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Forestomach fermentation and microbial communities of alpacas (*Lama pacos*) and sheep (*Ovis aries*) fed maize stalk-based diet

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ABSTRACT. The aim of the study was to investigate the differences of forestomach digestion and microbial communities between alpacas and sheep fed maize stalk-based diet. Six alpacas and six sheep were housed in metabolic crates, and were fed low-quality diet (30% maize-based concentrate and 70% rubbed maize stalk) twice a day. The animals were adapted to diet for 18 days, followed by 3 days of sampling. It was shown that alpacas had lower ($P < 0.001$) feed intake than sheep, but presented similar apparent digestibility in the total tract in comparison with sheep. The concentrations of propionate, valerate, isobutyrate and isovalerate in alpaca forestomach were higher ($P < 0.001$) than those in sheep, whereas acetate and acetate:propionate ratio was lower ($P < 0.001$) in alpacas than in sheep. The concentrations of ammonia-N and microbial protein in alpaca forestomach were 23 and 33% lower than those in sheep, respectively. For ruminal microbes, the proportions of fibrolytic bacteria *Clostridium* and *Pseudobutyrivibrio* were higher ($P < 0.05$) in alpacas than those in sheep, but the proportion of proteolytic bacteria *Selenomonas* was lower ($P < 0.05$) in alpacas. In conclusion, the forestomach fermentation characteristics in alpacas and sheep fed low-quality maize stalk diet were significantly different, and this phenomenon may result from the different composition of ruminal microbes (such as carbohydrate degrading bacteria and proteolytic bacteria).

Introduction

Alpacas (*Lama pacos*) and llamas (*Lama glama*) are domesticated species of South American camelid (SAC), and have been imported by many countries in recent decades (Davies et al., 2007). As the important economically species for wool and meat production (San Martin and Bryant, 1989), alpacas and llamas were investigated extensively, especially their forestomach characteristics and nutritional strategies (Pei et al., 2013; Ortiz-Chura et al., 2018). It is described (Vallenas et al., 1973; San Martin and Bryant, 1989) that alpacas and llamas have similar anaerobic fermentation process and end-product volatile fatty acids (VFA) production, but lower energy and protein requirements in comparison to true ruminants.

It was reported (San Martin and Bryant, 1989; Dulphy et al., 1994) that SAC show stronger low-quality food digestion capacity than sheep, and it was speculated that this phenomenon was due to a higher ruminal retention time for the solid phase, and a more efficient nitrogen recycling. Higher pH stability of forestomach may be another reason why alpacas exhibit better digestion capacity of low-quality food than sheep (Eckerlin and Stevens, 1973). In addition, the differences in forestomach fermentation characteristics including ammonia-N (NH_3-N), redox potential, osmolarity, surface potential, and

forestomach pressure between alpacas and sheep might influence digestive efficiency of both animals (Liu et al., 2009).

Except for physiological factors, rumen microbial community may be also a part of differences in forestomach digestion (Pei et al., 2013). We have previously shown that there were apparent differences in the bacterial diversity and abundance in alpaca forestomach and sheep rumen fed alfalfa (Pei et al., 2010). Besides, lower population of methanogens and higher percentage of cellulolytic fungi have been also found in alpaca forestomach when compared with sheep rumen fed fresh alfalfa as a forage source (Pei et al., 2013).

Unfortunately, the studies on the forestomach digestion coupled with microbiota descriptions in alpacas and sheep, especially under low-quality maize stalk diet, are scarce. So, the aim of the study was to comprehensively investigate the forestomach fermentation parameters and microbial communities of alpacas and sheep fed maize stalk-based diets.

Material and methods

Animals and experimental design

Six male alpacas (12 ± 2 months old, 29.5 ± 7.1 kg) and six male sheep (12 ± 2 months old, 27.9 ± 2.7 kg), were used in the study. The animals were housed in metabolic crates (1.2 × 1.6 m) with expanded metal flooring and fed low-quality diet (30% maize-based concentrate and 70% rubbed maize stalk). The composition and nutritional value of diet are shown in Table 1. Maize stalk was harvested during September and October, and chopped manually at 3–4 cm length before being fed to animals. Animals were offered diets twice a day (07:00 and 19:00) *ad libitum* and had free access to fresh water. Experimental period included 18 days of adaptation to the diet and then 3 days of sampling. The experiment was conducted at the Shanxi Agriculture University, and the protocol was approved by the Animal Care and Use Committee of the Shanxi Agriculture University.

