

# Effect of different feeding methods on rumen microbes in growing Chinese Tan sheep

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**ABSTRACT** - We evaluated the difference between rumen bacteria in Tan sheep fed either by grazing or in a feedlot. The aim was to provide a theoretical basis for ruminant nutrition and meat quality based on rumen fermentation. Twenty-four three-month-old Tan sheep were randomly and equally divided into two groups, the grazing group and ration group. Five sheep of each group were selected for slaughter at six months of age. Ruminal contents were collected and assessed to identify rumen bacteria, based on 16S rDNA sequencing analysis. A total of 17 phyla were identified, among which *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were dominant in both groups. The abundance of *Firmicutes* was higher in grazing group than in the ration group, while that of *Proteobacteria* was opposite. Besides the dominant phyla differences, the abundance of *Fibrobacteres*, *Tenericutes*, *Elusimicrobia*, and *Cyanobacteria* was significantly higher in the grazing group compared with the ration group. At genus level, a total of 174 genera were identified. The abundance of *Rikenellaceae\_RC9\_gut\_group*, *Dialister*, *Lachnospiraceae\_NA*, *Catonella*, *Ruminococcaceae\_UCG-014*, *Lachnospiraceae\_NK3A20\_group*, and *Fibrobacter* in the grazing group was higher than in the ration group. However, the abundance of *Succinivibrionaceae\_NA* was lower in the grazing group, and *Succinivibrionaceae\_UCG-001* showed a decreasing trend in the grazing group. The two feeding methods may influence the rumen bacterial composition, including the abundance of dominant bacteria, as well as the cellulolytic- and carbohydrate-degrading bacteria in the rumen of Tan sheep.

**Keywords:** feedlot, grazing, rumen microorganism, Tan sheep

## Introduction

Tan sheep are an endemic breed to northwestern China, mainly found in the arid desert areas. The wool of Tan sheep is popular in both domestic and overseas markets for its delicate curl, soft texture, and snow-white appearance. The meat of Tan sheep is also considered top grade, because it is tender, without a goaty flavor (Kang et al., 2013). Owing to environmental concerns in recent years (Chen et al., 2013), feeding methods for Tan sheep have started to shift from full-forage grazing in desert and arid grasslands to the feedlot, which has also caused a change in the composition of animal diets (Morand-Fehr et al., 2007).

The rumen, as an important digestive organ of ruminants, has an abundance of microorganisms, the composition of which is mainly influenced by the animal diet (Henderson et al., 2015). A previous study showed that diet composition can alter the content and composition of rumen bacteria (Bas et al., 2003). Furthermore, rumen microbes affect fermentation that is associated with fat metabolism and nitrogen storage, which are closely related to ruminant digestion and a range of production traits such as feed efficiency and milk yield and components (Schären et al., 2018; Spanghero et al., 2017). Fat storage, an

important factor of meat quality and flavor, is positively linear with the abundance of *Firmicutes* and negatively linear with that of *Bacteroidetes* in the porcine gut (Guo, 2009). Therefore, improving the composition of rumen microorganisms has become a focus of recent ruminant research. However, only a few studies have evaluated the total rumen bacteria of Tan sheep.

The population of rumen bacteria in goats usually becomes stable around six months of age (Guo, 2015). As a result, the present study sampled the rumen content of six-month-old Tan sheep fed at three months of age to determine the effects of different feeding methods on bacterial populations in the developing rumen, based on the 16S rDNA sequencing technique. The objective was to provide a theoretical basis for animal nutrition and meat quality control in Tan sheep. We hypothesized that the diversity and variety of rumen bacteria can be altered with different feeding methods.

## Material and Methods

The experimental procedure was approved by the Institutional Animal Care and Use Committee (NXU1074901).

