Postprint: Effects of Mannan Oligosaccharide on Rumen Microbiota Structure in Dairy Cows Based on 16S rDNA High-Throughput Sequencing Analysis

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Abstract

This study aimed to investigate the effects of mannan oligosaccharides on rumen bacterial community structure in dairy cows using 16S rDNA high-throughput sequencing technology. Four early-lactation dairy cows with similar lactation stages and identical parity were selected and randomly assigned to two groups. The control group was fed a basal diet, whereas the experimental group received the basal diet supplemented with 60 g/head of mannan oligosaccharides via oral administration. A 2×2 crossover design was adopted, with each period consisting of a 14-day adaptation phase followed by a 7-day sampling phase. The results demonstrated: 1) Compared with the control group, dietary supplementation of mannan oligosaccharides highly significantly increased rumen fluid acetate concentration ($P<0.01$), while the acetate/propionate ratio was elevated by 8.47% ($P>0.05$). 2) At the phylum level, the abundance of Cyanobacteria was highly significantly reduced ($P<0.01$), and the relative abundance of Armatimonadetes was highly significantly increased ($P<0.01$). At the genus level, the relative abundance of Anaerobiospirillum was significantly increased ($P<0.05$), that of Clostridium was significantly decreased ($P<0.05$), and that of Pyramidobacter was highly significantly decreased ($P<0.01$). The relative abundances of Ruminococcus, Pseudobutyrivibrio, and Lachnospira increased by 24.34%, 12.83%, and 31.80%, respectively ($P>0.05$), the relative abundance of Prevotella decreased by 4.66% ($P>0.05$), the relative abundance of unannotated Veillonella increased by 143.11% ($P>0.05$), and the relative abundance of Desulfovibrio increased by 4.88% ($P>0.05$). Under the experimental conditions, mannan oligosaccharide supplementation decreased rumen bacterial diversity and markedly influenced cellulose-degrading and hemicellulose-degrading bacterial communities; it promoted a significant increase in rumen fluid acetate concentration but exerted no significant effect on rumen fluid pH.

Full Text

Effects of Mannan Oligosaccharides on Rumen Bacterial Community Structure in Dairy Cows Based on 16S rDNA High-Throughput Sequencing Technology

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Abstract: This experiment investigated the effects of mannan oligosaccharides on rumen microflora in dairy cows using 16S rDNA high-throughput sequencing technology. Four healthy Chinese Holstein lactating cows with similar parity and lactation stage were randomly divided into two groups. The control group received a basal diet, while the experimental group received the basal diet supplemented with 60 g/cow of mannan oligosaccharides. A 2 × 2 crossover design was employed, with each period lasting 21 days (14-day pre-trial and 7-day sampling). The results showed that: (1) Mannan oligosaccharides significantly increased ruminal acetic acid concentration compared to the control group ($P < 0.01$), and the acetic acid/propionic acid ratio also increased, though not significantly ($P > 0.05$). (2) At the phylum level, mannan oligosaccharides significantly decreased the relative abundance of Cyanobacteria ($P < 0.01$) and significantly increased that of Armatimonadetes ($P < 0.01$). At the genus level, the abundance of Anaerobiospirillum significantly increased ($P < 0.05$), while that of Clostridium significantly decreased ($P < 0.05$). The abundance of Pyramidobacter also significantly decreased ($P < 0.01$). The abundances of Ruminococcus, Pseudobutyrivibrio, and Lachnospira increased by 24.34%, 12.83%, and 31.80%, respectively, but these differences were not significant ($P > 0.05$). The abundance of Prevotella decreased by 4.66%, while Veillonellaceae_NA and Desulfovibrio increased by 143.11% and 4.88%, respectively, though not significantly ($P > 0.05$). In conclusion, mannan oligosaccharide reduced rumen bacterial diversity in dairy cows, notably affecting cellulolytic and hemicellulolytic bacterial communities while significantly increasing ruminal acetic acid concentration. The regulation of ruminal pH likely results from the combined effects of starch-degrading bacteria and lactate-utilizing bacteria, though the specific mechanism requires further investigation.

Keywords: dairy cows; rumen; mannan oligosaccharides; 16S rDNA; bacterial flora

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1.1 Experimental Materials

Mannan oligosaccharides were purchased from Henan Sanhua Biotechnology Co., Ltd. with a purity of 99%.