Table 1. Ingredient and nutrient levels of alpaca and sheep, % dry matter (DM)

Indices	Amount
Ingredient	
maize stalk, cracked	70.0
maize grain, ground	5.1
soyabean meal	17.7
rapeseed meal	2.85
soyabean oil	2.85
premix[a]	1.5
Nutrient	
crude protein, %	13.22
neutral detergent fibre, %	44.79
acid detergent fibre, %	32.62
metabolizable energy (ME), MJ/kg DM[b]	11.16

[a] contained per kg of diet: g: NaCl 1.5, mg: $CuSO_4$ 22.8, $ZnSO_4$ 98.7, $MnSO_4$ 90.7, KI 1, $FeSO_4$ 326, Na_2SeO_3 0.7, $CoCl_2$ 0.6, IU: vit. A 3000, vit. D 500, vit. E 300; [b] calculated using the formula: ME (MJ/kg DM) = 11.78 + 0.00654 crude protein + (0.000665 ether extract)^2 – crude fibre (0.00414 ether extract) – 0.0118 ash

Measurements and collection of samples

Feed offered and refusals were weighted daily to calculate feed intake of the animals. The samples of feed refusals were collected once a day and then composited by period. The faeces were collected by harness-collection bag sets and were dried in an oven at 55 °C for 48 h, and then composited by period. The feeds and faeces were ground to pass a 1-mm screen with a mill (FZ102, Shanghai Hong Ji instrument Co., Ltd., Shanghai, China) for chemical analysis. Urine was collected into a container with 50 ml 12 M sulphuric acid and the volume was recorded daily.

Approximately 100 ml of forestomach fluid was taken anaerobically *via* oesophagus using a stomach tube (outer diameter 1 cm, inner diameter 0.8 cm, length 200 cm) connected to a 100-ml syringe, from several sites (the front and middle of the ventral sac and the cranial sac) within the forestomach after morning feeding (0, 3, 6 and 9 h), on three consecutive days at the end of each experimental period. The samples were filtered through cotton gauze (4-sheets) and 'ruminal fluid' was obtained. Then the 'ruminal fluid' was used to the microbial community determination, pH, VFA and NH_3-N analyses. The pH values were measured with an electric pH meter (Sartorius AG, Goettingen, Germany). Filtrate (5 ml) was preserved by adding 1 ml of 250 g/l (w/v) meta-phosphoric acid, and 1 ml of 20 g/l (w/v) H_2SO_4 to determine VFA and NH_3-N, respectively.

Chemical analysis

The dry matter (DM) content of samples was determined by oven drying (135 °C, 3 h), the ash content was determined by incineration at 550 °C for 5 h (AOAC, 1990). The organic matter (OM) content was calculated based on the difference between DM and ash content. The crude fibre (CF) was determined using the Weende method (Henneberg and Stohmann, 1859) and ether extract (EE) with the method of AOAC (1990). The acid detergent fibre (ADF) contents were determined according to the

methods described by Van Soest et al. (1991). The neutral detergent fibre (NDF) was determined according to Mertens (2002).

The nitrogen concentration in the samples was determined by the Kjeldahl method (AOAC, 1990) and multiplied by 6.25 to obtain crude protein (CP) concentration. The VFA was separated and determined by a gas chromatography (GC122; Shanghai Jingke instrument Co., Ltd., Shanghai, China) with 2-ethylbutyric acid as the internal standard. The concentration of NH_3-N, MCP as well as the content of phosphorus and calcium were determined using the method of AOAC (1990).

DNA extraction, PCR amplification and 16S rRNA gene sequencing

Microbial DNA was extracted from forestomach fluid based on the bead-beating method described by Zoetendal et al. (1998). Microbial DNA was amplified using the 517F/926R primers set (517F: 5'-GCCAGCAGCCGCGGTAA-3', 926R: 5'-CCGTCAATTYYTTTRAGTTT-3'). PCR product was purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences; Union City, CA, USA). Purified PCR products were sequenced using an Illumina MiSeq platform according to standard protocols.

