Effects of diet on ruminal methanogenic archaea composition and diversity in cashmere goats

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Abstract

Diet is the most direct way to affect the rumen microbial community. The objective of this study was to examine the effects of different diets on ruminal methanogen composition in cashmere goats. Twenty-four cashmere goats were randomly divided into four groups that were fed alfalfa hay (AH), alfalfa hay plus concentrate (AHC), corn stalks (CS), and corn stalks plus concentrate (CSC). The concentration of ammonia-N for the group fed AHC was substantially higher than in the other groups. The concentrations of total VFAs, acetate, and propionate in AH and AHC groups were higher than in the CS and CSC groups. The abundance of archaea was markedly different among different groups based on a high-throughput sequencing method. The abundance of *Candidatus*_Methanomethylophilus was 21.28% in the AH group, which was higher than the other groups. *Unclassified*_c_Thermoplasmata was the dominant methanogen in the AH and AHC groups, with abundances of 48.09% and 44.97%, respectively; *Methanobrevibacter* was the dominant methanogen in the CS and CSC groups, with abundances of 41.22% and 20.73%, respectively. A positive correlation was identified between pH and *Methanosphaera*; a negative relationship was observed between acetate and *Unclassified*_o_Thermoplasmatales. In conclusion, the methanogen communities in cashmere goats varied on different diets. In the alfalfa hay-based diets, the Methanomassiliicoccales-affiliated groups were the dominant methanogens in the rumen of the goats. However, when fed the corn stalk-based diets, *Methanobrevibacter* was the dominant methanogen in the rumen fluid. The results were related to dietary composition, especially crude protein content.

Introduction

Ruminants provide high-quality protein for humans, such as beef, mutton, and dairy products. The rumen of ruminants has the unique ability to utilize various feeds, which is mainly attributed to the

complex, diverse, nonpathogenic microorganisms inhabiting the rumen, including bacteria, fungi, protozoa, and archaea (Kittelmann *et al.,* 2013). Methanogens are a phylogenetically diverse group of archaea. The function of methanogens is to produce methane via the hydrogenotrophic pathway. This process keeps a low H_2 pressure in the rumen to promote the digestion of various raw materials by fungi, protozoa, and bacteria (Moss *et al.*, 2000). To a much lesser extent, methane can also be produced in the rumen through the utilization of methyl groups (methylotrophic pathway) and less commonly from acetate (Leahy *et al.*, 2010). The most studied methanogenesis pathway in the rumen is the hydrogenotrophic pathway, which accounts for >90% of the total methane production from the rumen. However, methane cannot be used by ruminants and is emitted into the atmosphere through eructation. This accounts for approximately 6% of global anthropogenic greenhouse gas emissions (Gerber *et al.*, 2013). Furthermore, enteric methane emissions are an important energy loss in the process of ruminant livestock feeding, accounting for 2–12% of the gross energy consumed by ruminants (Johnson & Johnson, 1995).

Enteric methane production is affected by many factors, including diet, feed intake, the digestibility of the diet in the rumen, the rumen passage rate of the diet, rumen pH, and animal genotype and phenotype (Beauchemin *et al.*, 2020). Previous studies on beef cattle, dairy cows, goats, and sheep have shown that dietary manipulation can affect the methanogen community composition and is an effective way to reduce enteric methane emissions in ruminant production systems (Yang *et al.*, 2012; Pedreira *et al.*, 2013; Philippeau *et al.*, 2017; Lima *et al.*, 2019).

Forage type is recognized to be an important factor in the composition of microbial communities, rumen fermentation, and rumination time in ruminants (Liu *et al.*, 2016). One type of forage, corn stalks, is abundant and low-cost but is typically considered poor-quality roughage. A second type, alfalfa, is well known for its high quality and plays an essential part in the production of ruminants. Due to differences in the physical structure and chemical properties, alfalfa and corn stalks in the rumen produce different emissions of enteric methane. However, the difference in the composition of colonized ruminal methanogen communities of the two forages may also be an important factor. Cashmere goats are an important ruminant species in many regions of the world, including China, Russia, India, and Mongolia. However, little is known about the effect of alfalfa hay- or corn stalk-based diets on the composition and diversity of methanogenic archaea in cashmere goats.

