# Postprint: Study on Rumen Bacterial Community Diversity in Degradation of Different Forages

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**Date:** 2018-12-25T00:00:00+00:00

## Abstract

This experiment aimed to investigate the diversity of rumen bacterial communities degrading different forages using high-throughput sequencing technology. Alfalfa, oat grass, Leymus chinensis, and rice straw were selected as experimental materials. Each forage was ground, weighed (3.0 g), placed into nylon bags, and incubated in the rumen. After 24 h of degradation, all bags were retrieved, washed, and total DNA was extracted. Sample preparation and sequencing analysis were performed according to MiSeq high-throughput sequencing requirements. The results showed that: 1) At the genus level, sequence alignment identified 91 genera, with the dominant bacterial genera on all four forages being Butyrivibrio, Prevotella, Fibrobacter, and Treponema. 2) Weighted principal coordinate analysis based on UniFrac revealed that the first and second principal components contributed 52.89% and 19.43% of the variation, respectively. 3) The numbers of operational taxonomic units (OTUs) in alfalfa, oat grass, Leymus chinensis, and rice straw were 5,778, 6,984, 5,220, and 6,018, respectively, with 2,913 OTUs shared among the four forage groups, accounting for 32.38% of the total bacterial OTUs. In conclusion, significant differences in microbial community structural diversity were observed among the four different forages under the same degradation time.

## Full Text

## Diversity of Ruminal Bacterial Communities in the Degradation of Different Forages

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## Abstract

This study aimed to investigate the diversity of ruminal bacterial communities degrading different forages using high-throughput sequencing technology. Alfalfa, oat hay, *Leymus chinensis*, and rice straw were selected as experimental materials. Each forage was ground, and 3.0 g samples were placed in nylon bags and incubated in the rumen for 24 h. After incubation, the bags were removed, washed, and total DNA was extracted. Sample preparation and sequencing were performed according to the requirements of the Miseq high-throughput sequencing platform. The results showed that: (1) At the genus level, sequence alignment identified 91 genera, with *Butyrivibrio*, *Prevotella*, *Fibrobacter*, and *Treponema* being the dominant genera across all four forages. (2) Based on weighted principal coordinate analysis (PCoA) using UniFrac, the first and second principal components contributed 52.89% and 19.43% of the variation, respectively. (3) The numbers of operational taxonomic units (OTUs) in alfalfa, oat hay, *Leymus chinensis*, and rice straw were 5,778, 6,984, 5,220, and 6,018, respectively. The four forage groups shared 2,913 OTUs, accounting for 32.38% of the total bacterial OTUs. In conclusion, the diversity of microbial community structures differed significantly among the four forages when degraded under the same conditions.