1.2 Experimental Animals and Diets

Four multiparous (second parity) Chinese Holstein lactating cows with 20 days in milk, daily milk yield of approximately 30 kg, and body weight of approximately 550 kg were selected as experimental animals. Diets were formulated according to NRC (2001) dairy cattle feeding standards, with a concentrate-to-forage ratio of 40:60 (DM basis). The composition and nutrient levels of the basal diet are shown in Table 1 . Animals were fed total mixed rations (TMR) three times daily with ad libitum access to feed and water.

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

Item	Content
Ingredients	
Alfalfa hay	
Leymus chinensis	
Corn silage	
Corn	
Soybean meal	
Wheat bran	
Whole cottonseed	
Premix[1]	
CaHPO	
NaCl	
MgCl	
Total	
Nutrient levels[2]	
Crude protein (CP)	
Net energy for lactation (NEL, MJ/kg)	
Neutral detergent fiber (NDF)	
Acid detergent fiber (ADF)	

[1] The premix provided the following per kg of diet: VA 14,000 IU, VD 7,500 IU, VE 43 mg, Se 0.6 mg, Cu 30 mg, Fe 358 mg, Mn 260 mg, salinomycin sodium salt 20 mg, zinc bacitracin 100 mg, sulfuric acid bacillus 39 mg.
[2] NEL was a calculated value [8], while others were measured values.

1.3 Experimental Design

Cows were randomly divided into two groups: a control group fed the basal diet and an experimental group fed the basal diet supplemented with 60 g/cow of mannan oligosaccharides administered via oral infusion. A 2 × 2 crossover design was used, with each period lasting 21 days (14-day pre-trial and 7-day sampling).

1.4.1 Rumen Fluid Collection

Rumen fluid was collected using an oral catheter. A nose clip was used to secure the cow's head, and a rigid tube (hard plastic with inner diameter exceeding that of the sampling hose) was inserted into the mouth to a depth not exceeding the pharynx, leaving approximately 20 cm exposed externally for manual fixation by staff. A flexible hose (approximately 2.5 m) was then gradually passed through the rigid tube into the rumen. The cow' s head was lowered to allow rumen fluid to flow naturally through the chewing process.

1.4.2 Rumen Fluid Processing

Collected rumen fluid was transferred to the laboratory and filtered through four layers of cheesecloth. The filtered fluid was then mixed in equal volumes. pH was measured directly using a colorimeter. For volatile fatty acid (VFA) analysis, 20 mL of rumen fluid was transferred to a 50 mL centrifuge tube, with a portion then moved to a 10 mL tube containing 3 mL of 25% metaphosphoric acid and 0.6% 2-ethylbutyric acid, and stored at -20 °C. VFA concentrations were determined by gas chromatography [11] using an Agilent-6890N gas chromatograph. An additional 50 mL of rumen fluid was stored in centrifuge tubes at -80 °C for bacterial community analysis. A total of 8 samples were collected: 4 from the mannan oligosaccharide-supplemented group (MT-1, MT-2, MT-3, MT-4) and 4 from the control group (CK-1, CK-2, CK-3, CK-4).

1.5.1 DNA Extraction

DNA was extracted using a commercial kit purchased from Nanjing Jiancheng Bioengineering Institute, following the manufacturer' s protocol.

1.5.2 PCR Amplification

Prior to PCR amplification, DNA sample purity and concentration were assessed by agarose gel electrophoresis. Appropriate amounts of samples were diluted in centrifuge tubes, and the diluted genomic DNA was used as template. The target fragment for rumen fluid DNA amplification was the V3+V4 region (approximately 468 bp) using primers 341F-806R [10]. Primer sequences were: 341F: 5 -CCTAYGGGRBGCASCAG-3 ; 806R: 5 -GGACTACNNGGGTATCTAAT-3 .

1.6 PCR Product Pooling and Purification

PCR products were examined by electrophoresis on 2% agarose gels. Based on concentration measurements, products were pooled at equal concentrations. After thorough mixing, the pooled PCR products were re-examined on 2% agarose gels and purified using a recovery kit provided by Nanjing Jiancheng Bioengineering Institute.