A range of molecular technologies have been used to determine the diversity and phylogeny of methanogens. Unlike the traditional method involving Sanger sequencing of 16S rRNA, the methyl coenzyme M reductase (*mcr*A) gene is an alternative strategy to detect the presence, abundance, and activity of methanogens under various conditions (Tomkins *et al.*, 2015). The *mcr*A gene is highly conserved phylogenetically and encodes the methyl coenzyme, M reductase, which catalyses the terminal step of methanogenesis in all methanogenic archaea (Friedrich 2005). Sirohi *et al.* (2013) reported that diversity analysis using the *mcr*A gene provides better insight into ruminal methanogens than the 16S rRNA gene. Therefore, the objective of the present study was to examine the effects of alfalfa hay- and corn stalk-based diets on fermentation profile and the composition and diversity of methanogens in rumen fluid of cashmere goats.

Materials and methods

All animal procedures were conducted following the 'Laboratory Animal Guideline for Ethical Review of Animal Welfare,' National Standard of the People's Republic of China (GB/T 35892-2018). The care and use of animals fully complied with local animal welfare laws, guidelines, and policies.

Twenty-four healthy, 8-month-old cashmere ewes (average body weight 25.2 ± 1.8 kg) were selected and randomly divided into four treatments consisting of six replicates with one goat per replicate. The dietary treatments were as follows: alfalfa hay-fed group (AH), alfalfa hay plus concentrate-fed group (AHC), corn stalk-fed group (CS), and corn stalk plus concentrate-fed group (CSC). The ratio of concentrate to forage in AHC and CSC groups was 25:75. Animals were individually fed twice a day, at 08:00 and 17:00. All animals had *ad libitum* access to water. The Feed Research Institute of the Chinese Academy of Agricultural Sciences was entrusted with the analysis of conventional indices of feeds. The compositions and nutrient levels of the diets are shown in Table 1. Before the start of the experiment, the goats were dewormed, and the animal shed was fully disinfected. The experiment lasted for 40 days, with a 20-day adaptation period and a 20-day formal experimental period.

Table 1 Composition and nutrient levels of diets (dry matter basis; %)

Items	Index	Treatment			
		AH	AHC	CS	CSC
Composition of diets	Alfalfa	100.00	75.00	-	-
	Corn stalk	-	-	100.00	75.00
	Corn	-	15.00	-	15.00
	Soybean meal	-	3.75	-	3.75
	Distillers dried grains	-	2.00	-	2.00
	Wheat bran	-	2.50	-	2.50
	CaHPO$_4$	-	0.25	-	0.25
	NaCl	-	0.50	-	0.50
	Premix[a]	-	1.00	-	1.00
	Total	100.00	100.00	100.00	100.00
Nutritional level	Dry matter	95.50	94.50	94.40	93.70
	Gross energy[b] (MJ·kg^{-1})	17.30	17.13	16.50	16.50
	Crude protein	13.95	14.25	4.52	7.18
	Ether extract	4.04	3.94	3.35	3.43
	Neutral detergent fibre	57.76	46.59	71.32	56.76
	Acid detergent fibre	41.11	31.95	42.92	33.31
	Calcium	0.62	0.57	0.52	0.50
	Phosphorus	0.16	0.21	0.05	0.12

Note: [a]Per kilogram of premix provided the following: iron (Fe) 0.05 g, copper (Cu) 0.25 g, zinc (Zn) 0.6 g, manganese (Mn) 1 g, selenium (Se) 5 mg, cobalt (Co) 25 mg, vitamin (VA) 100 000 IU (International Unit), vitamin D (VD3) 40 000 IU, vitamin E (VE) 800 mg. [b]Gross energy was measured value. AH = Alfalfa hay-fed; AHC = alfalfa hay plus concentrate-fed; CS = corn stalk-fed; CSC = corn stalk plus concentrate-fed

On day 20 of the formal experimental period, an oral stomach tube was used to collect ruminal fluid from the 24 animals for analysis of ruminal fermentation parameters and diversity of methanogens.

For each animal, 100 mL of ruminal fluid was collected and the initial 50 mL was discarded to avoid contamination by saliva; the last 50 mL of ruminal fluid was retained. The ruminal fluid was filtered with four layers of cheesecloth, and 2 mL of the strained ruminal filtrate was taken to measure the pH value immediately, using a portable pH meter (Starter 300; Ohaus Instruments Co. Ltd., Shanghai, China). A total of 4 mL of the filtrate was aliquoted into cryogenic vials, quickly frozen in liquid nitrogen, and stored at −80 °C until microbial DNA extraction. The concentrations of VFAs were determined using a previously reported method (Hoskin *et al.*, 1995). An amount of 10 mL of the filtrate was centrifuged at 12 000 × g at 4 °C for 5 min, and the supernatant from each sample was then transferred to 2 mL tube. After adding 25% (w/v) metaphosphoric acid solution to the fluid at a volume ratio of 1:5, the mixture was vortexed for 10 s and centrifuged at 2 000 × g for 20 min to remove the precipitate. The supernatant was then applied to a GC-8A gas chromatograph (Shimadzu Corp., Kyoto, Japan) to measure the concentrations of the main VFAs. A 5 mL sample of each ruminal filtrate sample was centrifuged at 3 500 × g for 10 min, and the supernatants were used to determine the ammonia-N (NH_3-N) concentrations using the methods described by Chaney & Marbach (1962).