**Keywords:** forage; bacterial community; high-throughput sequencing

The rumen of ruminant animals harbors a vast array of microorganisms including bacteria, fungi, and protozoa. Forage fiber can be degraded by rumen microbes into volatile fatty acids to provide energy for the host. Therefore, in-depth investigation of the microbial mechanisms underlying fiber degradation is crucial for efficient utilization of forage resources by ruminants. Different forages exhibit distinct tissue structures: leguminous forages such as alfalfa are primarily composed of easily degradable collenchyma, chlorenchyma, and phloem, whereas gramineous forages (e.g., oat hay and *Leymus chinensis*) and rice straw consist mainly of sclerenchyma, parenchyma, vascular bundles, and phloem. Previous research has demonstrated that microbial adhesion to and degradation of cell walls is closely associated with forage structure and type [1]. Among rumen microbes involved in fiber degradation, bacteria play a decisive role, particularly those tightly adherent to forage particles [2]. The three predominant fiber-degrading bacteria are *Ruminococcus flavefaciens* (R. flavefaciens), *Ruminococcus albus* (R. albus), and *Fibrobacter succinogenes* (F. succinogenes) [3-5]. Studies have shown that bacteria can rapidly colonize ryegrass surfaces, with the highest bacterial adhesion occurring in parenchyma and phloem tissues, especially by R. flavefaciens [6]. Akin et al. [7] employed electron microscopy to reveal that 70% of fiber-degrading bacteria colonizing fescue and orchard grass were F. succinogenes and R. flavefaciens. Koike et al. [8] used competitive PCR to examine the adhesion and degradation of orchard grass stems by the three major fiber-degrading bacteria in sheep. After 5 min of incubation, populations of F. succinogenes and the two *Ruminococcus* species (R. flavefaciens and R. albus) reached 10 and 10 cells per gram of stem dry matter (DM), respectively. At 24 h, F. succinogenes and R. flavefaciens peaked at 10 and 10 cells/g DM, respectively, while R. albus peaked at 10 cells/g DM at 48 h. However, previous studies have primarily focused on quantifying a limited number of major fiber-degrading bacteria using techniques such as microscopy and quantitative PCR [9]. Research employing high-throughput sequencing to investigate the diversity of bacterial communities embedded within forage cell walls remains scarce. Therefore, this study selected alfalfa, oat hay, *Leymus chinensis*, and rice straw—commonly used forages in Chinese dairy farms—as experimental materials. Using the nylon bag technique and Miseq high-throughput sequencing technology, we explored differences in fiber-degrading bacteria among different forages from the perspective of microbial community structure diversity, providing a theoretical foundation for further investigation into the mechanisms underlying differential forage degradation rates and the effects of diet on rumen microbiota formation.

### 1.2 Experimental Materials

Experimental materials consisted of early-blooming alfalfa, early-heading oat hay and *Leymus chinensis* with similar growth status and plant height, and post-harvest rice straw, all obtained from the experimental farm. Leaves and sheaths were removed from the base of the plants, and the stems were isolated for use as experimental material.

### 1.3 Experimental Methods

The four forage samples were dried in a 65°C oven for 48 h to determine initial moisture content, then passed through a 40-mesh sieve for analysis of crude protein, crude fat, organic matter, neutral detergent fiber, and acid detergent fiber contents. Non-fibrous carbohydrate content was calculated accordingly. Ground samples of alfalfa, oat hay, *Leymus chinensis*, and rice straw (2 mm sieve) were weighed (3.0 g each) and placed in nylon bags (8 cm × 12 cm, 300 mesh pore size). Six replicate bags were prepared for each forage, with two replicates per cow. All 24 nylon bags were inserted into the rumen of three dairy cows before morning feeding, with nylon strings attached to the cannula caps for fixation. After 24 h of incubation, all bags were removed and placed in ice water to halt fermentation. The two replicate samples from each forage were pooled, washed with cold water until the rinse water became clear, and stored at -80°C in a low-temperature freezer (BCD-208K ACJN, Qingdao Haier Co., Ltd.). For DNA extraction, forage samples were thawed on ice, and total

DNA was extracted using the OMEGA kit (M2327-02). Plant tissue pulverized with liquid nitrogen was placed in a 1.5 mL sterile centrifuge tube, immediately mixed with CPL buffer, and vortexed. The mixture was incubated in a 65°C water bath for 15 min, followed by addition of chloroform:isoamyl alcohol (24:1). After high-speed vortexing and centrifugation, the supernatant was collected. RNase, CXD buffer, and absolute ethanol were added sequentially. After 15 s of vortexing, the mixture was transferred to an adsorption column and centrifuged. The collection tube and liquid were discarded, and the column was transferred to a new collection tube. Absolute ethanol dilution buffer was added, followed by centrifugation, then ethanol wash buffer and another centrifugation step. The column was transferred to a new 1.5 mL centrifuge tube, pre-warmed double-distilled water was added for incubation, and DNA was eluted by centrifugation for 3 min. Extracted DNA samples were analyzed using a nucleic acid-protein analyzer (DU640, Beckman, USA) to determine absorbance at 260 and 280 nm and DNA concentration. DNA quality was assessed by electrophoresis on 1% agarose gels using a DYY-6C electrophoresis apparatus (Beijing Liuyi Instrument Factory).

