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Postprint: Rumen Bacterial Diversity in Holstein Dairy Cows at Different Physiological Stages

Authors: Zijian Li, Li Dabiao, Gao Min, Wang Dian, Lan Rubing

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Abstract

This experiment aimed to investigate the rumen bacterial diversity of Holstein dairy cows at different physiological stages. Four multiparous Holstein dairy cows in each of the prepartum, early lactation, mid-lactation, and late lactation stages were selected, totaling 16 cows. Rumen fluid was collected to determine rumen fermentation parameters and extract microbial DNA, and the Illumina Miseq PE300 platform was used to determine rumen bacterial composition. The results showed that: 1) The concentrations of rumen microbial protein, acetate, butyrate, total volatile fatty acids, and the acetate/propionate ratio in prepartum cows were significantly higher than those in lactating cows ($P<0.05$). The rumen propionate concentration in prepartum cows was significantly lower than that in lactating cows ($P<0.05$). 2) At the phylum level, the dominant phyla in the rumen of prepartum cows were Bacteroidetes and Firmicutes, while the dominant phyla in the rumen of lactating cows were Bacteroidetes, Proteobacteria, and Firmicutes. The relative abundances of Bacteroidetes, SR1 bacteria, Cyanobacteria, Tenericutes, TM7 bacteria, and Fibrobacteres in the rumen of prepartum cows were significantly higher than those in lactating cows ($P<0.05$), while there were no significant differences among the different stages of lactation ($P>0.05$). The relative abundance of Proteobacteria in the rumen of prepartum cows was significantly lower than that in lactating cows ($P<0.05$). 3) At the family level, the dominant family in the rumen of prepartum cows was Prevotellaceae, while the dominant families in the rumen of lactating cows were Succinivibrionaceae and Prevotellaceae. The relative abundances of Prevotellaceae, Ruminococcaceae, Rikenellaceae, BS11 bacteria, RF16 bacteria, SR1 bacteria, Gastranaerophilales, Christensenellaceae, TM7 bacteria, RF9 bacteria, and Fibrobacteraceae in the rumen of prepartum cows were significantly higher than those in lactating cows ($P<0.05$); while those of Succinivibrionaceae, Lachnospiraceae, and Veillonellaceae were significantly lower than those in lactating cows ($P<0.05$). In conclusion, as cows transitioned from the prepartum period to lactation, rumen bacterial diversity decreased significantly; the differences in

rumen bacterial composition among the various lactation stages were relatively small.

Full Text

Rumen Bacterial Diversity in Holstein Dairy Cows at Different Physiological Stages

LI Zijian[1], LI Dabiao[1]*, GAO Min[2], WANG Dian[3], LAN Rubing[3]

[1]College of Animal Science, Inner Mongolia Agricultural University, Hohhot 010018, China
[2]Animal Nutrition Institute, Agriculture and Animal Husbandry Academy of Inner Mongolia, Hohhot 010031, China
[3]Inner Mongolia Youran Co. Ltd., Hohhot 010107, China