Microbial DNA was extracted from rumen fluid samples using a QIAamp Fast DNA Stool Mini Kit (Qiagen, CA, USA). The integrity of the extracted DNA was detected using 1% agarose gel electrophoresis; the concentration and purity of the extracted DNA were determined using a NanoDrop 2000 UV-vis (ultraviolet-visible) spectrophotometer (Thermo Scientific, Wilmington, NC, USA). DNA samples that met the requirements were subjected to MiSeq sequencing. The *mcr*A-specific primers used in the present study were MLrF (5′-ACTCCTACGGGAGGCAGCA-3′) and MLrR (5′-GGACTACHVGGGTWTCTAAT-3′). The polymerase chain reaction (PCR) reaction volume was 20 µL, including 10 ng of template DNA, 0.8 µL of each primer (forward and reverse) with a concentration of 5 µmol L^{-1}, 4 µL of 5× FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.4 µL of FastPfu polymerase, 0.2 µL of bovine serum albumin, and ddH$_2$O. The PCR protocol was 95 °C for 3 min; 27 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; then 72 °C for 10 min. The PCR products were subjected to gel purification using an AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, USA), and quantified using QuantiFluor™-ST (Promega, Fitchburg, WI, USA). The purified amplicons were pooled on an Illumina MiSeq PE300 instrument (Illumina, San Diego, CA, USA) and were equimolar and paired-end sequenced (2 × 300) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd (Shanghai, China).

The raw FASTQ files were demultiplexed and quality-filtered using QIIME (version 1.9.1) using the following criteria: (i) the 300-bp reads were truncated at any site that had an average quality score <20 over a 50-bp sliding window, and truncated reads <50 bp were discarded; (ii) the paired-end reads were merged into a sequence with a minimum overlap length of 10 bp; (iii) the allowed maximum mismatch ratio of the overlap region of the spliced sequence was 0.2, and the nonconforming sequence was screened; and (iv) samples were distinguished according to the barcode and primer at the head and tail ends of the sequence, and the sequence direction was adjusted. The allowable number of barcode mismatches was 0, and the maximum number of primer mismatches was 2.

UPARSE software (Highly Accurate OTU Sequences from Microbial Amplicon Reads, version 7.0.1090, http://www.drive5.com/uparse/) was used to cluster the sequences into operational

taxonomic units (OTUs; based on similarity >97%) and chimeric sequences were identified and removed using UCHIME (Chimera Prediction for Amplicon Sequencing). OTUs were used for alpha diversity (Coverage, Sobs, Ace, Chao, Shannon, and Simpson) analysis. OTUs were taxonomically analysed using the Ribosomal Database Project (RDP) Classifier algorithm (version 2.11 http://sourceforge.net/projects/rdp-classifier/) against the FunGene Database, using a confidence threshold of 70% (Amato *et al.*, 2013). The rarefaction curve analysis using Mothur v.1.21.1 was performed to reflect the sequencing depth. The composition of the methanogen community was compared by principal coordinate analysis (PCoA) using the Bray-Curtis dissimilarity metric, with sequence identity ≥97% at the OTU level to perform visual calculation and analysis of the beta diversity of samples.

The data ruminal fermentation parameters were analysed in a completely randomized design using the Proc Mixed procedure of SAS (SAS Inst. Inc., Cary, NC, USA). According to this statistical model:

$$Y_{ij} = \mu + T_i + P_j + T_i \times P_j + \varepsilon_{ij}$$

where: Y_{ij} was the dependent, continuous variable; μ was the overall mean; T_i was the fixed effect of diet treatment (i = alfalfa hay, alfalfa hay plus concentrate, corn stalk, and corn stalk plus concentrate); P_j was the fixed effect of the pen (j = 1, 2, 3, 4, 5, and 6); $T_i \times P_j$ was the fixed effect of the interaction between diet and pen, and ε_{ij} was the residual error. The mixed model included fixed effects of diet and random effects of the pen. The pen was considered the experimental unit and the repeated measurement. Data means significance was declared at $P < 0.05$.

