presented in . Taxa not belonging to ciliates (including Neocallimastigomycota, Streptophyta, and Microsporidia) and the genus *Diplodinium* (due to low abundance) were excluded from the table. At the class level, no significant differences were observed between the two time points (P>0.05), with Ciliophora, Litostomatea being the dominant class in both samples. At the family level, the relative abundance of Isotrichidae was significantly higher in sample A (14.2% vs. 1.9%, P<0.05), while Ophryoscolecidae was significantly more abundant in sample F (42.8% vs. 31.8%, P<0.05).

At the genus level, nine rumen ciliate genera were identified across all samples, with community composition illustrated in [Figure 4: see original paper]. Among known ciliate genera, *Polyplastron* was the most abundant (20.9% in sample A; 25.4% in sample F), with no significant difference between samples (P>0.05). Significant differences between samples were detected for *Isotricha*, *Ophryoscolex*, *Diploplastron*, and *Enoploplastron*. Specifically, *Isotricha* (14.1% vs. 1.9%) and *Diploplastron* (2.8% vs. 1.5%) were significantly more abundant in sample A (P<0.05), whereas *Ophryoscolex* (6.7% vs. 12.5%) and *Enoploplastron* (0.3% vs. 2.5%) were significantly more abundant in sample F (P<0.05). Both samples contained a high proportion of unclassified genera (35.4% in sample A; 35.6% in sample F, not listed in the table). These results demonstrate substantial differences in ciliate community structure between the two sampling time points.

Discussion

3.1 Rumen Eukaryotic Biodiversity in Chuanzhong Black Goats

Alpha diversity analysis revealed that the average number of OTUs per sample was 275, substantially lower than that reported for rumen bacteria using the same technology (962–2,499 OTUs) [15,19-20]. Similarly, alpha diversity indices were much lower than those for rumen bacteria: Chao index (836–2,687) [15,18,21], ACE index (330–841) [18-19], and Shannon index (3.85–8.28) [19-21], indicating that bacterial richness and diversity far exceed those of eukaryotes in the rumen. The Shannon indices obtained in this study (3.024 and 3.089) were higher than those reported by Wang et al. [22] using PCR-DGGE (0.824), demonstrating the superior resolution of high-throughput sequencing for community diversity analysis.

Kittelmann et al. [23] investigated rumen eukaryotic communities in domestic sheep using 454 pyrosequencing and reported highly variable Simpson indices (0.004–0.767). The alfalfa pasture grazing group (sampled via stomach tube) had a Simpson index of only 0.004, the grain-fed group (sampled via slaughter) had 0.096, while summer and winter grazing groups (sampled via permanent fistula) showed similar values (0.711 and 0.731). Our results (Simpson indices of 0.765 and 0.784) differed markedly from the lower values reported by Kittelmann et al. [23], likely due to dietary differences. Rumen eukaryotes play crucial roles in feed digestion, and dietary composition is a key factor influencing community diversity [21,24]. Kobayashi [25] suggested that dietary monotony may reduce rumen microbial diversity. Additionally, differences in sequencing platforms, animal breeds, and feeding regimens may contribute to these discrepancies.

No significant differences in alpha diversity indices were observed between the two time points, suggesting that rumen eukaryotic biodiversity remains relatively stable over short periods.

3.2 Rumen Ciliate Community Structure in Chuanzhong Black Goats

A substantial proportion of sequences (35.4% and 35.6% in samples A and F, respectively) remained unclassified at the genus level, highlighting the vast unexplored diversity of rumen eukaryotes. Nine ciliate genera were identified across all samples: *Isotricha*, *Dasytricha*, *Polyplastron*, *Ophryoscolex*, *Diploplastron*, *Enoploplastron*, *Eudiplodinium*, *Diplodinium*, and *Entodinium*. *Polyplastron* was the dominant genus, which contrasts with most previous studies reporting *Entodinium* as the predominant genus in goats, including studies on Xuhuai white goats (81.3%) [26], Inner Mongolian goats (74.25%) [27], Hulunbuir grassland sheep (77.1%) [28], Spanish ibex (74–85%) [29], and domestic sheep (40%) [23]. In our study, *Entodinium* accounted for only 1.2% and 0.6% of total ciliates, possibly due to dietary differences. Our forage-based diet consisted of southern grasses, which have lower crude protein and fat content but higher crude fiber compared to other regional forages [30]. *Polyplastron* exhibits high xylanase and glucanase activities, whereas *Entodinium* has weak fiber-degrading capac-

ity [31], suggesting that high-fiber forage may favor *Polyplastron* dominance. Additionally, feeding regimen may influence these patterns: Mishima et al. [32] reported that free-ranging Tanzanian zebu cattle had significantly lower *Entodinium* abundance (7.0–25%) than most domesticated ruminants (80–99%). Coleman [14] noted that grazing facilitates protozoal colonization, with lambs acquiring adult-like protozoal communities within 3–6 months under normal grazing conditions. Franzolin et al. [33] compared two feeding systems in water buffalo and found that grazing animals had superior total ciliate counts and community composition compared to stall-fed animals. Host age also affects rumen microbiota; Hungate [11] reported that acidic rumen contents in young ruminants impede protozoal colonization. Furthermore, high-throughput sequencing may introduce bias, as Kittelmann et al. [34] suggested that smaller ciliate genera (*Entodinium*, *Epidinium*, and *Diplodinium*) may be underestimated, while larger genera (*Metadinium*, *Eremoplastron*, *Eudiplodinium*, *Ostracodinium*, and *Polyplastron*) may be overestimated.

Comparison of the two time points revealed that *Polyplastron* remained the most abundant genus without significant changes. However, significant differences were observed for *Isotricha*, *Diploplastron*, *Ophryoscolex*, and *Enoploplastron*. These shifts likely reflect dietary influences, as long-term high-fiber forage feeding may promote fiber-digesting genera such as *Ophryoscolex* while reducing starch-digesting genera like *Isotricha* [35-36]. Dietary composition appears to be the primary driver of these changes, though the small sample size may limit the generalizability of our findings regarding temporal dynamics of ciliate community structure.

Conclusion

This study characterized the rumen ciliate community structure of Chuanzhong black goats at two time points using high-throughput sequencing. The results demonstrate that *Polyplastron* is the dominant genus in young Chuanzhong black goats and that diet significantly influences ciliate community composition. Rumen eukaryotic richness and diversity remained stable over the short term, showing no significant differences between sampling periods. Furthermore, a substantial proportion of rumen eukaryotes remain unclassified, warranting further investigation into their structure and function.

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Note: Figure translations are in progress. See original paper for figures.