Corresponding author: LI Dabiao, E-mail: dkyldb@imau.edu.cn

Abstract

This study aimed to investigate rumen bacterial diversity in Holstein dairy cows at different physiological stages. Sixteen multiparous Holstein cows were selected, with four cows each in the prepartum period (7–15 days before calving), early lactation (30–55 days postpartum), mid-lactation (82–105 days postpartum), and late lactation (153–181 days postpartum). Rumen fluid was collected to determine fermentation parameters and extract microbial DNA, and the Illumina Miseq PE300 platform was used to characterize rumen bacterial composition. The results showed that: (1) Rumen microbial protein, acetate, butyrate, total volatile fatty acid concentrations, and the acetate/propionate ratio were significantly higher in prepartum cows than in lactating cows ($P < 0.05$), while rumen propionate concentration was significantly lower ($P < 0.05$). (2) At the phylum level, Bacteroidetes and Firmicutes were dominant in prepartum cows, whereas Bacteroidetes, Proteobacteria, and Firmicutes dominated in lactating cows. The relative abundances of Bacteroidetes, SR1, Cyanobacteria, Tenericutes, TM7, and Fibrobacteres were significantly higher in prepartum cows ($P < 0.05$), while Proteobacteria abundance was significantly lower ($P < 0.05$). No significant differences were observed among lactation stages ($P > 0.05$). (3) At the family level, Prevotellaceae was dominant in prepartum cows, while Succinivibrionaceae and Prevotellaceae dominated in lactating cows. The relative abundances of Prevotellaceae, Ruminococcaceae, Rikenellaceae, BS11, RF16, SR1, Gastranaerophilales, Christensenellaceae, TM7, RF9, and Fibrobacteraceae were significantly higher in prepartum cows ($P < 0.05$), whereas Succinivibrionaceae, Lachnospiraceae, and Veillonellaceae were significantly lower ($P < 0.05$). In conclusion, rumen bacterial diversity decreased significantly when cows transitioned from the prepartum period to lactation, with minimal differences in bacterial composition among lactation stages.

Keywords: Holstein dairy cows; physiological stage; rumen bacteria; diversity

Introduction

The rumen harbors a vast and diverse microbial community that degrades dietary fiber into nutrients utilizable by the host. Previous studies have shown that rumen microorganisms in adult cattle primarily consist of bacteria, fungi, protozoa, and archaea, with bacteria accounting for 95% of the total population at concentrations reaching 10^{11} cells per milliliter of rumen fluid [**?**, **?**]. These bacteria play a crucial role in digesting and converting feed into short-chain fatty acids and microbial protein (MCP) [**?**]. Rumen bacterial composition may shift across different physiological stages, and understanding these changes is important for elucidating the relationship between rumen metabolism and animal performance. Guo [**?**] reported that in goats from 7 days to 3 months of age, the relative abundance of Bacteroidetes gradually increased then decreased, stabilizing after 6 months, while Proteobacteria showed the opposite trend. Jami et al. [**?**] found significant differences in rumen microbiota composition between newborn calves and adult cattle, with bacterial diversity increasing with age. Gao et al. [**?**] investigated intestinal microbiota structure in Holstein cattle during nursing, weaning, growing, youth, lactation, and dry periods, finding that Bifidobacterium abundance was significantly higher during lactation than other stages at the phylum level. However, Dong [**?**] concluded that under the same dietary conditions, different physiological stages (lactation vs. dry period) had no significant effect on rumen microbial flora. This study employed high-throughput sequencing to analyze rumen bacterial diversity in Holstein cows at different physiological stages, providing a foundation for understanding the relationship between rumen microbiota and lactation performance.

Materials and Methods

1.1 Experimental Animals

The experimental animals were selected from a demonstration farm in Hohhot, Inner Mongolia. Sixteen healthy multiparous Holstein cows were chosen, with four cows each in the prepartum period (7–15 days before calving), early lactation (30–55 days postpartum), mid-lactation (82–105 days postpartum), and late lactation (153–181 days postpartum). All lactating cows produced more than 30 kg of milk daily. The cows were 4–5 years old with 3–4 parities. Within each group, body condition and calving dates were similar.

1.2 Experimental Design

This experiment used a single-factor completely randomized design. The four groups were designated as Group I (prepartum), Group II (early lactation), Group III (mid-lactation), and Group IV (late lactation). Cows had free access to water and were fed in groups three times daily at 06:00, 14:00, and 21:00. Diets for each stage were provided by the farm and fed as total mixed rations (TMR). Diet composition and nutrient levels are shown in Table 1 .

1.3 Sample Collection

In mid-June 2017, rumen digesta were collected 6 hours after morning feeding using an oral sampler. The samples were filtered through four layers of cheesecloth, and the filtrate was retained for determination of rumen fermentation parameters and bacterial diversity analysis.

1.4 Determination of Rumen Fermentation Parameters

Rumen pH was measured immediately after collection using a PHS-3S high-precision pH meter. Ammonia nitrogen (NH -N) concentration was determined according to the method of Feng et al. [**?**]. Microbial protein (MCP) concentration was measured using the Bradford method [**?**]. Volatile fatty acid (VFA) concentrations were determined following the method of Qin [**?**].

