Effects of Dietary Concentrate-to-Forage Ratio on Rumen Bacterial and Methanogen Microbiota in Lactating Water Buffaloes (Postprint)

Authors: Lin Bo, Liang Xin, Li Lili, Wei Shengju, Li Ping, Zou Caixia

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Abstract

This experiment aimed to investigate the effects of dietary concentrate-to-forage ratio on rumen bacterial and methanogenic archaeal communities in lactating water buffaloes. Fifteen healthy crossbred lactating water buffaloes were selected and randomly allocated into 3 groups (n=5) based on similar body weight and feed intake, and fed mixed diets with concentrate-to-forage ratios of 0:100 (all-forage group), 35:65 (low-concentrate group), and 50:50 (medium-concentrate group). The trial lasted for 40 days, with the first 10 days as an adaptation period. On day 1 post-experiment, rumen fluid was collected via oral intubation to extract microbial DNA, and the Illumina Miseq PE250 platform was employed to characterize rumen bacterial and methanogenic archaeal community composition. The results demonstrated: 1) At the phylum level, Bacteroidetes (45%~60%), Firmicutes (13%~27%), and Proteobacteria (13%~18%) were the predominant bacterial phyla in water buffalo rumen. Compared with the low-concentrate and medium-concentrate groups, the all-forage group elevated the proportions of Bacteroidetes, Flavobacteriota, and SR1 bacteria, while reducing the proportions of Firmicutes, Verrucomicrobiota, Actinobacteriota, and Chloroflexi bacteria. At the family level, Prevotellaceae (15%~32%) and Flavobacteriaceae (8%~21%) were the dominant bacterial families. Compared with the medium-concentrate group, the all-forage and low-concentrate groups increased the proportion of Prevotellaceae while decreasing the proportions of Flavobacteriaceae, Lachnospiraceae, Ruminococcaceae, Coriobacteriaceae, and Bifidobacteriaceae. The medium-concentrate group exhibited higher rumen bacterial diversity relative to the all-forage group. 2) Within the water buffalo rumen, over 90% of methanogenic archaea were attributed to the genus Methanobrevibacter, followed by Thermoplasma. Alterations in dietary concentrate-to-forage ratio did not influence the dominant status of Methanobrevibacter in the rumen. Collectively, the all-forage diet tended to

enhance the proportion of fiber-degrading associated bacteria in water buffalo rumen, yet diminished rumen bacterial diversity; variations in dietary concentrate-to-forage ratio exerted no significant effect on the genus-level composition of rumen methanogenic archaea in water buffaloes.

Full Text

Dietary Forage to Concentrate Ratio Affects Ruminal Bacterial and Methanogen Community Composition of Lactating Water Buffaloes

LIN Bo[1], LIANG Xin[2], LI Lili[2], WEI Shengju[2], LI Ping[1], ZOU Caixia[1,2*]

(1. College of Animal Science and Technology, Guangxi University, Nanning 530004, China;
2. Key Laboratory of Buffalo Genetics, Breeding and Reproduction of Ministry of Agriculture, Key Laboratory of Buffalo Genetics, Breeding and Reproduction of Guangxi, Buffalo Research Institute, Chinese Academy of Agricultural Sciences, Nanning 530001, China)

