Effect of Dietary Neutral Detergent Fiber Level on Rumen Bacterial Structure and Composition in Goats (Postprint)

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Abstract

This study aimed to investigate the effects of dietary neutral detergent fiber (NDF) levels on rumen bacterial structure and composition in goats using high-throughput sequencing technology. Six goats were selected for a 3×3 Latin square experiment, and divided into low (35.01%, LN group), medium (40.10%, MN group), and high NDF level groups (45.16%, HN group) based on dietary NDF levels, with 2 goats per group. The feeding trial was conducted in 3 periods, each lasting 20 d, including a 14-d preliminary period and a 6-d formal experimental period. After the formal experimental period, rumen contents were collected from the goats, bacterial total DNA was extracted, the V4 region of 16S rRNA was amplified using bacterial universal primers, the amplicons were subjected to high-throughput sequencing on the Illumina HiSeq 250PE platform, and the sequencing results were analyzed using bioinformatics software such as QIIME 1.8.0. The results showed: 1) The rumen fluid ammonia nitrogen (NH3-N) concentration in the HN group was extremely significantly lower than that in the LN and MN groups ($P<0.01$); the rumen fluid acetate/propionate ratio in the LN group was significantly lower than that in the HN group ($P<0.05$), but there was no significant difference between the MN group and the other two groups ($P>0.05$). 2) There were no significant differences in Chao1 index and Shannon index among groups ($P>0.05$); the observed species index in the LN group was significantly higher than that in the other two groups ($P<0.05$), while there was no significant difference between the other two groups ($P>0.05$). 3) At the phylum level, there were no significant differences in the relative abundance of all bacteria among the three groups ($P>0.05$); at the genus level, the relative abundances of Prevotellaceae UCG-001, Prevotellaceae UCG-003, and Ruminococcaceae UCG-014 in the HN group were significantly higher than those in the other two groups ($P<0.05$); the relative abundances of Ruminococcaceae NK4A214 group and Ruminococcaceae UCG-005 in the HN group were significantly higher than those in the LN group ($P<0.05$); the relative abundances

of SP3-e08 and Lachnoclostridium 10 in the HN group were significantly lower than those in the other two groups (P<0.05); the relative abundance of Succiniclasticum in the LN group was significantly higher than that in the other two groups (P<0.05); the relative abundances of Lysinibacillus, Bacillus, and Phyllobacterium in the MN group were significantly higher than those in the other two groups (P<0.05), and the relative abundance of Victivallis was extremely significantly higher than that in the other two groups (P<0.01). In conclusion, when dietary NDF levels varied from 35.01% to 45.16%, they significantly affected rumen fluid NH3-N concentration and acetate/propionate ratio, and significantly influenced the relative abundances of various bacterial genera such as Prevotellaceae UCG-001 and Prevotellaceae UCG-003 in the rumen.

Full Text

Effects of Dietary Neutral Detergent Fibre Level on Structure and Composition of Rumen Bacteria in Goats

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Abstract

This study investigated the effects of dietary neutral detergent fibre (NDF) level on rumen bacterial structure and composition in goats using high-throughput sequencing technology. Six healthy male Nubian black goats (average age: 8 months; average body weight: 28.33 ± 3.77 kg) were allocated to a 3 × 3 Latin square design with three dietary NDF levels: low (LN group, 35.01%), medium (MN group, 40.10%), and high (HN group, 45.16%), with two goats per group. The feeding trial consisted of three periods, each lasting 20 days (14-day pretrial and 6-day trial period). Following each trial period, rumen contents were collected for bacterial total DNA extraction. The V4 region of the 16S rRNA gene was amplified using bacterial universal primers and sequenced on the Illumina HiSeq 250PE platform. Bioinformatics analysis was performed using QIIME 1.8.0 and other software. The results showed that: (1) Rumen fluid ammonia nitrogen (NH -N) concentration in the HN group was significantly lower than in the LN and MN groups (P < 0.01). The acetate/propionate ratio in the LN group was significantly lower than in the HN group (P < 0.05), while the MN group showed no significant difference from the other two groups (P > 0.05). (2) No significant differences were observed among groups for the Chao1 and Shannon indices (P > 0.05). However, the observed species index in the LN group was significantly higher than in the other two groups (P < 0.05), which did not differ significantly from each other (P > 0.05). (3) At the phylum level, no significant differences in bacterial relative abundance were detected among the three groups (P > 0.05). At the genus level, the relative abundances of *Prevotellaceae* UCG-001, *Prevotellaceae* UCG-003, and *Ruminococcaceae* UCG-