1.5 Determination of Rumen Bacterial Diversity

1.5.1 DNA Extraction and PCR Amplification Total DNA was extracted using the E.Z.N.A.® soil kit (Omega Bio-tek, USA) according to the manufacturer' s instructions. DNA concentration and purity were assessed using a NanoDrop 2000, and extraction quality was verified by 1% agarose gel electrophoresis. The V3–V4 variable region was amplified using 338F/806R primers. The PCR program consisted of initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 10 min (ABI GeneAmp® 9700). The 20 µL reaction mixture contained 4 µL 5×FastPfu buffer, 2 µL 2.5 mmol/L dNTPs, 0.8 µL primers (5 µmol/L), 0.4 µL FastPfu polymerase, and 10 ng DNA template. Primer information is shown in Table 2 .

1.5.2 Illumina Miseq PE300 Platform Sequencing PCR products were recovered using 2% agarose gel and purified with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA), followed by Tris-HCl elution and verification by 2% agarose electrophoresis. Quantification was performed using QuantiFluor™-ST (Promega, USA). Purified amplicons were used to construct PE 2×300 libraries according to the Illumina MiSeq PE300 platform (Illumina, USA) standard protocol, and sequencing was performed on the Illumina Miseq PE300 platform (Shanghai Majorbio Bio-Pharm Technology Co., Ltd.).

1.5.3 Data Processing Raw sequences were quality-controlled using Trimmomatic software and assembled using FLASH software. Operational taxonomic units (OTUs) were clustered at 97% similarity using UPARSE software. Chimeras were removed using UCHIME software. Taxonomic classification was performed using the RDP classifier against the Silva database (SSU123) with a 70% confidence threshold.

1.6 Statistical Analysis

Data were analyzed using the GLM procedure in SAS 9.0 software for one-way ANOVA. Multiple comparisons were performed using Duncan' s method, with $P < 0.05$ considered statistically significant.

Results

2.1 Rumen Fermentation Parameters in Dairy Cows at Different Physiological Stages

Rumen fermentation parameters are presented in Table 3 . Rumen pH in Group I was significantly higher than in Groups III and IV ($P < 0.05$) but did not differ from Group II ($P > 0.05$), with no significant differences among Groups II, III, and IV ($P > 0.05$). NH -N concentration did not differ significantly among groups ($P > 0.05$). MCP concentration in Group I was significantly higher than in Groups II, III, and IV ($P < 0.05$), and Group II was significantly higher than Groups III and IV ($P < 0.05$), with no difference between Groups III and IV ($P > 0.05$). Acetate, butyrate, total VFA (TVFA) concentrations, and acetate/propionate ratio in Group I were significantly higher than in Groups II, III, and IV ($P < 0.05$). Conversely, propionate concentration in Group I was significantly lower than in the lactation groups ($P < 0.05$).

Among lactation stages, acetate concentration and acetate/propionate ratio in Group IV were significantly higher than in Groups II and III ($P < 0.05$), with no difference between Groups II and III ($P > 0.05$). Propionate concentration in Group III was significantly higher than in Group IV ($P < 0.05$) but did not differ from Group II ($P > 0.05$). Butyrate concentration in Group III was significantly lower than in Groups II and IV ($P < 0.05$), with no difference between Groups II and IV ($P > 0.05$). TVFA concentration did not differ significantly among Groups II, III, and IV ($P > 0.05$).