Twenty-four healthy three-month-old Tan ewes of similar weight ( $17.16 \pm 0.58$  kg) were randomly divided into the grazing group (G6) and ration group (R6), (12 ewes in each group). The pasture was available for *ad libitum* feeding of the sheep in the grazing group. The pasture included 40% *Astragalus adsurgens*, 20% *Lespedeza davurica*, 5% *Sophora alopecuroides*, 10% *Caragana korshinskii* 10%, *Glycyrrhizae radix*, and 10% *Achnatherum splendens*. The sheep in the ration group were fed roughage supplemented with concentrate in feedlots (Table 1). Both groups had free access to water.

At six months of age, five sheep from each group were slaughtered. The rumen fluid of the slaughtered sheep was filtered through four layers of gauze and collected separately. A volume of 50 mL of each sample, which contained digested plant particles and rumen fluid, were stored in CO<sub>2</sub>-containing centrifugal tubes and kept on ice for no longer than 30 min, before being stored in a refrigerator at -80 °C. Sample handling that entails cooling on ice and at -80 °C has little effect on the sample integrity or subsequent analyses (Wu et al., 2010). A total of 10 samples were tested for rumen bacteria. The DNA extraction was then conducted as previously described (Denman and McSweeney, 2006) using a FastDNA Kit and FastPrep Instrument. The final samples were either stored at 4 °C for a short term, or -80 °C for a long term.

After genomic DNA was extracted from the rumen samples, we conducted PCR amplification for the pre-experiments. Agarose gel electrophoresis was used to detect the purity and concentration of DNA samples before PCR amplification. After extracting genomic DNA from the samples, the V3 + V4 area of 16S rDNA was amplified. The primer sequence was as follows: 341F: CCTACGGGNGGGCWCAG; 806R: GGACTACHVGGTATCTAAT. The 16S rDNA was sequenced using the Illumina Hiseq2500 PE250 platform (Guangzhou Gene Denovo Technology Co., Ltd. in Guangzhou, China).

**Table 1 - Dietary composition and nutrition level of Tan sheep in the ration group**

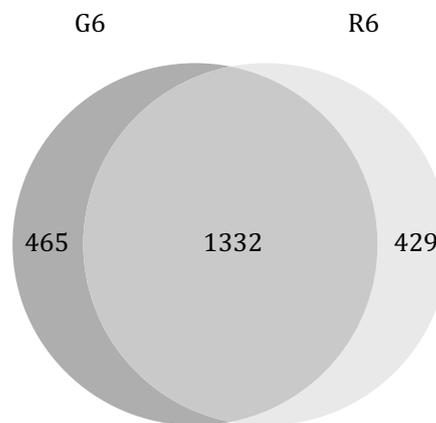
Ingredient	Content (%)	Dietary nutrition level	Value
Corn	27.6	Crude protein (%)	10.97
Soybean meal	2.5	Net energy (MJ/kg)	12.15
Bran	1.9		
<i>Caragana microphylla</i>	2.3		
Corn stalk	20.9		
Licorice	15.3		
Bitter soybean meal	16.3		
Alfalfa	7.5		

The operational taxonomic units (OTU) were obtained from the clustered effective tags of more than 97% similarity using the Uparse (version 9.2.64) software (Edgar, 2013), and then the abundances of OTU were calculated. Venn analysis was performed in R project VennDiagram package (version 1.6.16, Chen and Boutros, 2011). The alpha indexes were calculated in QIIME (version 1.9.1, Caporaso et al., 2010) and then processed by the Excel 2007 software and analyzed by the SAS software (Statistical Analysis System, version 8.2) using a completely random design. Principal component analysis (PCA) was performed in R project Vegan package (version 2.5.3, Oksanen et al., 2011). Anosim analysis was conducted by the Mothur software and calculated in R project Vegan package (version 2.5.3, Oksanen et al., 2011). The representative sequences were classified into bacteria based on SILVA database (version 132, Pruesse et al., 2007) and Greengene database (version gg\_13\_5, DeSantis et al., 2006), with the confidence threshold values ranging from 0.8 to 1. The abundance statistics of each taxonomy was visualized using Krona (version 2.6, Ondov et al., 2011).