For evaluation of ruminal methanogen diversity indices and the methanogen community structure at phylum and genus level, a one-way analysis of variance (ANOVA) and Duncan's multiple range tests were carried out in SAS (SAS Inst. Inc., Cary, NC, USA). Relative abundance data are presented as percentages, and the correlation between fermentation parameters and methanogens was examined using Spearman's correlations.

Results

The effects of diet on ruminal fermentation parameters are shown in Table 2. The concentration of NH_3-N in group AHC was higher than in the other groups ($P < 0.01$). The concentrations of total VFAs in group AH and AHC were higher than in group CS ($P < 0.05$). The acetate concentration in group AH and AHC was higher than that in group CSC ($P < 0.05$), and the concentration of propionate in group AH and AHC was higher than that in group CS and CSC ($P < 0.01$). The differences in other ruminal fermentation parameters were not significant ($P > 0.05$).

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Table 2 Effect of diet on ruminal fermentation parameters in cashmere goats

Items	Treatment				*P*-Value
	AH	AHC	CS	CSC	
pH	7.28±0.10	6.94±0.12	7.30±0.10	7.14±0.06	0.659
NH_3-N (mg·100mL^{-1})	8.18±0.76^b	11.90±1.27^a	6.76±0.32^b	8.01±0.35^b	0.001
Total VFAs (mmol·L^{-1})	48.15±3.83^a	48.43±3.33^a	33.03±3.89^b	37.71±3.20^{ab}	0.016
Acetate (mmol·L^{-1})	36.65±2.25^a	34.50±2.30^{ab}	25.91±3.63^{bc}	24.17±3.46^c	0.028
Propionate (mmol·L^{-1})	7.64±0.34^a	8.49±0.45^a	4.79±0.60^b	5.81±0.81^b	0.001
Butyrate (mmol·L^{-1})	3.94±0.39	4.45±0.31	3.37±0.58	4.21±0.46	0.378
Acetate : Propionate	4.66±0.27	4.41±0.53	5.72±0.82	4.30±0.71	0.370

Note: [a-c] Same row with different superscripts indicates a significant difference (*P* <0.01). AH = Alfalfa hay-fed; AHC = alfalfa hay plus concentrate-fed; CS = corn stalk-fed; CSC = corn stalk plus concentrate-fed

After sequence processing, a total of 410 247 high-quality sequences were obtained. The number of sequences for each sample ranged from 11 488 to 23 455. Rarefaction curves of methanogens based on the Shannon index at the OTU level are shown in Figure 1. The curves tended to be flat, indicating that the amount of sequencing data was large enough to reflect the diversity of the methanogens in the sample. Table 3 shows the richness and diversity indices of methanogens in the rumen of cashmere goats fed different diets. The Good's coverage of each group was >0.99, indicating that the sampling and sequencing reflected the diversity of the methanogens that were present. The Simpson index in group AHC was higher than that in group AH and CS (*P* < 0.05). However, the differences in the Sobs, Shannon, Ace, or Chao indices were not significant (*P* >0.05). A Venn diagram was constructed at the OTU level based on the sequences obtained from ruminal fluid samples from cashmere goats (Figure 2). A total of 106 OTUs were obtained from all samples, of which 47 OTUs existed in all groups (defined as core OTUs). The core OTUs comprised approximately 44.34% of the total OTUs. In addition, 10, 5, 4, and 3 OTUs were uniquely identified in groups AH, AHC, CS, and CSC, respectively. The ordination plot showed apparent spatial separation among the AH, AHC, CS, and CSC group based on methanogen communities (Figure 3).

Table 3 Measures of alpha diversity of methanogen communities in each group

Items	Treatment				*P*-Value
	AH	AHC	CS	CSC	
Sobs	37.83±2.27	36.33±3.17	41.50±2.73	36.83±3.89	0.64
Shannon	2.40±0.15	1.74±0.19	2.46±0.11	2.10±0.28	0.06
Simpson	0.15±0.03^b	0.33±0.07^a	0.14±0.01^b	0.23±0.06^{ab}	0.04
Ace	49.66±4.27	51.36±5.41	47.86±3.23	43.42±4.29	0.62
Chao	44.22±2.35	47.54±4.52	50.56±4.16	40.49±4.20	0.32
Coverage	0.995±0.00	0.994±0.00	0.995±0.00	0.995±0.00	0.20