2.2 Quality Control and Diversity Indices of Rumen Bacterial High-Throughput Sequencing

After quality control, the number of clean reads, OTUs, and diversity indices are shown in Table 4 . A total of 871,196 high-quality bacterial 16S rRNA gene sequences were obtained from 16 samples, with an average of 54,450 clean reads per sample and no significant differences among groups ($P > 0.05$). The average OTU number was 1,039, with Group I significantly higher than Groups II, III, and IV ($P < 0.05$), Group II significantly higher than Groups III and IV ($P < 0.05$), and no difference between Groups III and IV ($P > 0.05$). Sequencing coverage exceeded 0.99 in all groups, meeting analytical requirements. Ace and Chao1 indices in Group I were significantly higher than in Groups II, III, and IV ($P < 0.05$), with Group II significantly higher than Groups III and IV ($P < 0.05$), and Group III significantly higher than Group IV ($P < 0.05$). Shannon index followed the same pattern as OTU number: Group I > Group II > Groups

III and IV ($P < 0.05$), with no difference between Groups III and IV ($P > 0.05$). Simpson index in Group I was significantly lower than in Groups II, III, and IV ($P < 0.05$), with no differences among the latter three groups ($P > 0.05$).

2.3 Effects of Physiological Stage on Rumen Bacterial Flora

At the phylum level (Table 5), bacterial composition differed among groups. The dominant phyla in Group I were Bacteroidetes and Firmicutes, while Groups II, III, and IV were dominated by Bacteroidetes, Proteobacteria, and Firmicutes. The relative abundances of Bacteroidetes, SR1, Cyanobacteria, Tenericutes, TM7, and Fibrobacteres in Group I were significantly higher than in Groups II, III, and IV ($P < 0.05$), with no significant differences among the lactation groups ($P > 0.05$). Conversely, Proteobacteria abundance in Group I was significantly lower than in Groups II, III, and IV ($P < 0.05$). Firmicutes abundance did not differ significantly among groups ($P > 0.05$).

At the family level (Table 6), bacterial composition also varied among groups. Prevotellaceae was dominant in Group I, while Succinivibrionaceae and Prevotellaceae dominated in Groups II, III, and IV. The relative abundances of Prevotellaceae, Ruminococcaceae, Rikenellaceae, BS11, RF16, SR1, Gastranaerophilales, Christensenellaceae, TM7, RF9, and Fibrobacteraceae in Group I were significantly higher than in Groups II, III, and IV ($P < 0.05$). In contrast, Succinivibrionaceae, Lachnospiraceae, and Veillonellaceae in Group I were significantly lower than in the lactation groups ($P < 0.05$). Erysipelotrichaceae abundance in Group I was significantly lower than in Groups III and IV ($P < 0.05$), while S24-7 abundance was significantly higher than in Groups III and IV ($P < 0.05$). Acidaminococcaceae abundance did not differ significantly among groups ($P > 0.05$).

Among lactation stages, no significant differences were observed for Prevotellaceae, Succinivibrionaceae, Lachnospiraceae, Ruminococcaceae, Veillonellaceae, RF16, SR1, Christensenellaceae, TM7, or RF9 ($P > 0.05$). Rikenellaceae, S24-7, BS11, and Fibrobacteraceae abundances in Group II were significantly higher than in Groups III and IV ($P < 0.05$), with no difference between the latter two groups ($P > 0.05$). Erysipelotrichaceae abundance in Group II was significantly lower than in Groups III and IV ($P < 0.05$). Gastranaerophilales abundance in Group II did not differ from Groups III and IV ($P > 0.05$), but Group IV was significantly higher than Group III ($P < 0.05$).

Figure 1 [Figure 1: see original paper] shows the partial least squares discriminant analysis (PLS-DA) at the OTU level, where spatial distance between sample points represents dissimilarity. Principal components COMP1 and COMP2 explained 42.6% and 7.35% of sample variation, respectively. Group I was separated from the other three groups by COMP1, Group II was separated from Groups III and IV by COMP2, and Groups III and IV showed some similarity.