## Results

After removing the low-frequency and insignificant tags, effective tags were spliced and clustered into OTU. The results of OTU clustering of the different samples were analyzed, and a Venn diagram (Figure 1) was constructed, based on the common and unique OTU information. The OTU of the sheep in the grazing group was 1797, while that in the ration group was 1761 (Figure 1), without any significant difference between the groups. A total of 1332 common OTU were shared between the two groups.

Alpha diversity indexes (Table 2), Chao1, ACE, Shannon, and Simpson were used to analyze species diversity and abundance. The Chao1 and ACE indexes predict the species of microorganisms (the number of OTU) in the sample based on the number of measured tags, OTU, and their relative proportions. The Shannon index reflects the species diversity according to OTU homogeneity and richness. The Simpson index refers to the probability that the randomly sampled species in two successive evaluations belong



G6 - grazing group; R6 - ration group.

**Figure 1** - Venn diagram of microorganism operational taxonomic units between groups.

**Table 2** - Alpha indexes of rumen bacteria of Tan sheep in the grazing and ration groups

Item	Grazing	Ration	P-value
ACE	1419.354±23.573	1297.686±97.432	0.260
Chao1	1400.832±32.038	1272.312±89.579	0.214
Shannon	5.804±0.131	4.830±0.561	0.129
Simpson	0.947±0.014	0.836±0.048	0.064
Good's coverage	0.997±0.000a	0.997±0.000b	0.033

Data are presented as mean ± standard error.  
a,b - P<0.05.

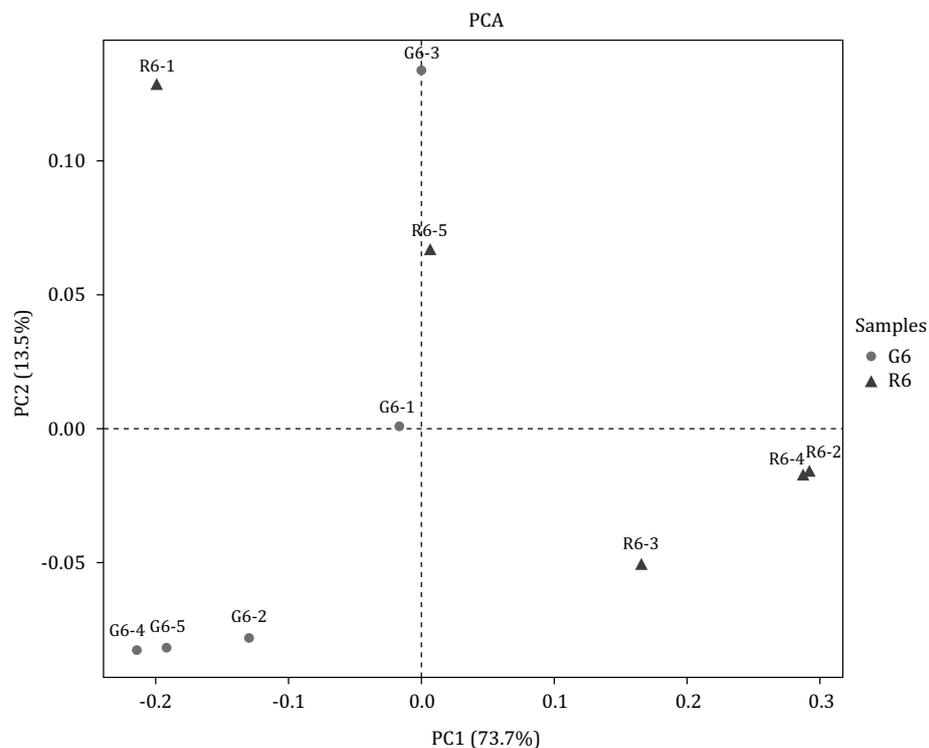
to different species. The Shannon and Simpson indexes both reflect the synthesis of species richness and evenness.