014 in the HN group were significantly higher than in the other two groups ($P < 0.05$). The relative abundances of *Ruminococcaceae* NK4A214 group and *Ruminococcaceae* UCG-005 in the HN group were significantly higher than in the LN group ($P < 0.05$). Conversely, the relative abundances of SP3-e08 and *Lachnoclostridium* 10 in the HN group were significantly lower than in the other two groups ($P < 0.05$). The relative abundance of *Succiniclasticum* in the LN group was significantly higher than in the other groups ($P < 0.05$). The MN group exhibited significantly higher relative abundances of *Lysinibacillus*, *Bacillus*, and *Phyllobacterium* compared to the other groups ($P < 0.05$), while *Victivallis* abundance was extremely significantly higher ($P < 0.01$). In conclusion, dietary NDF levels ranging from 35.01% to 45.16% significantly affect rumen NH -N concentration, acetate/propionate ratio, and the relative abundance of multiple bacterial genera including *Prevotellaceae* UCG-001 and *Prevotellaceae* UCG-003 in goats.

Keywords: neutral detergent fiber; bacteria; high-throughput sequencing; fermentation parameters

Introduction

Dietary fiber constitutes a major proportion of ruminant diets and plays an irreplaceable role in promoting rumen motility and maintaining normal rumen pH. Although numerous studies have investigated the physiological functions of fiber, previous definitions were neither scientific nor unified. Neutral detergent fibre (NDF) is now widely recognized as the best indicator of dietary fiber level in animal nutrition because it encompasses nearly all fiber components [1]. NDF not only maintains gastrointestinal health but also provides substantial energy through its degradation products. However, ruminants lack the innate ability to digest NDF and rely entirely on rumen microorganisms for fiber utilization. The structure and composition of rumen microbiota determine the host' s capacity to digest and utilize NDF, while dietary NDF serves as a crucial substrate for microbial growth and reproduction.

Previous studies on dietary fiber level effects on rumen microbiota have primarily employed traditional culture techniques and fingerprinting methods such as denaturing gradient gel electrophoresis [2–6]. Culture-based methods only capture microorganisms that can grow in artificial media, yet approximately 99% of rumen microbes remain unculturable. Fingerprinting techniques are time-consuming, labor-intensive, and have low resolution, detecting only about 10% of dominant microorganisms. Both approaches severely underestimate rumen microbial diversity, leaving the relationship between dietary NDF level and rumen microbial structure incompletely understood. To address these limitations, this study employed Illumina HiSeq 250PE high-throughput sequencing technology to comprehensively elucidate the effects of dietary NDF level on rumen bacterial structure and composition in goats. These findings will enhance our

understanding of how rumen microorganisms adapt to nutritional changes and how nutrient levels affect microbial diversity, providing a foundation for future strategies to promote fiber degradation through rumen microbial manipulation.

1.1 Experimental Animals and Management

Six healthy male Nubian black goats with an average age of 8 months and average body weight of (28.33 ± 3.77) kg were used in this study. Diets were formulated according to the Chinese Feeding Standard of Meat-Producing Sheep and Goats (NY/T 816–2004) to achieve a daily weight gain of 0.1 kg per animal. A 3 × 3 Latin square design was employed with three dietary NDF levels: low (LN group, 35.01%), medium (MN group, 40.10%), and high (HN group, 45.16%), with two goats per group. Diet composition and nutrient levels are presented in Table 1 . Dry matter (DM), crude protein (CP), calcium (Ca), and phosphorus (P) were analyzed according to Chemists [7], while NDF and acid detergent fiber (ADF) were determined using the method of Van Soest et al. [8]. Experimental goats were housed individually and fed twice daily at 09:00 and 17:00, with free access to water.