Discussion

Previous studies have demonstrated that rumen bacterial diversity and abundance change across physiological stages in ruminants. Guo [?] investigated rumen bacterial abundance and diversity in goats from 7 days to 1.5 years of age, finding that OTU number, Chao1 index, and Shannon index were highest at 7 days (indicating maximum species richness and diversity), lowest at 3 months for Shannon index, and lowest at 1 year for Chao1 index. In contrast, Jami et al. [?] found that OTU number and Shannon index gradually increased in Holstein cattle from 1 day to 2 years of age. Our diversity analysis revealed that OTU number, Ace index, Chao1 index, and Shannon index were significantly higher in prepartum cows than in lactating cows, while Simpson index was significantly lower. These differences may be attributed to variations in diet composition and nutrient levels between the prepartum period and lactation. During lactation, dietary composition and nutrient levels changed minimally among stages, particularly between early and mid-lactation, where NEL (6.85 vs. 6.92 MJ/kg), CP (16.91% vs. 17.38%), and NDF (28.91% vs. 28.69%) were similar. However, rumen bacterial richness (Ace and Chao1 indices) and diversity (Shannon index) were significantly higher in early lactation than in mid-lactation, suggesting that hormonal changes during different lactation stages significantly influence rumen microbiota. Furthermore, comprehensive analysis of nutrient levels and MCP concentrations across physiological stages showed that despite similar nutrient levels between early and mid-lactation, MCP concentration was significantly higher in early lactation, indicating that lactation stage itself affects rumen microbial communities.

Rumen bacterial composition differs substantially across physiological stages in ruminants. Guo [?] reported that in goats, Bacteroidetes abundance first increased then decreased with age, while Proteobacteria showed the opposite trend, and Firmicutes remained stable. Jami et al. [?] noted that Bacteroidetes abundance gradually increased while Proteobacteria decreased in Holstein cattle from 1 day to 2 years, with Firmicutes first decreasing then increasing. During pregnancy, uterine growth affects gastric and intestinal motility, causing gastrointestinal dysfunction [?] and altering microbial composition. Studies have shown that gut bacterial diversity decreases during pregnancy [?, ?, ?]. Kong et al. [?] found that in Huanjiang mini-pigs, the relative abundances of Corynebacterium, Acinetobacter, and Allobaculum decreased significantly during pregnancy. However, some evidence suggests minimal differences in gut bacterial composition between specific physiological stages. Koren et al. [?] reported that gut bacterial composition in women during the first trimester was similar to the non-pregnant state, and Jost et al. [?] found minimal differences between late pregnancy and early lactation in women. Our study identified Bacteroidetes, Proteobacteria, and Firmicutes as dominant rumen phyla across physiological stages, with Firmicutes abundance remaining stable, consistent with Guo [?]. Bacterial composition was similar across lactation stages, aligning with Koren et al. [?].

Diet composition directly influences rumen bacterial composition. Previous research has shown that increasing dietary concentrate proportion reduces fiber-degrading bacteria and fungi, and the resulting low rumen pH inhibits fiber-degrading bacterial growth [**?**, **?**]. Mao et al. [**?**] reported that cows fed high-concentrate diets had higher proportions of Firmicutes and Actinobacteria but lower Proteobacteria and Bacteroidetes. Kocherginskaya et al. [**?**] found that Proteobacteria dominated under high-concentrate feeding conditions. Lin et al. [**?**] observed that increasing concentrate proportion in buffalo diets elevated Firmicutes and Proteobacteria while reducing Bacteroidetes; at the family level, Prevotellaceae, Lachnospiraceae, and Ruminococcaceae increased with concentrate proportion. In our study, Proteobacteria was the most abundant phylum during lactation, consistent with previous reports. The significantly higher Proteobacteria and lower Bacteroidetes abundances in lactating versus prepartum cows indicate that high-concentrate diets during lactation favor Proteobacteria proliferation while inhibiting Bacteroidetes. At the family level, Prevotellaceae was dominant across all stages but was significantly more abundant in the prepartum period. This may be because Prevotellaceae contains numerous highly active hemicellulose-degrading bacteria with strong protein and carbohydrate fermentation capacity [**?**], and its higher abundance in high-forage diets suggests a close association with fiber digestion. Conversely, the significantly higher Succinivibrionaceae abundance during lactation may be due to the presence of numerous starch- and protein-degrading bacteria that proliferate under high-concentrate conditions.

In conclusion, rumen bacterial diversity decreased significantly when cows transitioned from the prepartum period to lactation, with minimal differences in bacterial composition among lactation stages.

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