Bioinformatics and statistical analysis

Operational taxonomic units (OTUs) were clustered with a 97% similarity cut-off using UPARSE (version 7.1, http://drive5.com/uparse/), and chimeric OTUs were identified and removed using UCHIME. Mothur was used to calculate the alpha diversity including ACE, Chao1, Shannon, Simpson and coverage. Principal coordinate analysis (PCoA) was conducted using the unweighted UniFrac distance method (Lozupone and Knight, 2005). Analysis of molecular variance (AMOVA) was conducted using the programme MOTHUR v.1.29.0.

All statistical analyses were performed using SPSS (SPSS Statistics 21, SPSS Inc., Chicago, IL, USA) software packages. The statistical model on animal species was:

$$Y_i = \mu + A_j + P_f + e_{ijkf},$$

where: Y_i – abundance or relative abundance of a given classification of microorganisms, sequences, or OTU; μ – mean; A_j – fixed effect of animal species j; P_f – fixed effect of experimental period f; and e_{ijkf} – experimental error. Post-hoc multiple comparisons were made to compare the means using Fisher's least significant difference (LSD). Differences were considered significant when $P < 0.05$.

Results

Apparent digestibility in the total tract

The DM intake was 38% lower in alpacas (547.8 g/day) than in sheep (880.4 g/day). The digestibilities of DM, OM, CP, EE, NDF and ADF were similar in alpacas and sheep (Table 2). The digestibility of phosphorus was higher ($P = 0.081$) in alpacas than in sheep, while that of calcium was lower ($P = 0.067$) in alpacas.

Table 2. Diet digestibility in the total tract of alpacas (*Lama pacos*) and sheep (*Ovis aries*) fed maize stalk diet

Indices	Alpaca	Sheep	SE	*P*-value
Intake, g/day				
dry matter	547.8	880.4	14.28	0.001
Digestibility				
dry matter	65.1	67.1	2.94	0.509
organic matter	68.0	69.3	2.85	0.663
crude protein	72.5	71.2	11.19	0.910
ether extract	92.6	92.8	0.96	0.840
neutral detergent fibre	57.4	55.8	6.20	0.798
acid detergent fibre	46.6	47.7	5.78	0.899
Calcium	31.6	41.4	4.80	0.067
Phosphorus	41.7	25.5	8.35	0.081

SE – standard error

Forestomach fermentation characteristics

Forestomach fermentation characteristics in alpacas and sheep fed maize stalk are shown in Table 3. The fermentation profiles of propionate, valerate, isobutyrate and isovalerate in alpacas were higher ($P < 0.001$) than those in sheep, whereas acetate and acetate:propionate ratio (A/P ratio) was lower ($P < 0.001$) in alpacas than in sheep. The concentrations of ammonia-N and microbial protein in alpaca forestomach were 23 and 33% lower than those in sheep, respectively.

Table 3. Forestomach fermentation characteristics of alpacas (*Lama pacos*) and sheep (*Ovis aries*) fed maize stalk diet

Indices	Alpaca	Sheep	SE	*P*-value
Total volatile fatty acids, mM mol/100mol	66.29	66.72	1.110	0.851
acetate (A)	63.72	66.12	0.236	0.001
propionate (P)	24.04	22.83	0.155	0.001
butyrate	8.15	8.35	0.104	0.339
valerate	1.34	1.01	0.026	0.001
isobutyrate	1.07	0.80	0.014	0.001
isovalerate	1.66	0.89	0.044	0.001
A/P ratio	2.66	2.92	0.026	0.001
Microbial crude protein, mg/ml	0.64	0.95	0.043	0.001
NH_3-N, mg/100ml	11.63	15.14	0.628	0.010
pH	6.76	6.72	0.026	0.498

SE – standard error

However, the fermentation profiles of pH values, total VFA, and butyrate were similar in alpacas and in sheep.

Forestomach microbial community

In this study, 32 211 and 33 038 high-quality sequences per sample from alpaca forestomach and sheep rumen, respectively were obtained (Table 4). The number of OTUs per sample was similar in alpacas (1139) and sheep (1103). Microbial community diversity indices ACE, Chao1 and Shannon were slightly, but not significantly, higher ($P > 0.10$) in alpacas than in sheep (Table 4). However, the Simpson indices were lower ($P > 0.10$) in alpacas than in sheep.