Note: [a-b] Same row and different superscripts indicate a significant difference (*P* <0.01). AH = Alfalfa hay-fed; AHC = alfalfa hay plus concentrate-fed; CS = corn stalk-fed; CSC = corn stalk plus concentrate-fed.
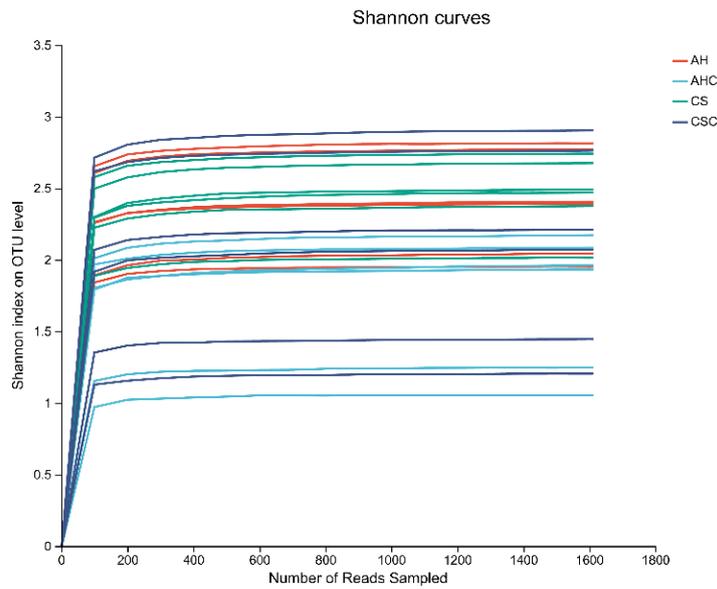
Figure 1 The operational taxonomic unit (OTU) rarefaction curves of the rumen fluid methanogen communities when feeding diets composed of alfalfa hay (AH), alfalfa hay plus concentrate (AHC), corn stalk (CS), and corn stalk plus concentrate (CSC) to cashmere goats
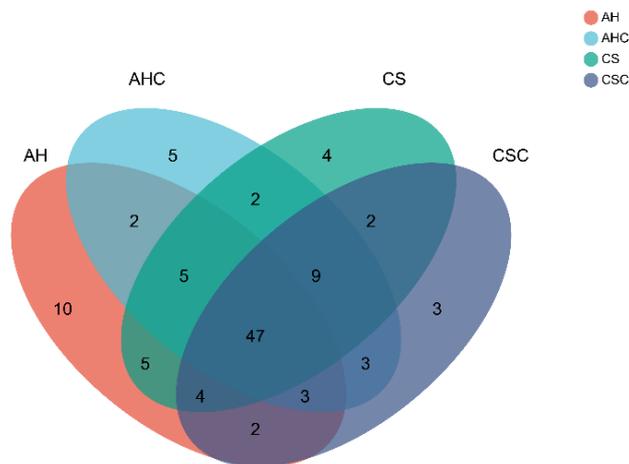

Figure 2 Venn diagram representation of the shared and exclusive operational taxonomic units at 97% similarity level of rumen microbes when feeding diets composed of alfalfa hay (AH), alfalfa hay plus concentrate (AHC), corn stalk (CS), and corn stalk plus concentrate (CSC) to cashmere goats
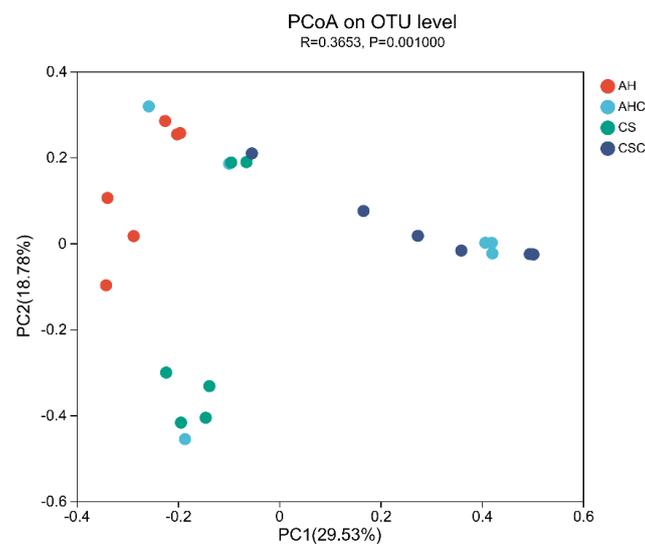
Figure 3 Principal coordinate analysis (PCoA) of methanogen operational taxonomic units (OTUs) in rumen fluid from cashmere goats fed diets composed of alfalfa hay, (AH), alfalfa hay plus concentrate (AHC), corn stalk (CS), and corn stalk plus concentrate (CSC). Principal component 1 (PC1) and principal component 2 (PC2) explain 29.53% and 18.78% of the variance, respectively.