Universal primers targeting the V3 region of bacterial 16S rDNA were designed: 338F (5 -ACTCCTACGGGAGGCAGCAG-3 ) and 533R (5 -TTACCGCGGCTGCTGGCAC-3 ). PCR amplification was performed in a 25 L reaction mixture containing 2.5 L 10× PCR Buffer, 1 L of each primer (10 mol/L), 2 L dNTP Mix (2.5 mmol/L), 0.125 L DNA polymerase (5.0 U/ L), 20 ng DNA template, and ddH O to volume. The thermal cycling program consisted of initial denaturation at 94°C for 5 min, followed by 21 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min and storage at 4°C. PCR products were verified by electrophoresis on 1.5% agarose gels, then purified using the Axygen DNA gel extraction kit (Axy Prep DNA Gel Extraction kit, AP-GX-500). Purified PCR products were quantified using a Biotek microplate reader (FLX800, BioTek Instruments, USA). To prevent uneven representation in multiplex sequencing, all samples were mixed in equal amounts according to sequencing requirements to create a homogeneous mixture. After quality control, the PCR mixture was used to construct an Illumina sequencing library following the standard Illumina TruSeq DNA library preparation protocol. Finally, barcoded Illumina Miseq sequencing was performed on the Illumina Miseq platform.

Raw sequence data underwent quality control to obtain valid sequences. Sequences shorter than 120 bp, containing ambiguous bases, with more than 2 mismatched primer bases, or with homopolymer runs exceeding 6 bases were discarded to obtain high-quality sequences. Sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity threshold and subjected to phylogenetic analysis.

Bacterial community richness and diversity were analyzed based on the generated OTU information. Richness indices were calculated using the Chao1 and

Ace indices, while diversity was assessed using the Shannon and Simpson indices. Higher Shannon values indicate greater community diversity, whereas higher Simpson values indicate lower diversity. Cluster heatmaps were generated at the genus level, and UniFrac analysis was performed to compare community differences among forages.

### 1.5 Statistical Analysis

Data were organized using Excel 2007. Statistical analysis was performed using the PDIFF module in SAS 9.0 software for analysis of variance and significance testing, with $P < 0.05$ and $P < 0.01$ considered as thresholds for significant and highly significant differences, respectively.

### 2.1 Nutritional Levels of Forages

The nutritional levels of the four forages are presented in Table 2 . The results indicate that nutritional levels varied among forages. While dry matter and organic matter contents were comparable across all four forages, alfalfa had substantially higher crude protein content compared to oat hay, *Leymus chinensis*, and rice straw. Conversely, alfalfa contained lower neutral detergent fiber and crude fiber contents than the gramineous forages (oat hay, *Leymus chinensis*, and rice straw).

**Table 1** Composition and nutrient levels of the diet (DM basis) %

*Note: The diet composition table appears to be incomplete in the original text.*

Following sequencing, the 12 samples generated a total of 591,378 valid sequences, which yielded 562,727 high-quality sequences after quality control, with an average length of 161 bp. The richness and diversity of bacterial communities during forage degradation are presented in Table 3 . Species richness indices ranged from 5,603 to 7,293 for the Chao index and 7,455 to 9,792 for the Ace index, with no significant differences among groups ($P > 0.05$). Forage type had a highly significant effect on the Simpson diversity index ($P < 0.01$). Alfalfa exhibited the highest Simpson index, which was significantly greater than other forages ($P < 0.01$), while *Leymus chinensis* showed the lowest Simpson index, significantly lower than other forages ($P < 0.01$). These results indicate that bacterial diversity varied among forages, with *Leymus chinensis* having the highest species diversity and alfalfa the lowest. Library coverage exceeded 94% for all groups, indicating that sequencing depth was adequate to accurately reflect bacterial community composition and structural diversity in the forages.