Abstract: This study investigated the effects of dietary forage to concentrate ratio on ruminal bacterial and methanogen community composition in lactating water buffaloes. Fifteen healthy hybrid lactating water buffaloes were selected and randomly assigned to three groups (n = 5) based on similar body weight and feed intake. The groups were fed mixed diets with concentrate to forage ratios of 0:100 (all-forage group), 35:65 (low-concentrate group), and 50:50 (medium-concentrate group). The experiment lasted 40 days, including a 10-day preliminary period. On day 1 following the experiment, rumen fluid was collected orally, microbial DNA was extracted, and ruminal bacterial and methanogen communities were analyzed using the Illumina MiSeq PE250 platform. The results showed: (1) At the phylum level, Bacteroidetes (45%–60%), Firmicutes (13%–27%), and Proteobacteria (13%–18%) were the dominant bacterial groups in buffalo rumen. Compared with the low- and medium-concentrate groups, the all-forage group increased the proportions of Bacteroidetes, Fibrobacteres, and SR1 bacteria while decreasing the proportions of Firmicutes, Verrucomicrobia, Actinobacteria, and Chloroflexi. At the family level, Prevotellaceae (15%–32%) and Fibrobacteraceae (8%–21%) were the dominant taxa. Compared with the medium-concentrate group, the all-forage and low-concentrate groups showed higher proportions of Prevotellaceae but lower proportions of Fibrobacteraceae, Lachnospiraceae, Ruminococcaceae, Coriobacteriaceae, and Bifidobacteriaceae. The medium-concentrate group exhibited greater bacterial diversity than the all-forage group. (2) Over 90% of ruminal methanogens in water buffaloes belonged to the genus *Methanobrevibacter*, followed by *Thermoplasma*. Dietary concentrate to forage ratio did not affect the dominant status of *Methanobrevibacter* in the rumen. In conclusion, an all-forage diet tended to increase the proportion of fiber-degrading bacteria but reduced ruminal bacterial diversity, while dietary concentrate to forage ratio had no significant effect on ruminal

methanogen composition at the genus level.

Keywords: water buffalo; concentrate to forage ratio; rumen; bacterial community; methanogen community

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Water buffalo is a typical roughage-fed ruminant, yet its rumen fermentation characteristics and microbial community composition remain poorly understood. Dietary forage to concentrate ratio is a primary determinant of rumen fermentation, and altering this ratio to study microbial community responses represents an important approach to elucidating ruminant rumen microbiota characteristics [1-2]. Mao et al. [3] reported that in dairy cows fed high-concentrate diets, the proportions of Firmicutes and Actinobacteria were higher, while Proteobacteria and Bacteroidetes were lower. Pitta et al. [4] found that in buffalo fed all-forage diets, *Ruminococcus* and Fibrobacteres were more abundant, whereas the 50:50 concentrate to forage ratio diet increased *Prevotella* proportions, suggesting that *Ruminococcus* and Fibrobacteres are important fiber-digesting bacteria. Our previous research also demonstrated that increasing the dietary concentrate to forage ratio reduced the populations of *Fibrobacter succinogenes* and *Butyrivibrio* [5]. Under high-roughage conditions, fiber-degrading bacteria such as *Butyrivibrio* and *Prevotella ruminicola* dominate, while high-concentrate conditions favor starch-loving bacteria including *Selenomonas ruminantium*, *Peptostreptococcus*, *Lactobacillus*, and *Ruminobacter amylophilus* [6-7]. However, few studies have examined changes in buffalo rumen microbiota under different concentrate to forage ratios, limiting our understanding of buffalo rumen microbial characteristics. Therefore, this study employed high-throughput sequencing technology to investigate ruminal bacterial and methanogen communities in buffalo fed different concentrate to forage ratios, aiming to clarify how dietary composition affects these communities and provide a theoretical basis for understanding buffalo rumen function.

1.1 Experimental Animals

The experiment was conducted at the Buffalo Breeding Demonstration Base of the Buffalo Research Institute in Guangxi. Fifteen healthy hybrid lactating water buffaloes [Guangxi local buffalo × (Nili-Ravi × Murrah)] were selected and randomly divided into three groups (n = 5) based on similar body weight and feed intake. Experimental animals were fed individually in separate stalls by designated personnel, following a roughage-before-concentrate feeding sequence. After feeding, buffaloes were allowed free movement and water access in exercise areas.

1.2 Experimental Design and Management

A single-factor design was employed. Three groups were fed mixed diets with concentrate to forage ratios of 0:100 (all-forage group), 35:65 (low-concentrate group), and 50:50 (medium-concentrate group). The total experimental period

was 40 days, including a 10-day preliminary period during which the concentrate to forage ratio was gradually transitioned to the target levels before the formal 30-day experimental period. Concentrate composition, feeding amounts of concentrate and forage, and nutrient intake levels are presented in Table 1 .

1.3 Rumen Fluid Collection

On day 1 following the experimental period, 100 mL of rumen fluid was collected from each buffalo via oral intubation. Samples were pooled by group, filtered through four layers of cheesecloth, and 50 mL of filtrate was lyophilized (Christ Alpha 1-4 LD plus, Christ Alpha, Germany) for subsequent DNA extraction.