1.7 Miseq Sequencing

Samples were sent to Guangzhou Gene Denovo Biotechnology Co., Ltd. for Miseq sequencing using the Miseq 2500 PE 250 platform.

1.8 Statistical Analysis

Data were initially processed using Excel 2007 and analyzed using two-stage crossover design analysis of variance in SAS 8.2 statistical software. Results are expressed as mean ± standard error. $P < 0.05$ was considered significant, and $P < 0.01$ was considered highly significant.

2.1 Effects of Mannan Oligosaccharides on Rumen Fermentation Parameters

As shown in Table 2 , ruminal pH in the experimental group increased by 2.46% compared to the control group ($P > 0.05$). Mannan oligosaccharide supplementation highly significantly increased ruminal acetic acid concentration ($P < 0.01$), while the acetic acid/propionic acid ratio increased by 8.47% ($P > 0.05$).

Table 2 Effects of mannan oligosaccharides on fermentation parameters in the rumen of dairy cows

Item	Control group	Experimental group	P-value
Acetic acid (mmol/L) Propionic acid (mmol/L) Butyrate acid (mmol/L) Acetic acid/propionic acid	54.60	64.28	<0.001

In the same row, values with no letter superscript indicate no significant difference ($P > 0.05$), different lowercase letters indicate significant difference ($P < 0.05$), and different uppercase letters indicate highly significant difference ($P < 0.01$). The same applies to Table 4 through Table 6 .

2.3.1 Operational Taxonomic Unit (OTU) Count

After Illumina Miseq sequencing, low-quality sequences were removed and reads were assembled. Using Mothur, sequences were clustered into OTUs at 97% similarity. Based on OTU clustering results, Venn graphs were constructed to illustrate shared and unique OTUs among samples. As shown in Figure 2 [Figure 2: see original paper], mannan oligosaccharide supplementation decreased rumen fluid bacterial diversity.

2.3.2.1 OTU Rarefaction Curve and OTU Shannon Rarefaction Curve

To determine whether the sequencing data adequately represented the bacterial community distribution in experimental and control groups, rarefaction curves

were constructed by plotting randomly sampled sequence numbers against OTU numbers. These curves indicate whether sequencing depth was sufficient to capture species diversity. As shown in Figures 3 [Figure 3: see original paper] and 4 [Figure 4: see original paper], OTU numbers changed dramatically at low sequencing depth, increasing substantially with greater depth. When sequencing depth reached 5,000 reads, the rarefaction curve continued to rise, indicating that novel bacteria remained to be discovered. However, the Shannon curve reached saturation at 5,000 reads, demonstrating that sequencing was adequate for bacterial diversity analysis.

2.3.2.2 Alpha Diversity Analysis

Alpha diversity analyzes species diversity within individual samples, including Chao1 index, ACE index, Shannon index, and Simpson index [11]. Chao1 and ACE indices predict microbial species richness (OTU numbers) based on tag abundance and OTU proportions, providing relative values derived from known results. The Shannon index comprehensively reflects both OTU richness and evenness; a larger Shannon index approaching 0 indicates greater species richness.

As shown in Table 3 , the experimental group exhibited lower ACE and Chao1 indices than the control group, indicating reduced bacterial richness. The Shannon index was lower and the Simpson index higher in the experimental group, demonstrating that mannan oligosaccharide supplementation decreased bacterial diversity. Coverage exceeded 0.93 for all samples, confirming adequate representation of rumen bacterial communities.

Table 3 Diversity indexes of samples

Item	Control group	Experimental group	P-value
Richness index			
ACE index			
Chao1 index			
Diversity index			
Shannon index			
Simpson index			
Coverage (%)	93.900	95.300	

2.4.1 Phylum Level

As shown in Figure 5 [Figure 5: see original paper] and Table 4 , a total of 29 phyla were identified in dairy cow rumen fluid, including Bacteroidetes, Firmicutes, Proteobacteria, Spirochaetes, Cyanobacteria, Verrucomicrobia, Fibrobacteres, Tenericutes, Planctomycetes, Actinobacteria, Elusimicrobia, Synergistetes, Euryarchaeota, Fusobacteria, Chloroflexi, Acidobacteria, and Arma-

timonadetes. Bacteroidetes, Firmicutes, and Proteobacteria accounted for 90% of the total bacterial community.