At the genus level, 11 of the 91 identified bacterial genera had relative abundances greater than 0.1% (Table 4 ), while the remaining genera were present at low relative abundances, indicating that forage-associated bacterial communities contained numerous low-abundance taxa. Among the genera with >0.1% relative abundance, *Butyrivibrio*, *Prevotella*, *Fibrobacter*, and *Treponema* were dominant across all forages. *Butyrivibrio* and *Prevotella* were particularly abundant, accounting for 8.88%-12.30% and 7.01%-9.29% of total sequences, respectively. Forage type had a highly significant effect on these dominant genera ($P < 0.01$).

Color gradients and similarity metrics were used to reflect the similarity and dissimilarity of community composition among forage groups at various taxonomic levels, with different colors representing relative OTU abundance proportions. As shown in Figure 1 [Figure 1: see original paper], the 12 samples clustered into two major groups, each containing four subclusters corresponding to the six replicate samples from each forage. This indicates that bacterial community structures attached to the same forage were highly similar and clustered together, while distinct differences existed among different forages.

In this study, we employed weighted principal coordinate analysis (PCoA) based on UniFrac to investigate differences in bacterial community structures degrading different forages. This method uses phylogenetic distances to measure intersample distances and characterize the magnitude of bacterial community structural differences. PCoA visualizes these differences in two- or three-dimensional space, where closer distances between points indicate greater similarity in microbial community structure. As shown in Figure 2 [Figure 2: see original paper], the first and second principal components of the UniFrac-based PCoA explained 52.89% and 19.43% of the variation, respectively. Bacterial community structures differed markedly among forages, while the three replicate samples from each forage clustered closely together, indicating high within-forage similarity.

By enumerating shared and unique OTUs among forage groups, a total of 8,997 OTUs were identified at the 97% similarity level. As illustrated in Figure 3 [Figure 3: see original paper], alfalfa, oat hay, *Leymus chinensis*, and rice straw contained 5,778, 6,984, 5,220, and 6,018 OTUs, respectively. The four forages shared 2,913 OTUs, representing 32.38% of the total bacterial OTUs. The proportions of unique OTUs in alfalfa, oat hay, *Leymus chinensis*, and rice straw were 6.07%, 10.61%, 4.85%, and 6.88%, respectively, following the order: oat hay > rice straw > alfalfa > *Leymus chinensis*. These results demonstrate that bacteria attached to different forages shared approximately one-third of OTUs, while each forage harbored a distinct proportion of unique OTUs.

## 3 Discussion

Sequence alignment revealed that *Butyrivibrio*, *Prevotella*, *Treponema*, and *Fibrobacter* were the dominant genera in forages, consistent with the dominant genera found in rumen fluid of cows fed these four forages [12]. Although the dominant genera did not differ in composition among forages, their relative abundances varied significantly or highly significantly among forage groups, primarily due to differences in nutritional composition, tissue structure, and microbial adhesion among forages, as different microorganisms respond differently to various substrates [12-13]. *Butyrivibrio*, a major fibrolytic genus within the phylum Firmicutes, plays an important role in fiber degradation. Studies have shown that

*Butyrivibrio* can utilize cellulose, starch, and other polysaccharides as fermentation substrates, and its abundance was significantly higher in alfalfa than in the other three forages, likely related to the higher fermentable carbohydrate content in alfalfa compared to the gramineous forages (oat hay, *Leymus chinensis*, and rice straw). *Prevotella* contains highly active hemicellulose-degrading bacteria [14] and is crucial for degradation of plant non-fibrous polysaccharides and proteins [15]. The combined proportion of *Butyrivibrio* and *Prevotella* in alfalfa (21.59%) was markedly higher than in oat hay (16.45%), *Leymus chinensis* (18.05%), and rice straw (18.96%), possibly associated with the higher protein and fermentable carbohydrate content in alfalfa, though this requires further investigation. Although *Ruminococcus* and *Fibrobacter* are less abundant than *Butyrivibrio* and *Prevotella* in the rumen, they also play important roles in forage fiber degradation. Additionally, this study identified up to 80 low-abundance genera. Despite their low abundance, these taxa may secrete highly active cellulases, including *Eubacterium*, *Pseudobutyrivibrio*, and *Oscillibacter*, which contribute significantly to fiber degradation [16].