Favorable coverage was more than 99% in both groups (Table 2), which suggests that the indexes could fully reflect the situation of rumen bacteria. The values of the ACE index and the Chao1 index of the grazing group were higher than those of the ration group; however, this difference was not significant ( $P = 0.260$  and  $0.214$ , respectively). The value of the Shannon index of the grazing group was higher than that of the ration group; however, this difference was not significant ( $P = 0.129$ ). The Simpson index of the grazing group tended to be closer to 1 than that of the ration group ( $P = 0.064$ ).

The PCA plot was based on species abundance of the OTU list and evaluated the distance between samples by reducing dimensionality. The more similar the sample compositions were, the closer the distance reflected on the PCA plot. By evaluating the variance decomposition, the PCA (Figure 2) could identify the main elements and structures in data and simplify the complex relationships of sample composition, by reflecting the two eigenvalues of the abscissa and ordinate.

The abscissa was the first principal factor (73.7%) and the ordinate was the second principal factor (13.5%) (Figure 2). The samples in the grazing group were in close proximity to each other when considering the first factor, while two samples (G6-1 and G6-3) were separately distributed based on the second factor. Three samples (R6-2, R6-3, and R6-4) were concentrated, and the other two (R6-1 and R6-5) were separately distributed in the ration group. In addition, some samples in different groups (e.g., G6-3 and R6-5) showed a clustering trend.

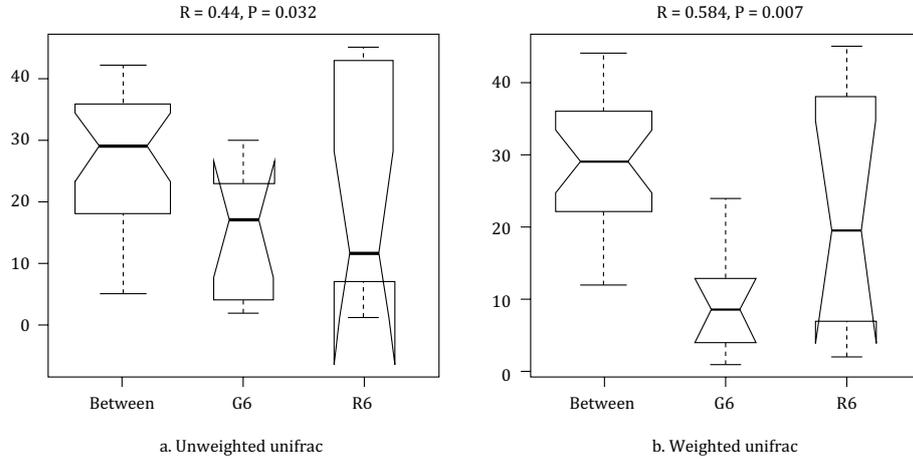
Anosim analysis is a non-parametric test for microbial community structure. It determines whether a significant difference exists between groups compared to within groups. Unweighted UniFrac only considers whether there are changes in the species composition. Weighted UniFrac synthesizes both the changes in species composition and abundance. The medians of the grazing and ration groups differed (Figure 3), indicating statistical significance, whether the analysis was weighted or not ( $P < 0.05$ ). Animal-to-animal variation was higher in the ration group than in the grazing group.



G6 - grazing group; R6 - ration group.