The relative abundance of the various methanogenic archaea identified using the *mcr*A gene sequencing approach is shown in Table 4. Taxonomy-based analysis revealed that two phyla and six genera were detected across the 24 samples. At the phylum level, the relative abundance of Euryarchaeota in group AH and CS was higher than in group CSC ($P <0.05$). The relative abundance of Unclassified_d_Unclassified in group CSC (33.09%) was higher than that in group AH (0.70%) (P <0.05) and CS (8.60%) ($P <0.05$). At the genus level, the relative abundance of *Candidatus*_Methanomethylophilus in group AH (21.28%) was higher than group AHC (6.92%), CS (4.00%), and CSC (3.49%) ($P <0.05$).

The correlation heatmap was based on Spearman's correlation coefficient for the relationship between the methanogenic archaea community (at the genus level) and ruminal fermentation variables (Figure 4). The pH value was positively ($P <0.05$) related to the presence of *Methanosphaera*. A negative relationship ($P <0.05$) was observed between acetate and *Unclassified*_o_Thermoplasmatales.

Table 4 Relative abundances of methanogenic archaea at different classification levels based on *mcr*A sequences from Cashmere goats fed diets composed of Alfalfa hay, (AH), alfalfa hay plus concentrate (AHC), corn stalk (CS), and corn stalk plus concentrate (CSC)-(The proportion of total methanogenic archaea higher than 1%)

Items	Treatment				*P*-Value
	AH	AHC	CS	CSC	
Phylum					
Euryarchaeota	99.30±0.18[a]	77.41±11.66[ab]	91.40±1.44[a]	66.91±8.15[b]	0.02
Unclassified_d_Unclassified	0.70±0.18[b]	22.59±11.66[ab]	8.60±1.44[b]	33.09±8.15[a]	0.02
Genus					
*Unclassified*_c_Thermoplasmata	48.09±7.50	44.97±12.89	31.08±1.59	26.42±6.79	0.21
Methanobrevibacter	14.74±6.09	18.61±6.48	41.22±7.73	20.73±6.70	0.16
*Unclassified*_d_Unclassified	0.70±0.18[b]	22.59±11.66[ab]	8.60±1.44[b]	33.09±8.15[a]	0.02
Methanosphaera	14.88±4.60	5.61±2.89	13.22±5.36	13.78±5.63	0.51
*Candidatus*_Methanomethylophilus	21.28±6.29[a]	6.92±2.15[b]	4.00±1.94[b]	3.49±1.21[b]	0.04
*Unclassified*_o_Thermoplasmatales	0.08±0.07	1.21±0.55	1.77±0.31	2.43±1.19	0.12

Note: [a-b] The same row different superscripts mean significant difference ($P < 0.01$). AH = Alfalfa hay-fed; AHC = alfalfa hay plus concentrate-fed; CS = corn stalk-fed; CSC = corn stalk plus concentrate-fed.
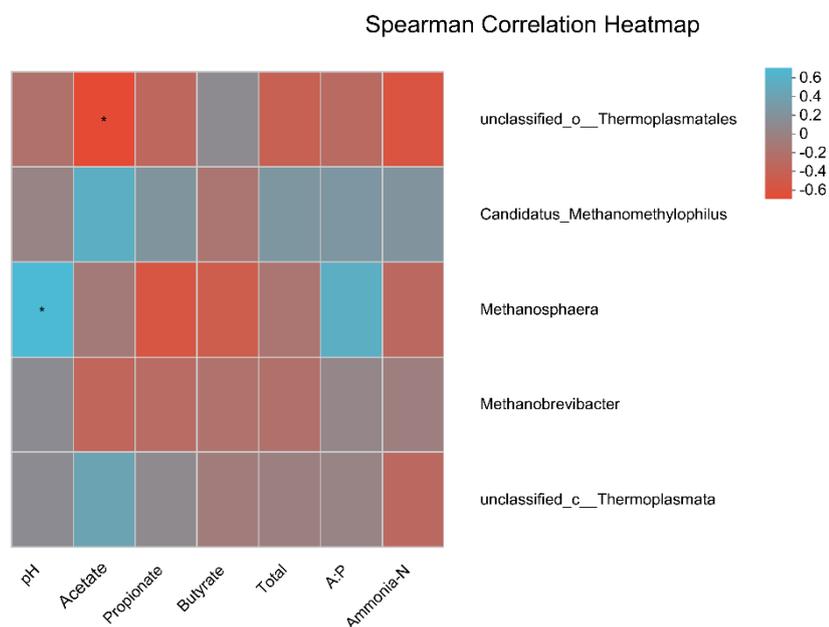

Spearman Correlation Heatmap

Figure 4 Environmental factors associated with the methanogenic archaea community at the genus level. The total was the group of volatile fatty acids (VFA) detected in the rumen fermentation. A:P was the acetate to propionate ratio. The units for acetate, propionate, butyrate, and total are mmol·L^{-1}. The significant difference between any two parameters within this figure was represented by using * ($P <0.05$) or ** ($P <0.01$).