1.2 Experimental Design and Sample Collection

The feeding trial consisted of three periods, each lasting 20 days (14-day pre-trial and 6-day trial period). Following each period, rumen contents were collected at 2 hours after morning feeding on the next day using methods described in references [9–10]. A 10-mm diameter plastic tube connected to a vacuum pump was inserted through the oral cavity into the rumen to extract approximately 50 mL of rumen contents. After pH measurement using a portable pH meter, samples were immediately placed in nitrogen-filled bags on ice. The bags were repeatedly agitated to ensure solid-phase microbes were thoroughly transferred to the liquid phase. The contents were then filtered through four layers of cheesecloth to obtain rumen fluid, which was snap-frozen in liquid nitrogen and stored at -80 °C until analysis.

1.3 Rumen Fermentation Parameter Analysis

Ammonia nitrogen (NH -N) concentration was determined using the method of Broderick et al. [11]. Rumen contents were first preprocessed, followed by preparation of NH -N standard curves to establish linear regression equations. Preprocessed rumen fluid (80 L) was added to test tubes, followed by sequential addition of 40 L methanol, 2.5 mL phenol, and 2.0 mL alkaline sodium hypochlorite solution. After incubation at 37 °C for 10 minutes and standing at room temperature for 10 minutes, absorbance was measured at 650 nm using a microplate reader (Spectramax M2, Molecular Devices, USA). Sample NH -N concentrations were calculated using the linear regression equation.

Volatile fatty acid (VFA) concentration was analyzed by gas chromatography (CP-3800, Varian, USA) according to Li et al. [12]. Rumen contents were preprocessed, then 0.2 mL metaphosphoric acid and 40 L crotonic acid were added to standard intermediate solutions, mixed, and held at 4 °C for 30 minutes. After centrifugation at 12,000 × g for 10 minutes, 0.1 mL supernatant was mixed with 0.9 mL methanol and filtered through a 0.22- m organic membrane. The filtrate was analyzed for acetate, propionate, and butyrate concentrations.

1.4 DNA Extraction and High-Throughput Sequencing

Total microbial DNA was extracted from 200 L rumen fluid samples using a stool genomic DNA extraction kit (Tiangen Biotech, Beijing, China). The V4 region of the bacterial 16S rRNA gene was amplified using universal primers (515F: GTGCCAGCMGCCGCGGTAA; 806R: GGACTACHVGGGTWTCTAAT) [13]. PCR amplification was performed in a 50 L reaction mixture containing: 1 L dNTP Mixture (10 mmol/L), 1.25 L each of forward and reverse primers (10 mol/L), 1 L total DNA (50 ng/ L), 0.25 L Taq DNA Polymerase (5 U/ L, with Mg^2), 5 L 10×Taq Buffer, and double-distilled water to 50 L. Thermal cycling conditions were: initial denaturation at 95 °C for 2 min; 30 cycles of denaturation (95 °C, 30 s), annealing (55 °C, 30 s), and extension (72 °C, 30 s); final extension at 72 °C for 5 min; and cooling to 10 °C. Qualified PCR products were sent to Novogene Bioinformatics Technology (Beijing, China) for high-throughput sequencing on the Illumina HiSeq 250 PE platform.

1.5 Bioinformatics Analysis

Raw sequencing data were initially quality-filtered using QIIME 1.8.0 software to remove low-quality sequences, barcodes, and primer sequences [14]. Sequences were then assembled in Mothur software following the method of Yáñez-Ruiz et al. [15]. Assembled sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using the Uparse module in QIIME 1.8.0, with the most abundant sequence in each OTU selected as the representative sequence. Representative sequences were aligned against the RDP database (Release 11.1, http://rdp.cme.msu.edu/) to construct OTU tables. Taxonomic annotation was performed using RDP Classifier at all microbial classification levels, and bar charts of phylum-level composition were generated. Alpha diversity indices (Chao1, Shannon, observed species) were calculated based on the OTU table, rep_set.tree file, and maximum sampling depth after removing chimeras and singletons. Rarefaction curves were plotted for each sample, and shared genus analysis was performed using R software to generate clustering heatmaps based on shared genus composition and proportions.