Table 4. The diversity of bacterial communities in forestomach fluid of alpacas and sheep

Indices	Alpaca	Sheep	SE	*P*-value
Reads	32211	33038	2833	0.776
OTUs	1139	1103	74.05	0.436
ACE indices	1304	1280	45.11	0.615
Chao1 value	1307	1289	48.17	0.717
Shannon indices	5.46	5.29	0.10	0.139
Simpson indices	0.014	0.017	0.0042	0.237
Coverage, %	99.31	99.29	0.10	0.842

SE – standard error

Table 5. Bacterial phyla and selected genera in forestomach bacterial community of alpaca and sheep, % of total sequences

Indices	Alpaca	Sheep	SEM	*P*-value
Bacteroidetes	62.733	64.717	1.676	0.422
Prevotella	31.495	34.390	2.045	0.6991
Rikenellaceae RC9	21.392	18.830	1.8619	0.5177
Prevotellaceae UCG-001	2.907	3.708	0.3390	0.2559
Prevotellaceae UCG-003	3.037	4.186	0.3315	0.08146
others	3.902	3.608	-	-
Firmicutes	32.180	31.324	1.547	0.704
Christensenellaceae R-7	2.881	3.358	0.2692	0.4019
Ruminococcaceae UCG-011	2.266	1.649	0.2431	0.2189
Erysipelotrichaceae UCG-004	1.759	3.475	0.8422	0.3313
Lachnospiraceae UCG-004	1.640	3.025	0.3676	0.05414
Saccharofermentans	0.898	0.884	0.07104	0.9291
Quinella	1.081	0.157	0.272	0.037
Butyrivibrio	0.925	0.645	0.1002	0.1727
Ruminococcus	1.056	0.863	0.1361	0.093
Pseudobutyrivibrio	0.768	0.312	0.107	0.013
Selenomonas	0.404	0.624	0.083	0.003
Clostridium	0.290	0.144	0.039	0.024
others	18.716	16.406	-	-
Spirochaetae	1.889	0.954	0.212	0.011
Treponema	1.673	0.489	0.162	<0.001
others	0.216	0.465	-	-
Proteobacteria	1.278	0.867	0.111	0.026

SEM - standard error

In total, 18 phyla were identified in all samples (Table 5). Bacteroidetes (62.73 and 64.72%) were the most dominant and more numerous than Firmicutes (32.18 and 31.12%) in both alpacas and sheep (Table 5). The other phyla were of low-relative-abundance for the percentage under 2% in the total bacterial communities. In addition, the relative abundances of Spirochaetes, Proteobacteria and Nitrospirae in alpacas were higher ($P < 0.05$) than that in sheep, while the relative abundances of Chloroflexi ($P = 0.013$) and Fibrobacteres ($P = 0.073$) in alpacas were lower than that in sheep.

At the genus level, a total of 251 genera were detected in the forestomach of alpaca and sheep. Sixteen genera were discovered only in alpacas, and eleven genera were found only in sheep. In addition, the proportions of *Treponema*, *Quinella* and *Pseudobutyrivibrio* were higher ($P < 0.05$) in alpacas than those in sheep, but the proportion of *Selenomonas* was lower ($P < 0.05$) in alpacas (Table 5, Figure 1).