Discussion

Diets are substrates for rumen microbial fermentation; their nutritional composition and physical properties directly affect rumen fermentation patterns in ruminants. NH_3-N is an important parameter to reflect the rumen fermentation index. The concentrations of ammonia in the rumen represent a balance between formation (by microbial degradation of protein, amino acids, urea, and nucleic acids) and removal (by synthesis of microbial biomass, absorption across the rumen epithelium, and by outflow to the omasum) (Knight *et al.*, 2011). In the current study, the concentration of NH_3-N in group AHC was substantially higher than in groups AH, CS, and CSC. In addition, the concentrations of the propionate, acetate, and total VFAs were higher in group AH and AHC than in group CS and CSC. These results may be related the higher readily degradable fraction of carbohydrates and protein in alfalfa (Wang *et al.*, 2019). Compared with corn stalks, alfalfa hay contains a higher proportion of nonfibrous carbohydrates and a lower proportion of neutral detergent fibre (NDF). In this study, the NDF content in group AH (57.76%) was lower than in group CS (71.32%). Mccaughey *et al.* (1999) studied the effects of different types of forage on beef cattle methane production and found that, compared with pure gramineous forage grass, mixed forage of alfalfa and gramineous forage grass had a lower fibre content and higher rumen flow rate, and the methane production was reduced by 9%. When the proportion of NDF in dairy cow diets was increased from 31.5% to 38%, the daily methane production increased by 23% (Dong *et al.*, 2019). Therefore, reducing the content of NDF is an effective method to reduce methane production from ruminants. According to previous reports, methane production from concentrate-based diets is lower than that from forage-based diets (Pedreira *et al.*, 2013). The reason is that fermentation of the starch in the rumen will produce more propionate, and the increased level of propionate can compete with methanogens for H_2, thereby reducing methane production. Furthermore, starch has a faster digestion and fermentation rate than cellulose, which will lower the rumen pH, thereby inhibiting the growth of methanogens (Beauchemin *et al.*, 2020). In the present work, because of the small proportion of concentrate added to the diets, the rumen pH value in groups AH, AHC, CS, and CSC did not differ substantially.

The diet, as a substrate for microbial fermentation, its physical properties, and nutritional composition directly affects methanogenic archaea diversity. In this experiment, by using a high-throughput sequencing method based on the *mcr*A gene, we investigated the α-diversity and β-diversity indices of ruminal methanogens in cashmere goats on different diets. The Simpson index observed in this study was substantially influenced by the diet, and the PCoA chart showed apparent spatial separation among the AH, AHC, CS, and CSC groups based on methanogen communities. These data indicate that the different diets had a marked effect on the composition of methanogen communities in the rumen. In previous studies, similar results were observed by Franzolin *et al.* (2012), when analyzing the ruminal methanogenic archaea communities of water buffalo fed different diets. The different diets had a marked effect on the ruminal methanogen population structure. Dong *et al.* (2019) performed high-throughput sequencing based on *mcr*A and found that changes in the liquid diet, solid diet, and weaning time of Holstein's calves led to changes in the composition of the methanogens in the rumen.

Typically, archaeal communities account for only 3–4% of the rumen microbiome, while Euryarchaeota are the dominant archaea in the rumen (Kumar *et al*., 2015). Wang *et al*. (2017) reported that Euryarchaeota accounted for 82% of the composition of methanogens in the ruminal fluid of goats, which is in accordance with the methanogen densities found in this study. In the present study, the mean relative abundance of Euryarchaeota accounted for approximately 84% of the total sequences. In addition, the relative abundance of Euryarchaeota in group AH was higher than in groups CS and CSC. Shi *et al*. (2014) and Danielsson *et al*. (2017) reported weak or no relationships between the methane production of individual animals and the total abundance of archaea in their rumen. By contrast, it seems that the composition of the methanogenic archaeal community is highly correlated with methane production. Wallace *et al*. (2015) applied metagenomics on the rumen microbial community of dairy cows and found that the relative abundance of *Methanobrevibacter* in the rumen of dairy cows with high methane production was higher than that of dairy cows with low methane production. In the present study, the dominant methanogenic archaeal identified in the rumen of the cashmere goats varied among the different treatments. *Unclassified*_c_Thermoplasmata was the overwhelming dominant methanogen in group AH and AHC at the genus level, whereas *Methanobrevibacter* was the overwhelming dominant methanogen in group CS and CSC. Therefore, we conclude that the differences in methane emissions between diets with corn stalk as roughage and those with alfalfa hay as roughage may be related to *Methanobrevibacter.* Moreover, the relative abundance of *Unclassified*_c_Thermoplasmata was highest in groups AH and AHC. This may be caused by the variable production of methylated substrate compounds (methylamine and methanol) in the rumen, which is mediated by alfalfa hay. Peng *et al*. (2021) reported that the methanol-utilizing archaea, Thermoplasmata, was enriched on alfalfa, which had the highest pectin content of all the four substrates they used (alfalfa stems, bagasse, reed canary grass, and xylan). A primary degradation product of pectin is methanol and this probably explains the enrichment of Thermoplasmata on alfalfa.