1.6 Statistical Analysis

Experimental data were analyzed using the following general linear model:

$$Y_{ij(k)} = \mu + \alpha_i + \beta_j + \gamma(k) + \varepsilon_{ij(k)} \quad (i = j = k = 1, 2, 3)$$

where Y represents the observed value of a parameter, μ is the overall mean, α, β, and γ represent experimental period, goat group, and dietary NDF level, respectively, and ε is random error. Differences among groups were tested using the ANOVA module in SPSS 21.0 software, with NDF level as the main effect and no interaction with the other two factors. Duncan' s multiple range test was used for post-hoc comparisons. Results are expressed as mean ± standard deviation. Statistical significance was declared at $P < 0.05$ and extreme significance at $P < 0.01$.

2.1 Effects of Dietary NDF Level on Rumen Fermentation Parameters

As shown in Table 2 , rumen fluid pH did not differ significantly among the three groups ($P > 0.05$), although it tended to increase with dietary NDF level. The HN group exhibited extremely significantly lower NH -N concentration compared to the LN and MN groups ($P < 0.01$), while the latter two groups showed no significant difference ($P > 0.05$). No significant differences were observed among groups for acetate, propionate, butyrate, or total volatile fatty acid (TVFA) concentrations ($P > 0.05$). However, the acetate/propionate ratio tended to increase with dietary NDF level, reaching statistical significance between the LN and HN groups ($P < 0.05$).

2.2.1 Sequencing Depth and OTU Analysis

A total of 1,125,746 high-quality sequences (clean data) were obtained, with an average of (62,541 ± 9,024) sequences per sample. Clustering yielded 17,198 OTUs, with 1,012 OTUs shared among all three groups. Pairwise shared OTUs were 1,197 between LN and MN groups, 1,083 between LN and HN groups, and 1,083 between MN and HN groups (Figure 1 [Figure 1: see original paper]).

2.2.2 Rarefaction Curves and Alpha Diversity Analysis

Rarefaction curves for all samples are presented in Figure 2 [Figure 2: see original paper]. At the sequencing depth of 30,154 reads, all curves plateaued, indicating that the sequencing depth adequately covered the microbial diversity in each sample. Alpha diversity indices calculated at this depth are summarized in Table 3 . No significant differences were detected among groups for Chao1 or Shannon indices ($P > 0.05$). However, the observed species index in the LN group was significantly higher than in the other two groups ($P < 0.05$), which did not differ significantly from each other ($P > 0.05$).

2.2.3 Rumen Bacterial Structure and Composition

Taxonomic annotation identified 23 phyla, 44 classes, 71 orders, 121 families, and 225 genera. At the phylum level, no significant differences in relative abundance were observed among groups ($P > 0.05$). *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were the dominant phyla across all groups, followed by *Lentisphaerae* and *Tenericutes* (Figure 3 [Figure 3: see original paper]).

At the genus level, *Prevotella* 1 and *Rikenellaceae* RC9 gut group were the top two dominant genera in all groups. After pooling genera with relative abundance below 1% into an "others" category (215 genera), the genus-level composition is shown in Figure 4 [Figure 4: see original paper]. Table 4 lists genera with significantly different relative abundances among groups. The relative abundances of *Prevotellaceae* UCG-001, *Prevotellaceae* UCG-003, and *Ruminococcaceae* UCG-014 increased with dietary NDF level and were significantly higher in the HN group ($P < 0.05$). Similarly, *Ruminococcaceae* NK4A214 group and *Ruminococcaceae* UCG-005 increased with NDF level, with the HN group significantly higher than the LN group ($P < 0.05$). In contrast, SP3-e08 and *Lachnoclostridium* 10 decreased with NDF level, showing significantly lower abundance in the HN group ($P < 0.05$). The LN group had significantly higher *Succiniclasticum* abundance ($P < 0.05$), while the MN group showed significantly higher abundances of *Lysinibacillus*, *Bacillus*, and *Phyllobacterium* ($P < 0.05$) and extremely significantly higher *Victivallis* abundance ($P < 0.01$). Additionally, the *Eubacterium ruminantium* group was significantly higher in the LN group compared to the HN group ($P < 0.05$).