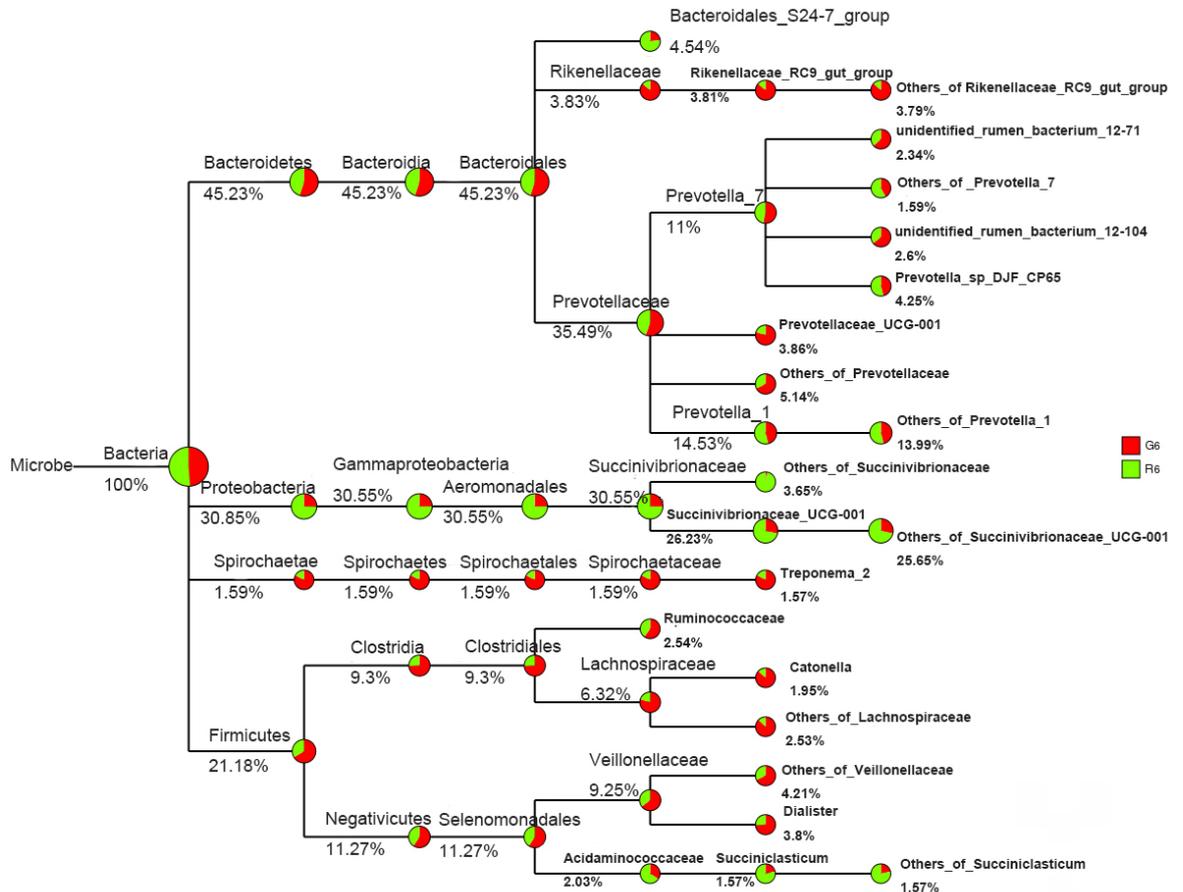
**Figure 2** - Principal component analysis (PCA) plots.

The species taxonomic tree shows those species with an abundance greater than 1%, as selected by the Perl + SVG software, based on species annotation of the OTU. Seven levels of microbial species are displayed: kingdom, phylum, class, order, family, genus, and species. As the taxonomic tree (Figure 4)



G6 - grazing group; R6 - ration group.

Figure 3 - Unifrac Boxplots.



G6 - grazing group; R6 - ration group.

Figure 4 - Species taxonomic tree.

shows, most *Proteobacteria* were in the ration group at the phylum level, while most *Spirochaetae* and *Firmicutes* were in the grazing group. The abundance of *Bacteroidetes* was similar between the groups. In addition, in the *Bacteroidetes* phylum, most of the *Bacteroidetes\_S24\_7\_group* were in the ration group at the family level, while most *Rikenellaceae* were in the grazing group. In the *Negativicutes* class, *Firmicutes* phylum, most *Acidaminococcaceae* were in the ration group at the family level, while most *Veillonellaceae* were in the grazing group.