1.4.1 Rumen Fluid DNA Extraction

DNA extraction followed the method described by Rius et al. [8]. Briefly, 25 mg of lyophilized rumen fluid was placed in a centrifuge tube containing 0.7 g zirconia beads (0.1 mm), 200 L of 20% sodium dodecyl sulfate (SDS), 282 L of QIAquick Buffer A, 268 L of QIAquick Buffer PB (Qiagen, Germany), and 550 L of saturated phenol/chloroform/isoamyl alcohol solution (25:24:1). After sealing, the tube was homogenized in a Mini-Beadbeater-16 (Biospec, USA) for 2 min, then centrifuged at 16,000 × g for 20 min at 4°C. The supernatant (500 L) was mixed with 650 L of Buffer PB and purified using the QIAquick PCR purification kit (Qiagen, Germany) to obtain DNA samples.

1.4.2 Sequencing Analysis of Buffalo Rumen Microbial Communities

Amplification and processing of 16S rRNA gene fragments for ruminal bacteria and methanogens followed the protocol of Kittelmann et al. [9]. PCR primers consisted of Illumina MiSeq PE250 sequencing adapters, a 2-base linker, and sample barcodes. Bacterial primers were 515f/806r, and methanogen primers were 519f/915r (Table 2). PCR amplification was performed using Promega PCR mix in a 50 L volume, with two replicate tubes per sample for each microbial group. The thermocycling program included initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s. After amplification, replicate PCR products from the same sample were pooled, electrophoresed, and gel-purified using a gel extraction kit (Tiangen Biotech). DNA concentration was precisely measured using a NanoDrop 2000 spectrophotometer (Thermo, Germany) to 0.1 ng/ L accuracy. Purified PCR products from each sample were then mixed at a bacteria:methanogen concentration ratio of 5:1. Finally, all samples were pooled and sent to Guangzhou Haiji Biotechnology Co., Ltd. for sequencing on the MiSeq PE250 platform.

1.4.3 Sequence Clustering and Annotation

Sequencing data were analyzed using QIIME 1.5. After quality control to remove adapter contamination, low-complexity, and low-quality reads, clean reads

were assembled and primers were trimmed for subsequent analysis [10]. Operational taxonomic units (OTUs) were annotated and clustered at 97% sequence similarity against reference databases. OTU clustering and annotation were performed by comparing with bacterial and archaeal sequences in the Ribosomal Database Project (RDP) database. Bacteria were classified to phylum and family levels, while methanogens were classified to genus level.

1.5 Statistical Analysis

All data were analyzed using the GLM procedure of SAS 8.02 software for one-way ANOVA. Duncan' s multiple range test was used for post-hoc comparisons, with $P < 0.05$ considered statistically significant.

2.1 High-Throughput Sequencing Quality Control and Diversity Analysis

After quality control, high-throughput sequencing data for ruminal bacteria and methanogens are summarized in Table 3 . For ruminal bacteria, the average number of clean reads at the species level was 26,613, with an average of 969 OTUs and sequencing coverage of 0.89–0.90. The average Shannon diversity index was 7.58, and the average Chao1 diversity index was 1,233. Except for the significantly higher Shannon index in the medium-concentrate group compared with the all-forage group ($P < 0.05$), no significant differences were observed among groups for bacterial parameters ($P > 0.05$). For ruminal methanogens, the average number of clean reads was 8,607, with 136 OTUs and sequencing coverage of 0.74–0.93. The average Shannon diversity index was 1.25, and the average Chao1 diversity index was 237. No significant differences were detected among groups for any methanogen parameters ($P > 0.05$).

2.2 Effects of Dietary Forage to Concentrate Ratio on Ruminal Bacterial Community

Ruminal bacterial composition at phylum and family levels under different dietary ratios is presented in Table 4 . At the phylum level, the dominant bacterial phyla in buffalo rumen were Bacteroidetes, Firmicutes, and Proteobacteria. The all-forage group showed significantly higher proportions of Bacteroidetes, Fibrobacteres, and SR1 bacteria compared with the low- and medium-concentrate groups ($P < 0.05$), but significantly lower proportions of Firmicutes, Verrucomicrobia, Actinobacteria, and Chloroflexi compared with the medium-concentrate group ($P < 0.05$).