In the present study, the acetate concentration was negatively related to *Unclassified*_o_Thermoplasmatales, possibly because Thermoplasmatales reduce methanol or other methylated compounds for methanogenesis. Extensive evidence has demonstrated that the formation of acetate results in the production of additional methanogenic substrates (formate and H_2) (Danielsson *et al*., 2017). Most previous studies that have investigated the structure of the methanogen community have found that *Methanobrevibacter* phylotypes are the dominant methanogens in the rumen of different ruminants worldwide (Fouts *et al*., 2012; Seedorf *et al*., 2015). However, less research has reported that the archaeal community is dominated by the methanogenic archaea variably classified as the members from the seventh order of Thermoplasmatales, i.e., Methanomassiliicoccales (previously called Rumen Cluster C, RCC) (Borrel *et al*., 2014; Noel *et al*., 2016). Iino *et al*. (2015) reported that the Methanomassiliicoccales mainly produce methane through the methylotrophic pathway and can use methylamine and methanol as substrates for methanogenesis. Methanomassiliicoccales belong to a branch of Thermoplasmata (Tajima *et al*., 2001). *Unclassified*_c_Thermoplasmata and *Candidatus*_Methanomethylophilus belong to the Methanomassiliicoccales-affiliated groups (Noel *et al*., 2016). The present study showed that *Unclassified*_c_Thermoplasmata, *Methanobrevibacter*, *Methanosphaera*, and *Candidatus*_Methanomethylophilus were the dominant methanogens in the rumen fluid of cashmere

goats. This result concurs with other studies on the composition of rumen methanogen communities in ruminants (Wang *et al.*, 2017; Zhu *et al.*, 2017). The current results showed that the relative abundance of *Candidatus*_Methanomethylophilus was higher in group AH than in the other groups, and *Unclassified*_c_Thermoplasmata was the overwhelming dominant methanogen in group AH and AHC. We speculate that the differences in methane production in group AH and group CS may be related to the relative abundance of Methanomassiliicoccales-affiliated groups, but further studies are needed to confirm this. In the present study, we also found the Methanomassiliicoccales-affiliated groups were the dominant methanogens found in the alfalfa hay-based diets, and *Methanobrevibacter* was the main methanogen found in the corn stalk-based diets. These results may be related to the chemical composition of the diet. Compared with the alfalfa hay-based diets, the corn stalk-based diets had lower crude protein content. However, there are few reports on the effects of dietary crude protein on the methanogenic archaea community. Furthermore, in the present study, the pH value was positively related to *Methanosphaera*. The rumen pH is a consequence of feed degradation and microbial function. Dong *et al.* (2019) also reported the pH value was substantially related to the presence of *Methanosphaera* in the rumen of Holstein bull calves. This concurs with the current study. However, further studies should be conducted to explore the relationship between pH value and *Methanosphaera*.

Conclusion

In summary, the ruminal fermentation parameters and the methanogen communities in the rumen of cashmere goats varied when fed different diets. With the alfalfa hay-based diets, the Methanomassiliicoccales-affiliated groups were the dominant methanogen found in the rumen fluid of the goats. However, when fed the corn stalk-based diets, *Methanobrevibacter* was the main methanogen found in the rumen fluid. These results are related to dietary composition, especially crude protein content. These analyses provide further insight into diets that can be used to mitigate enteric methane emissions in ruminant livestock production systems.

Authors' Contributions

KNL was responsible for laboratory analyses, data collection, analysis of results, and writing the manuscript. YRW contributed to laboratory analyses and data acquisition. RHN supervised the project planning and statistical analysis of the results and critically reviewed the manuscript.

Conflict of interest declaration

The authors declare there are no competing interests.

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