Compared to the control group, mannan oligosaccharide supplementation highly significantly decreased the relative abundance of Cyanobacteria ($P < 0.01$) and highly significantly increased that of Armatimonadetes ($P < 0.01$). The relative abundances of Spirochaetes, Fibrobacteres, Fusobacteria, and Acidobacteria were reduced, though not significantly ($P > 0.05$).

2.4.2 Genus Level

At the genus level, 219 genera were identified. As shown in Figure 6 [Figure 6: see original paper] and Table 5 , the 25 most abundant genera accounted for over 91% of total bacterial abundance. Thirteen genera exhibited relatively high abundance: *Prevotella*, NA, S24-7_NA, Succinivibrionaceae_NA, Lachnospiraceae_NA, Ruminococcaceae_NA, Prevotellaceae_NA, *Succiniclasticum*, *Ruminococcus*, *Butyrivibrio*, *Treponema*, *Shuttleworthia*, and *Coprococcus*. The relative abundance of *Pyramidobacter* was highly significantly lower in the experimental group ($P < 0.01$), while *Clostridium* was significantly lower ($P < 0.05$). The abundances of cellulose-degrading *Ruminococcus* and *Pseudobutyrivibrio* increased by 24.34% and 12.83%, respectively ($P > 0.05$). The hemicellulose-degrading genus *Anaerobiospirillum* was significantly higher in the experimental group ($P < 0.05$). Lachnospiraceae_NA abundance increased by 8.16% ($P > 0.05$). Starch-degrading *Prevotella* decreased by 4.66% ($P > 0.05$). Lactate-utilizing Veillonellaceae_NA increased by 143.11% ($P > 0.05$), and lactate-oxidizing *Desulfovibrio* increased by 4.88% ($P > 0.05$).

2.5 LEFse Analysis of Inter-group Differences

LEFse software was used to analyze differences between groups. The analysis first performed Kruskal-Wallis rank-sum tests across all groups, followed by Wilcoxon rank-sum tests for pairwise comparisons. Significant differences were then ranked by Linear Discriminant Analysis (LDA) scores, as shown in Figure 7 [Figure 7: see original paper]-A. This figure displays species with significantly different relative abundances between groups (LDA score > preset threshold), where bar length represents effect size (LDA score). Differences were mapped onto a known hierarchical classification tree to generate a cladogram (Figure 7 [Figure 7: see original paper]-B). In the cladogram, circles radiating from inner to outer represent taxonomic levels from phylum to genus (or species), with circle diameter proportional to relative abundance. Non-significant species are colored yellow. As shown in Figures 7-A and 7-B, the relative abundances of Armatimonadetes, RB046, SJA_176, *Kingella*, and Acholeplasmatales increased significantly, while *Pyramidobacter*, Cyanobacteria, 4C0d_2, YS2, and *Ruminicola* decreased significantly compared to the control group.

Discussion

Rumen microorganisms constitute an indispensable digestive microflora for ruminants, with the rumen providing a relatively stable environment for their survival. Rumen microbes primarily include bacteria, protozoa, and fungi, with bacteria being extremely abundant and diverse. Numerous studies have demonstrated that Bacteroidetes and Firmicutes dominate the gastrointestinal microbiota of mammals [12-15]. The present study found that mannan oligosaccharide supplementation reduced rumen bacterial diversity in dairy cows. In contrast, Min [16] reported that functional oligosaccharide combinations increased microbial species richness in the rumen liquid phase and promoted the growth of certain bacteria, enabling them to become dominant populations. Our study revealed that mannan oligosaccharides highly significantly decreased the relative abundance of Cyanobacteria while highly significantly increasing that of Armatimonadetes. Derakhshani [17] reported decreased Armatimonadetes abundance in postpartum dairy cows, possibly due to dietary changes, negative energy balance, and physiological stress, as postpartum diets contain higher concentrate-to-forage ratios with reduced fiber content. Armatimonadetes may utilize cellulose and hemicellulose as carbon sources, with cellulose primarily composed of -1,4-glucan. However, Lee [18] found that non-fiber carbohydrates significantly promoted Armatimonadetes growth. Our results indicate that mannan oligosaccharides promote Armatimonadetes proliferation. Mannan oligosaccharides are -glucans with primary structures of -1,3-glucan and -1,6-glucan, fundamentally different from the -1,4-glycosidic bonds in starch and the -1,4-glucan structure of cellulose.