At the family level, Prevotellaceae and Fibrobacteraceae were the dominant taxa. The all-forage and low-concentrate groups exhibited significantly higher proportions of Prevotellaceae but lower proportions of Fibrobacteraceae, Lachnospiraceae, Coriobacteriaceae, and Bifidobacteriaceae compared with the medium-concentrate group ($P < 0.05$). The proportion of Ruminococcaceae in

the all-forage group was also significantly lower than in the medium-concentrate group ($P < 0.05$).

Bar charts and heat maps illustrating ruminal bacterial composition at the family level are shown in Figure 1 [Figure 1: see original paper] and Figure 2 [Figure 2: see original paper], respectively. Although bacterial composition was generally similar among samples, distinct differences in proportions were observed between groups. Notably, the all-forage group showed higher proportions of Prevotellaceae, Streptococcaceae, and Aeromonadaceae, whereas the medium-concentrate group had higher proportions of Fibrobacteraceae, Ruminococcaceae, Lachnospiraceae, Bifidobacteriaceae, and Anaerolineaceae.

2.3 Effects of Dietary Forage to Concentrate Ratio on Ruminal Methanogen Community in Lactating Water Buffaloes

The effects of dietary forage to concentrate ratio on ruminal methanogen composition at the genus level are shown in Figure 3 [Figure 3: see original paper]. Six methanogen genera were identified, with the dominant taxa being *Methanobrevibacter*, *Thermoplasma*, and *Methanosphaera*, which accounted for approximately 95% of total methanogens. The proportion of *Methanobrevibacter* exceeded 90% in all groups. Dietary concentrate to forage ratio did not affect the relative composition of methanogen genera.

3.1 Effects of Dietary Forage to Concentrate Ratio on Ruminal Bacterial Community

Dietary forage to concentrate ratio is a primary determinant of rumen fermentation. Our previous research indicated that concentrate to forage ratio affects ruminal fermentation parameters in water buffaloes [5], likely through modulation of ruminal microbial communities and functions. Studies have shown that increasing dietary concentrate proportion gradually reduces fiber-degrading bacterial and fungal populations, and when concentrate reaches certain levels, low ruminal pH eventually inhibits fiber-degrading bacteria [7,11]. Kocherginskaya et al. [12] found that high-concentrate diets supported greater bacterial numbers and diversity, with Proteobacteria as the dominant phylum, whereas high-roughage diets favored low G+C Gram-positive bacteria. Wetzels et al. [13] reported that compared with 30% concentrate, 0% and 60% concentrate diets resulted in higher abundances of Firmicutes and SR1 on the rumen epithelium. Mao et al. [3] demonstrated that in dairy cows with subacute ruminal acidosis induced by high-concentrate feeding, Proteobacteria and Bacteroidetes proportions decreased significantly while Firmicutes and Actinobacteria increased. At the genus level, *Prevotella*, *Treponema*, and *Anaeroplasma* were less abundant in high-concentrate diets, whereas *Ruminococcus*, *Atopobium*, and Clostridiales were more abundant.

Our results showed that the 50:50 concentrate group had higher proportions of Firmicutes, Proteobacteria, and Actinobacteria at the phylum level, while the

all-forage group had higher proportions of Bacteroidetes, Fibrobacteres, and SR1 bacteria. These findings align with studies in dairy cows and goats [3,13], confirming that high-concentrate diets increase Firmicutes and Proteobacteria while decreasing Bacteroidetes. At the family level, the all-forage group showed the highest proportion of Prevotellaceae (31.6%), while the medium-concentrate group had the highest proportion of Fibrobacteraceae (20.5%). Prevotellaceae, the predominant family within Bacteroidetes, contains numerous highly active hemicellulose-degrading bacteria and is widely distributed in the rumen and hindgut, responsible for protein and carbohydrate degradation [14]. Its higher abundance in high-roughage diets indicates a close association with fiber digestion.