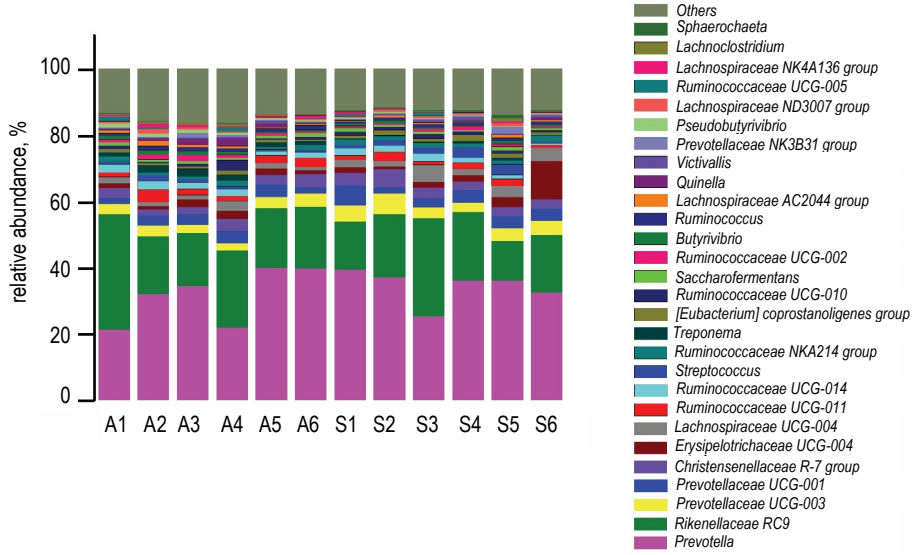

Figure 1. Relative abundances of bacterial taxa at the genus level in the forestomach of alpacas (A) and sheep (S)

At the species level, the proportions of *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* (0.013 and 0.174%, respectively) were higher ($P > 0.05$) in alpacas than those (0.004 and 0.106%, respectively) in sheep.

The PCoA results of overall diversity based on an unweighted UniFrac metric showed that bacterial communities in alpacas were slightly different from that of sheep, as shown by PC1, which accounted for 25.02% of the total variation (Figure 2).

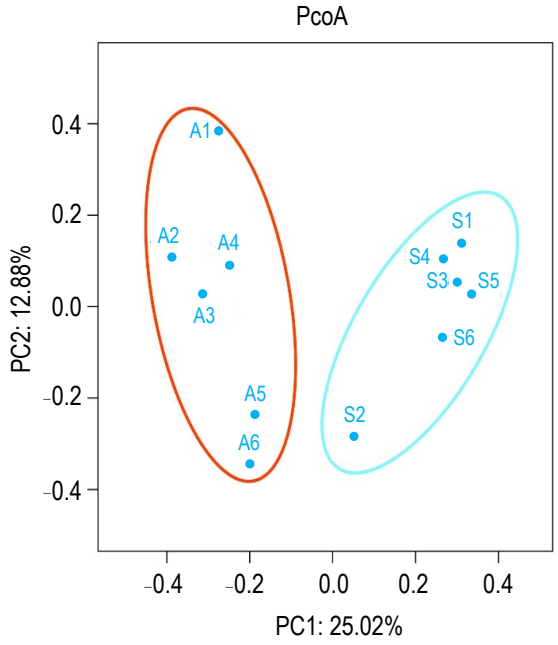

Figure 2. Principal coordinate analysis (PCoA) results showing relationships of bacterial communities in the forestomach of alpacas (A) and sheep (S). The PCoA plots were constructed using the unweighted UniFrac method

Discussion

It was found that alpacas had a lower DM intake than sheep when fed maize stalk as roughage. It is consistent with the results reported by Liu et al. (2009), who noted that feed intake (sorghum-sudan or fresh alfalfa) was lower in alpacas than in sheep. Lower DM intake values in alpacas probably result from smaller rumen volumes and lower particulate passage (San Martin, 1987). In addition, the lack of differences in nutrient digestibility (DM, OM, CP, EE, NDF and ADF) between alpacas and sheep in the present study is in line with previous findings (San Martin et al., 1982; Liu et al., 2009). However, numerous studies have suggested that SAC show an apparently better digestion capacity than sheep (San Martin and Bryant, 1989; Dulphy et al., 1997). The discrepancy between studies could be due to the CP content in diet. Greater digestion coefficients were found in alpacas than in sheep fed diets with less than 7.5% CP, but similar digestion coefficients were detected in those animals when dietary CP was above 10.5% (San Martin and Bryant, 1989). In the present study, the similar nutrient digestibility between alpacas and sheep may closely correlate with the content (13.22%) of dietary CP but not the roughage (maize stalk).

The total VFA concentration in the forestomach was similar in alpacas and sheep, as it was previously observed by Vallenas et al. (1973). Analysing concentrations of various kinds of VFA, lower acetate as well as A/P ratio were found in alpacas. However, Liu et al. (2009) found in alpacas similar A/P ratio but higher acetate than in sheep fed alfalfa or sorghum diet. This discrepancy can be probably linked to the properties of roughage. Maize stalk inherent complex lignocellulosic structures and contain higher proportion of lignin preventing the digestion by ruminal microbes, than alfalfa or sorghum (Himmel et al., 2007). In this study, higher Firmicutes/Bacteroidetes ratio was found in alpacas fed maize stalk-based diet. Firmicutes and Bacteroidetes are the most abundant phyla in ruminant forestomach (Petri et al., 2012; Plaizier et al., 2016), they are the major degraders of polysaccharides including cellulose, starch, hemicellulose, xylan and so on (Martens et al., 2011). The changes in the composition of microbiota may be leaded to lower acetate and higher propionate in alpacas.