A total of 17 phyla were identified in the rumen of Tan sheep: *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Spirochaetae*, *Cyanobacteria*, *Fibrobacteres*, *Verrucomicrobia*, *Synergistetes*, *Actinobacteria*, *Saccharibacteria*, *Elusimicrobia*, *Tenericutes*, *Lentisphaerae*, *Bacteria\_NA*, *Planctomycetes*, *Euryarchaeota*, and *SR1*. In terms of the abundance of rumen bacteria at the phylum level, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were the dominant bacteria in both groups (Table 3), which accounted for more than 85% of the total rumen bacteria.

The abundance of *Fibrobacteres* and *Tenericutes* was significantly higher in the grazing group than in the ration group ( $P < 0.01$ ). The abundance of *Firmicutes*, *Cyanobacteria*, and *Elusimicrobia* was significantly higher in the grazing group than in the ration group ( $P < 0.05$ ). The abundance of *Actinobacteria* showed a greater increasing trend in the grazing group than in the ration group; however, this difference was not significant ( $P = 0.068$ ). Furthermore, the abundance of *Proteobacteria* was significantly lower in the grazing group than in the ration group ( $P < 0.05$ ).

A total of 174 genera were identified and analyzed in the rumen fluid. Only the dominant bacteria (with an abundance of more than 5%), subdominant bacteria (with an abundance of 0.5%–5%), and bacteria that showed significant group differences are listed in Table 4. Eight dominant genera were identified in the grazing group: *Succinivibrionaceae\_UCG-001*, *Prevotella\_1*, *Prevotella\_7*, *Prevotellaceae\_NA*, *Rikenellaceae\_RC9\_gut\_group*, *Prevotellaceae\_UCG-001*, *Veillonellaceae\_NA*, and *Dialister*. Five dominant genera were identified in the ration group: *Succinivibrionaceae\_UCG-001*, *Prevotella\_1*, *Prevotella\_7*, *Bacteroidales\_S24-7\_group\_NA*, and *Succinivibrionaceae\_NA*. Moreover, the abundance of the *Rikenellaceae\_RC9\_gut\_group*, *Lachnospiraceae\_NA*, *Ruminococcaceae\_UCG-014*, *Lachnospiraceae\_NK3A20\_group*, *Erysipelotrichaceae\_UCG-004*, and *Fibrobacter* was significantly higher in the grazing group than in the ration group ( $P < 0.01$ ). In addition, the abundance of *Dialister*, *Catonella*, *Roseburia*,

**Table 3 - Phyla of rumen bacteria of Tan sheep in grazing and ration groups (%)**

Phylum	Grazing	Ration	P-value
<i>Bacteroidetes</i>	50.952±1.711	38.834±9.201	0.176
<i>Firmicutes</i>	28.357±5.943a	13.679±2.197b	0.041
<i>Proteobacteria</i>	16.423±5.397a	46.244±11.295b	0.035
<i>Spirochaetae</i>	2.704±1.835	0.496±0.211	0.204
<i>Cyanobacteria</i>	0.444±0.053a	0.228±0.056b	0.014
<i>Fibrobacteres</i>	0.376±0.059A	0.083±0.044B	0.003
<i>Verrucomicrobia</i>	0.267±0.143	0.138±0.061	0.478
<i>Synergistetes</i>	0.166±0.059	0.166±0.040	0.946
<i>Actinobacteria</i>	0.164±0.050	0.059±0.014	0.068
<i>Saccharibacteria</i>	0.045±0.009	0.030±0.014	0.414
<i>Elusimicrobia</i>	0.033±0.012a	0.006±0.001b	0.037
<i>Tenericutes</i>	0.029±0.005A	0.004±0.002B	0.001
<i>Lentisphaerae</i>	0.019±0.013	0.011±0.006	0.654
<i>Bacteria_NA</i>	0.013±0.003	0.018±0.003	0.225
<i>Planctomycetes</i>	0.007±0.007	<0.001	0.364
<i>Euryarchaeota</i>	0.002±0.000	0.003±0.001	0.115
<i>SR1</i>	0.001±0.000a	<0.001b	0.049

Data are presented as the mean ± standard error:  
a,b -  $P < 0.05$ ; A,B -  $P < 0.01$ .