However, Pitta et al. [4] reported different results in buffalo fed varying concentrate to forage ratios, finding higher abundances of *Ruminococcus* and Fibrobacteres in the all-forage group and *Prevotella* as the dominant genus in the 50:50 group. These discrepancies may stem from differences in experimental design: our study fed three groups simultaneously, whereas Pitta et al. used a sequential feeding design (all-forage → low-concentrate → medium-concentrate) within the same animals. Additionally, primer selection may contribute, as different primers can yield different bacterial community profiles [4], highlighting the importance of appropriate primer choice.

Our study found that the Shannon diversity index was significantly higher in the 50:50 group compared with the all-forage group, suggesting that balanced concentrate feeding tends to increase ruminal bacterial diversity. This result is consistent with Sun et al. [15], who reported increased bacterial diversity in goats when diets changed from all-forage to 30:70 concentrate to forage ratio. Kocherginskaya et al. [12] also observed greater bacterial numbers and diversity in goats fed high-concentrate diets. The higher bacterial diversity in our 50:50 group may be attributed to the coexistence of both starch- and fiber-degrading bacteria, whereas the all-forage group was dominated by specialized fiber-degrading bacteria with lower overall diversity. Therefore, feeding buffalo a balanced concentrate to forage diet helps maintain ruminal bacterial diversity and stability.

3.2 Effects of Dietary Forage to Concentrate Ratio on Ruminal Methanogen Community

Dietary forage to concentrate ratio significantly affects methane production in ruminants, with high-roughage diets producing more methane and supporting greater methanogen numbers than high-concentrate diets. However, few studies have examined effects on methanogen community composition. Existing research indicates that ruminal methanogens are dominated by *Methanobrevibacter*, followed by *Methanosphaera* and *Thermoplasma* [16]. Several Indian studies reported *Methanomicrobium* as the dominant methanogen in buffalo rumen, suggesting a distinct community composition compared with other ruminants [17-19]. Our study identified seven methanogen genera, with *Methanobrevibac-*

ter accounting for over 90% in all groups, consistent with Franzolin et al. [20] and with reports from other ruminants. The discrepancy with Indian studies may be related to geographic location, buffalo breed [21], or primer selection, as different methanogen primers can produce different community profiles [22].

Our findings indicate that dietary concentrate to forage ratio did not affect the dominant status of *Methanobrevibacter* in buffalo rumen. Therefore, the impact of dietary ratio on methane production may be primarily attributed to changes in methanogen abundance rather than community composition. Whether dietary shifts affect *Methanobrevibacter* species composition requires further investigation. Previous studies suggest that ruminal methanogen composition exhibits individual specificity [23] and is relatively resistant to changes in environment and diet.

In conclusion, an all-forage diet tends to increase the proportion of fiber-degrading bacteria but reduces ruminal bacterial diversity, while dietary concentrate to forage ratio has no significant effect on ruminal methanogen composition at the genus level.

References:

[1] Narenhua, Dong Hongmin, Chen Yongxing, et al. Effects of dietary concentrate to forage ratio on rumen fermentation characteristics [J]. Chinese Journal of Animal Science, 2011, 47(9): 49–54.
[2] Petri R M, Forster R J, Yang W, et al. Characterization of rumen bacterial diversity and fermentation parameters in concentrate fed cattle with and without forage [J]. Journal of Applied Microbiology, 2012, 112(6): 1152–1162.
[3] Mao S Y, Zhang R Y, Wang D S, et al. Impact of subacute ruminal acidosis (SARA) adaptation on rumen microbiota in dairy cattle using pyrosequencing [J]. Anaerobe, 2013, 24: 12–19.
[4] Pitta D W, Kumar S, Veicchiarelli B, et al. Bacterial diversity associated with feeding dry forage at different dietary concentrations in the rumen contents of Mehshana buffalo (*Bubalus bubalis*) using 16S pyrotags [J]. Anaerobe, 2014, 25: 31–41.
[5] Li P, Liang X, Wei S J, et al. Effects of dietary concentrate to forage ratio on rumen fermentation parameters and fiber-degrading bacterial populations in buffalo heifers [J]. Animal Husbandry and Veterinary Medicine, 2015, 47(7): 36–40.
[6] Yi Xuewu, Zhang Shirui. Research progress on dietary concentrate to forage ratio in dairy cows [J]. China Herbivore Science, 2006, 26(3): 51–55.
[7] Sun Yunzhang, Mao Shengyong, Yao Wen, et al. Co-culture fermentation characteristics and microbial community shifts of rumen fungi and fiber-degrading bacteria under different concentrate to forage ratios [J]. Acta Microbiologica Sinica, 2006, 46(3): 422–426.
[8] Rius A G, Kittelmann S, Macdonald K A, et al. Nitrogen metabolism and rumen microbial enumeration in lactating cows with divergent residual feed intake fed high-digestibility pasture [J]. Journal of Dairy Science, 2012, 95(9):