Microorganisms can rapidly adsorb to cell wall surfaces, but the rate and extent of cell wall degradation are influenced by microbial-substrate adhesion, feed physicochemical properties, and rumen digestion kinetics [17]. A study on microbial adsorption to different substrates (ryegrass leaf, ryegrass stem, and rice straw) in goat rumen over 24 h revealed no significant differences in microbial adhesion among fiber substrates during the first 6 h of rumen degradation, with total bacterial numbers stabilizing after 6 h. Denaturing gradient gel electrophoresis (DGGE) profiles demonstrated significant differences in microbial community structure between substrates degraded for 12 and 24 h compared to 6 h, indicating that substrate-associated microbial communities change over time [18]. Clone library analysis of adherent microorganisms also found *Treponema* only in alfalfa samples but not in orchard grass [8]. However, *Treponema* was detected in all four forages in this study, differing from the orchard grass findings, possibly due to variations in forage varieties, experimental animals, and dietary composition. Research has shown that bacteria can rapidly colonize ryegrass cell walls within 15 min, with R. flavefaciens preferentially adhering to the epidermis, parenchyma, and phloem margins of ryegrass leaves [19]. Studies using fescue and orchard grass as substrates demonstrated that adherent microorganisms were predominantly F. succinogenes and R. flavefaciens [7, 20], representing 0.1%-6.6% and 1.3%-2.9% of total bacteria, respectively [21-23]. These findings align with our results showing higher *Ruminococcus* abundance in gramineous forages (oat, *Leymus chinensis*, and rice straw) than in leguminous alfalfa, likely related to the higher cellulose content in gramineous forages. *Ruminococcus* can ferment cellulose and its hydrolysis product cellobiose, while alfalfa cellulose content was only 22.65%, lower than that of oat hay, *Leymus chinensis*, and rice straw. Microbial morphology was similar across different substrates, but microbial numbers differed significantly, peaking at 24 h with the highest abundance of rod-shaped cocci, diplococci, and Spirochaetes [24], which justified our selection of the 24 h degradation timepoint for microbial community analysis.

Results from bacterial community structure similarity analysis showed that the 12 samples formed two major clusters in the heatmap, with three replicates from each forage grouping into subclusters. This demonstrates that microbial community structures degrading the same forage were highly similar among replicates, while distinct differences existed among forages, consistent with the significant or highly significant differences observed at the genus level. Notably, the two gramineous forages, oat hay and *Leymus chinensis*, clustered together, indicating greater similarity in bacterial community structures degrading gramineous forages, likely related to their similar cell wall composition and nutritional profiles [25-26]. As shown in Table 2, oat hay and *Leymus chinensis* had comparable crude protein (5.89% vs. 5.54%), neutral detergent fiber (67.58% vs. 69.25%), and crude fiber (28.14% vs. 29.36%) contents. Similar nutritional composition and cell wall structure may be the primary reason for the comparable bacterial community structures in oat hay and *Leymus chinensis* [12]. Alfalfa and rice straw formed another cluster, showing greater similarity in bacterial community structure between them, which aligns with previous scanning electron microscopy and dry matter degradation studies reporting similar degradation patterns between alfalfa and rice straw [1,27]. In the UniFrac PCoA plot, which considers species abundance, the four forage groups were clearly separated, consistent with the heatmap clustering results.

### 4 Conclusion

High-throughput sequencing analysis of bacterial community structure differences in the degradation of four forages revealed that microbial community structure diversity attached to different forages differed significantly under the same degradation conditions. At the genus level, the dominant fiber-degrading bacteria were primarily *Butyrivibrio*, *Prevotella*, *Fibrobacter*, and *Treponema*.

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