**Table 4** - Genera of rumen bacteria of Tan sheep in the grazing and ration groups

Genus	Grazing	Ration	P-value
<i>Succinivibrionaceae_UCG-001</i>	15.741±5.327	37.973±10.320	0.065
<i>Prevotella_1</i>	13.876±2.146	14.236±5.875	0.964
<i>Prevotella_7</i>	11.749±3.724	10.967±5.142	0.930
<i>Prevotellaceae_NA</i>	6.958±2.162	3.295±0.466	0.103
<i>Rikenellaceae_RC9_gut_group</i>	6.631±0.926A	0.994±0.170B	<0.001
<i>Prevotellaceae_UCG-001</i>	6.610±3.257	1.416±0.621	0.127
<i>Veillonellaceae_NA</i>	5.733±2.159	2.700±0.288	0.184
<i>Dialister</i>	5.615±1.499a	2.049±0.660b	0.042
<i>Lachnospiraceae_NA</i>	4.426±1.221A	0.623±0.082B	0.009
<i>Catonella</i>	3.371±1.387a	0.501±0.178b	0.049
<i>Treponema_2</i>	2.681±1.835	0.478±0.213	0.267
<i>Bacteroidales_S24-7_group_NA</i>	2.172±0.725	6.247±4.088	0.384
<i>Ruminococcaceae_UCG-014</i>	1.405±0.278A	0.297±0.037B	0.002
<i>Lachnospiraceae_NK3A20_group</i>	0.898±0.131A	0.320±0.124B	0.008
<i>Bacteroidales_BS11_gut_group_NA</i>	0.792±0.474	0.409±0.112	0.516
<i>Succiniclasticum</i>	0.749±0.599	2.216±1.443	0.413
<i>Acidaminococcus</i>	0.710±0.257	0.212±0.095	0.078
<i>Erysipelotrichaceae_UCG-004</i>	0.640±0.102A	0.051±0.013B	<0.001
<i>Roseburia</i>	0.568±0.067a	0.279±0.114b	0.0412
<i>Prevotellaceae_UCG-003</i>	0.538±0.356	0.163±0.095	0.353
<i>Nodatum_group</i>	0.396±0.143a	0.033±0.006b	0.024
<i>Fibrobacter</i>	0.376±0.059A	0.083±0.044B	0.002
<i>Succinivibrionaceae_NA</i>	0.205±0.015A	6.912±1.730B	0.003

Data are presented as the mean ± standard error.  
a,b - P<0.05; A,B - P<0.01.

and *Nodatum\_group* in the grazing group was significantly higher than in the ration group (P<0.05). However, the abundance of *Succinivibrionaceae\_NA* in the grazing group was significantly lower than that in the ration group (P<0.01), and the abundance of *Succinivibrionaceae\_UCG-001* showed a decreasing trend in the ration group (P = 0.065).

## Discussion

The rumen provides a relatively stable living environment for rumen microorganisms, including bacteria, protozoa, and fungi, among which bacteria are the most abundant (Pitta et al., 2016a). In the present study, rumen bacteria were investigated under two feeding methods, full-forage grazing and feedlot feeding with high levels of concentrates. The analyses showed relatively high animal-to-animal variation, but significant group differences in some aspects. In terms of the rumen bacteria diversity, Kocherginskaya et al. (2001) identified greater diversity in high-grain diets relative to hay diets; however, this was not supported by the results of the current study. This might be due to differences in the nutrition level of the diets. In the present study, the diversity and evenness of rumen bacteria showed an increasing trend in the grazing group, according to the Simpson index. These results are consistent with those of Grilli et al. (2016).

Rumen fermentation is influenced by the species composition of bacteria (Oliveira et al., 2006; Ley et al., 2008; Singh et al., 2012). The phyla of rumen bacteria detected by gene sequencing mainly include *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Spirochaetae*, *Fibrobacteria*, and *Actinobacteria* (Huws et al., 2007; Pitta et al., 2016a). These findings are consistent with those of the present study, although the abundance of individual species showed a disparity.