In the present study, higher NH_3-N concentration was observed in sheep rumen than in alpaca forestomach when maize stalk was fed as forage source. This finding coincided with the results of previous study (Liu et al., 2009) in which higher NH_3-N concentration was noted in sheep fed alfalfa or sorghum-sudan diet. Higher concentration of NH_3-N in sheep might be due to the higher DM intake (major intake of N) than in alpacas (Ortiz-Chura et al., 2018), however, more studies are needed to confirm this hypothesis. Besides, the concentration of NH_3-N in the rumen could be associated to the degradation of dietary protein and NH_3-N absorption by ruminal microbes (Ushida et al., 1986; Belanche et al., 2012). In the present study, higher proportions of proteolytic bacteria *Selenomonas* spp., which significantly increased NH_3-N production (Liu et al., 2020), was found in sheep rumen. In addition, fibrolytic bacteria are highly dependent on NH_3-N availability as a source of N (Russel et al., 1992). In this study, higher abundance of fibrolytic bacteria,

such as *Butyrivibrio, Selenomonas*, *Clostridium*, *F. succinogenes* and *R. flavefaciens* were found in alpaca forestomach, and may be one of the reasons for lower NH_3-N concentration in alpacas. Except proteolytic and fibrolytic bacteria, protozoa and animal species may be also attributed to lower NH_3-N concentration in alpacas.

It is generally agreed that dietary composition was one of the major factors influencing types and numbers of forestomach microbial communities (Ley et al., 2008; Henderson et al., 2015). However, to date, little information is available on microbial populations present in forestomach of alpacas fed low-quality roughages. In this study, high-throughput sequencing was used to reveal the composition and biodiversity of the forestomach microbial community in alpacas and sheep fed maize stalk as roughage. It was shown that Bacteroidetes is the most abundant bacteria phylum in the forestomach bacterial community of alpacas and sheep. Additionally, the genus *Prevotella*, which was the dominated genus under Bacteroidetes, reached up to 31.5 and 34.4% of the total bacterial communities in alpacas and sheep, respectively. This finding is in agreement with the results of previous studies indicating that *Prevotella* was one of the most abundant genera in the forestomach (Bekele et al., 2010). This likely reflects that the dominant bacteria, such as *Prevotella*, *Butyrivibrio*, *Pseudobutyrivibrio* and *Selenomonas* are likely to be responsible for the majority of the transformation of ingested and used feed for microbial growth in the rumen of alpacas and sheep (Bekele et al., 2010).

It was believed that the host phylogeny influence gut bacterial diversity, and bacterial communities diversified with their hosts (Ley et al., 2008). Although they all belong to Artiodactyla, alpacas are classified as Camelidae, and sheep as Bovidae. In this study, significant differences in the diversity and richness of bacterial communities between alpacas and sheep were revealed by AMOVA analysis ($P < 0.0002$) and PCoA plot. For example, at the genus level, 16 genera were found only in alpacas as well as 11 genera were discovered only in sheep, and the major determinant for the difference of bacterial community composition in the forestomach may be animal species. Furthermore, significantly different ($P < 0.05$) abundance of some bacteria was found in alpacas and sheep, such as *Pseudobutyrivibrio, Selenomonas,* and *Treponema*. This phenomenon may be related to the discrepancy of forestomach environments. It is described that SAC showed faster liquid passage rate (Clemens and Stevens, 1980) and longer gastrointestinal retention time of digesta (Yao et al., 2015), which may influence the interaction of microorganisms and feed particles.

Conclusions

The apparent digestibility in the total tract was similar, but the forestomach fermentation characteristics were different in alpacas and sheep when offered low-quality maize stalk diet. The different forestomach fermentation patterns may result from the different composition of forestomach microbiota (such as carbohydrate degrading bacteria and proteolytic bacteria).

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