Roughage constitutes the primary feed source for ruminants, with ruminal fiber degradation by microorganisms rapidly converting fiber into absorbable nutrients to meet energy demands [19]. Bacteria and fungi play major roles in cellulose decomposition, with bacteria, protozoa, and fungi breaking down cellulose and hemicellulose into rumen-absorbable small molecules through enzymatic catalysis [20], among which cellulolytic bacteria are particularly important. This study found that mannan oligosaccharide supplementation increased the relative abundances of cellulose-degrading *Ruminococcus* and *Pseudobutyrivibrio* by 24.34% and 12.83%, respectively. Rumen fermentation parameters showed that ruminal pH increased by 2.46% in the experimental group, providing suitable conditions for fiber-degrading bacterial proliferation. Liu et al. [7] reported that functional oligosaccharide combinations increased the abundance of *Fibrobacter succinogenes*, a major ruminal fiber-degrading bacterium. Our study found that mannan oligosaccharides significantly increased the abundance of hemicellulose-degrading *Anaerobiospirillum* and increased Lachnospiraceae_NA abundance by 8.16%. Min [16] reported that mannan oligosaccharide supplementation in Jinjiang yellow cattle produced 12 specific bands in rumen bacterial PCR-DGGE analysis, two of which were identified as *Fibrobacter succinogenes*, thereby enhancing fiber degradation rates and improving roughage digestibility.

Prevotella is widely present in the dairy cow rumen for starch degradation [21].

In this study, mannan oligosaccharide supplementation decreased *Prevotella* abundance by 4.66%. *Prevotella* represented over 49% of total bacterial abundance, constituting the dominant ruminal bacterial genus. Min [16] reported that functional oligosaccharide supplementation in Jinjiang yellow cattle increased one *Prevotella* species, though no reports have documented changes in overall *Prevotella* abundance. Mannan oligosaccharides highly significantly reduced *Clostridium* abundance, which represented only approximately 0.5% of total bacteria, indicating it is not a dominant ruminal genus. Although not dominant, *Clostridium* is diverse in the rumen, including cellulose-degrading *Clostridium fibrinolysis*, *Clostridium perfringens*, and butyric acid-producing *Clostridium butyrate*. Some strains degrade protein [22], and *Clostridium butyrate* can be isolated from ruminants fed high-starch diets [23], hydrolyzing starch (but not cellulose) to produce butyrate, acetate, and lactate. Mannan oligosaccharides highly significantly increased ruminal acetic acid concentration and increased the acetic acid/propionic acid ratio by 8.47%. The study also found that lactate-utilizing Veillonellaceae_NA abundance increased by 143.11% and lactate-oxidizing *Desulfovibrio* increased by 4.88% compared to the control. Although acetic acid concentration increased highly significantly, ruminal pH only increased by 2.46%. Compared to volatile fatty acids like acetic and butyric acids, lactic acid is more acidic and contributes more significantly to ruminal pH reduction. Therefore, mannan oligosaccharide regulation of ruminal pH likely results from combined effects of starch-degrading bacteria and lactate-utilizing bacteria such as Veillonellaceae_NA, though the specific mechanism requires further investigation.

Conclusions

1. Under the conditions of this experiment, mannan oligosaccharide supplementation reduced rumen bacterial diversity in dairy cows. At the phylum level, Cyanobacteria abundance highly significantly decreased while Armatimonadetes abundance highly significantly increased. At the genus level, hemicellulose-degrading *Anaerobiospirillum* abundance significantly increased, while starch-degrading *Clostridium* abundance significantly decreased.

2. Mannan oligosaccharide supplementation increased the relative abundances of major fiber-degrading genera including *Ruminococcus*, *Pseudobutyrivibrio*, and *Lachnospira*, while decreasing starch-degrading *Prevotella* abundance and increasing lactate-utilizing Veillonellaceae_NA and lactate-oxidizing *Desulfovibrio* abundances, though these differences were not significant.

3. Mannan oligosaccharide supplementation significantly increased ruminal acetic acid concentration but had no significant effect on ruminal pH.

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