The dominant bacteria of the 17 phyla identified in the rumen of both groups were *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. The abundance of the dominant phyla of the grazing and feedlot-fed groups was as follows: *Bacteroidetes*, 51 and 39%, respectively; *Firmicutes*, 28 and 14%, respectively; and *Proteobacteria*, 16 and 46%, respectively. Significant differences were observed in the *Firmicutes* and *Proteobacteria* phyla, indicating the existence of a strong effect on the abundance of dominant rumen bacteria under different feeding conditions.

*Proteobacteria*, as an important phylum in rumen metabolism, tends to become co-dominant in ruminants fed starch-based diets (Pitta et al., 2016b). However, in the present study, *Proteobacteria* became the most abundant phylum in sheep fed a high concentrate diet. The *Fibrobacteres* are closely associated with the degradation of cellulose and lignin (Ransom-Jones et al., 2012). Their abundance in the grazing group was significantly higher than that in the ration group, which was consistent with the differences in fiber intake between the groups.

Roughage is a major feed component for ruminants. Fiber in roughage is degraded rapidly by rumen microorganisms into nutrients that provide energy for ruminants (Aschenbach et al., 2011). Bacteria and fungi, including cellulolytic bacteria, play an important role in enzymatic decomposition, by degrading cellulose and hemicellulose into small molecules that can be absorbed by the rumen (Zebeli et al., 2012).

The structure of rumen microflora is related to the dietary method (Yáñez-Ruiz et al., 2010). In one study of the effects on rumen flora of goats fed a high-grain diet (Liu et al., 2015), the results showed that such diets improve the abundance of *Succiniclaticum* and decreases the abundance of unclassified *Rikenellaceae* and unclassified *Erysipelotrichaceae*. *Ruminococcaceae*, *Fibrobacter*, and *Lachnospiraceae*, which are associated with cellulose and hemicellulose degradation (Biddle et al., 2013; Li et al., 2014).

In the present study, the abundance of the *Rikenellaceae\_RC9\_gut\_group*, *Lachnospiraceae\_NA*, *Ruminococcaceae\_UCG-014*, *Lachnospiraceae\_NK3A20\_group*, *Erysipelotrichaceae\_UCG-004*, and *Fibrobacter* in the ration group was significantly lower than that in the grazing group. Most of these species are associated with cellulose and hemicellulose degradation. Moreover, in the present study, the abundance of *Succinivibrionaceae\_NA* was significantly increased and that of *Succinivibrionaceae\_UCG-001* showed an increasing trend, when the feeding method was switched from grazing to feedlot feeding. The genera of the *Succinivibrionaceae* family detected in the rumen mostly play a role in the degradation of starch (Santos and Thompson, 2014), which could explain the results of this study.

## Conclusions

The different feeding methods employed in this study have no significant effect on the diversity of rumen bacteria in Tan sheep, but affect the structure of bacterial populations. The change in methods from grazing to feedlot feeding with higher levels of concentrate feed decreases the abundance of cellulolytic bacteria, but increases that of the *Succinivibrionaceae* family, which is associated with starch decomposition in this study.

## Conflict of Interest

The authors declare no conflict of interest.

## Author Contributions

Conceptualization: Z. Fu, X. Xu and L. Zhang. Data curation: Z. Fu, X. Xu and J. Zhang. Formal analysis: Z. Fu, X. Xu and J. Zhang. Funding acquisition: L. Zhang. Investigation: Z. Fu and J. Zhang. Methodology: Z. Fu, X. Xu and L. Zhang. Project administration: Z. Fu, X. Xu and L. Zhang. Resources: X. Xu and L. Zhang. Software: Z. Fu. Supervision: X. Xu and L. Zhang. Validation: L. Zhang. Visualization: J. Zhang. Writing-original draft: Z. Fu. Writing-review & editing: X. Xu and J. Zhang.

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