5024-5034.
[9] Kittelmann S, Seedorf H, Walters W A, et al. Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities [J]. PLoS One, 2013, 8(2): e47879.
[10] Caporaso J G, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data [J]. Nature Methods, 2010, 7(5): 335-336.
[11] Wang Hairong. Effects of different dietary concentrate to forage ratios and nitrogen sources on rumen fiber-degrading bacterial populations and fiber degradation in sheep [D]. PhD Thesis. Hohhot: Inner Mongolia Agricultural University, 2006: 2-3.
[12] Kocherginskaya S A, Aminov R I, White B A. Analysis of the rumen bacterial diversity under two different diet conditions using denaturing gradient gel electrophoresis, random sequencing, and statistical ecology approaches [J]. Anaerobe, 2001, 7(3): 119-134.
[13] Wetzels S U, Mann E, Metzler-Zebeli B U, et al. Pyrosequencing reveals shifts in the bacterial epimural community relative to dietary concentrate amount in goats [J]. Journal of Dairy Science, 2015, 98(8): 5572-5587.
[14] Matsui H, Ogata K, Tajima K, et al. Phenotypic characterization of polysaccharidases produced by four *Prevotella* type strains [J]. Current Microbiology, 2000, 41(1): 45-49.
[15] Sun Yunzhang. Molecular characterization of rumen microbial fermentation characteristics and bacterial community shifts under different substrates [D]. PhD Thesis. Nanjing: Nanjing Agricultural University, 2005: 6-9.
[16] St-Pierre B, Wright A D G. Diversity of gut methanogens in herbivorous animals [J]. Animal, 2013, 7(Suppl. 1): 49-56.
[17] Chaudhary P P, Sirohi S K. Dominance of *Methanomicrobium* phylotype in methanogen population present in Murrah buffaloes (*Bubalus bubalis*) [J]. Letters in Applied Microbiology, 2009, 49(2): 274-277.
[18] Singh K M, Pandya P R, Parnerkar S, et al. Molecular identification of methanogenic archaea from Surti buffaloes (*Bubalus bubalis*), reveals more hydrogenotrophic methanogens phylotypes [J]. Brazilian Journal of Microbiology, 2011, 42(1): 132-139.
[19] Singh K M, Ahir V B, Tripathi A K, et al. Metagenomic analysis of Surti buffalo (*Bubalus bubalis*) rumen: a preliminary study [J]. Molecular Biology Reports, 2012, 39(4): 4841-4848.
[20] Franzolin R, St-Pierre B, Northwood K, et al. Analysis of rumen methanogen diversity in water buffaloes (*Bubalus bubalis*) under three different diets [J]. Microbial Ecology, 2012, 64(1): 131-139.
[21] Wright A D G, Auckland C H, Lynn D H. Molecular diversity of methanogens in feedlot cattle from Ontario and Prince Edward Island, Canada [J]. Applied and Environmental Microbiology, 2007, 73(13): 4206-4210.
[22] Cheng Y F, Mao S Y, Liu J X, et al. Molecular diversity analysis of rumen methanogenic Archaea from goat in eastern China by DGGE methods using different primer pairs [J]. Letters in Applied Microbiology, 2009, 48(5): 585-

592.
[23] Zhou M, Hünerberg M, Beauchemin K A, et al. Individuality of ruminal methanogen/protozoa populations in beef cattle fed diets containing dried distillers' grain with solubles [J]. Acta Agriculturae Scandinavica, Section A: Animal Science, 2012, 62(4): 273-288.

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