2.2.4 Shared Genus Analysis

Thirty-five genera were shared across all samples. The major shared genera (>1% relative abundance) in descending order were: *Prevotella* 1 [(37.29 ± 7.27)%], *Rikenellaceae* RC9 gut group [(7.30 ± 0.54)%], *Prevotellaceae* UCG-003 [(2.31 ± 0.88)%], *Lachnospiraceae* ND3007 group [(2.19 ± 0.52)%], *Prevotellaceae* UCG-001 [(2.04 ± 0.93)%], *Succinivibrionaceae* UCG-002 [(1.90 ± 1.75)%], *Selenomonas* 1 [(1.79 ± 4.24)%], *Ruminococcus* 2 [(1.35 ± 1.77)%], *Succinivibrio* [(1.20 ± 0.58)%], and *Ruminococcaceae* UCG-014 [(1.13 ± 0.81)%]. These ten genera accounted for 56.50% of the total bacterial community. A clustering heatmap of shared genera is presented in Figure 5 [Figure 5: see original paper].

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3 Discussion

Dietary NDF regulates and maintains normal rumen fermentation, and different NDF levels affect fermentation patterns. Naren Batu et al. [16] investigated rumen fermentation in Inner Mongolian white cashmere goats fed six dietary NDF levels (49%, 52%, 55%, 59%, 62%, and 65%) and found significant effects

on rumen pH and NH -N concentration, but not on microbial crude protein or VFA concentrations. Similarly, Wang Hairong et al. [17] reported that increasing dietary NDF level (42.71%, 54.59%, 64.38%) significantly decreased NH -N concentration and increased acetate/propionate ratio in Sunit sheep, consistent with our findings.

The present study revealed that the relative abundances of *Ruminococcaceae* NK4A214 group, *Ruminococcaceae* UCG-005, and *Ruminococcaceae* UCG-014 increased with dietary NDF level. These bacteria belong to the family *Ruminococcaceae*, which are typical fiber-degrading bacteria that produce cellulases to degrade cellobiose and other fiber components [18–19]. Patra et al. [20] demonstrated that reduced *Ruminococcaceae* abundance decreases fiber digestibility, while Zhao et al. [21] confirmed a significant correlation between *Ruminococcaceae* and dietary NDF digestibility. Our results suggest that increased dietary NDF level enhances *Ruminococcaceae* proliferation, likely through substrate induction effects, further supporting the close relationship between this family and rumen fiber degradation.

The relative abundances of *Prevotellaceae* UCG-001 and *Prevotellaceae* UCG-003 also increased with dietary NDF level. These bacteria belong to the genus *Prevotella*, which has been shown to digest proteins and amino acids in vitro but lacks direct fiber-degrading capacity [22–24]. However, co-culture with fiber-degrading bacteria can indirectly promote fiber degradation [22–24]. Zhao et al. [21] demonstrated that dietary fiber digestibility affects the abundance of these bacteria in calves. Combined with our results, this suggests that *Prevotellaceae* UCG-001 and UCG-003 may be important cooperative fiber-degrading bacteria that benefit from fiber degradation processes, leading to their significantly higher abundance in the HN group.

Interestingly, *Succiniclasticum* abundance showed the opposite pattern, being significantly higher in the LN group. *Succiniclasticum* is a typical fiber-degrading bacterium that ferments fiber or cellobiose to produce succinate, acetate, and CO [25], and its abundance has been associated with fiber digestibility [26–27]. Our previous studies also found higher *Succiniclasticum* abundance in goats with high fiber digestibility. Theoretically, increased dietary fiber should promote *Succiniclasticum* growth, but the LN group showed the highest abundance. This unexpected result may be related to the higher starch content in the LN diet, as starch is a primary substrate for *Succiniclasticum* and may stimulate its growth more effectively than fiber.

Conclusion

This study demonstrates that dietary NDF levels ranging from 35.01% to 45.16% significantly affect rumen fermentation parameters and bacterial community structure in goats. Increasing NDF level decreased rumen NH -N concentration while increasing the acetate/propionate ratio. The dominant bacterial phyla

were *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. Thirteen bacterial genera showed significantly different relative abundances in response to dietary NDF level, with *Prevotellaceae* UCG-001, *Prevotellaceae* UCG-003, and *Ruminococcaceae* UCG-014 increasing with NDF level. These findings provide new insights into the adaptation of rumen microbiota to dietary fiber changes and offer a theoretical basis for optimizing fiber utilization in goat nutrition.

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