Response of the Rumen Microbiota of Sika Deer (Cervus nippon) Fed Different Concentrations of Tannin Rich Plants

Zhipeng Li1, André-Denis G. Wright2, Hanlu Liu1, Zhongyuan Fan1, Fuhe Yang1, Zhigang Zhang2*, Guangyu Li1*

1 Jilin Provincial Key Laboratory for Molecular Biology of Special Economic Animals, Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, Changchun, Jilin, China, 2 State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan, China, 3 School of Animal and Comparative Biomedical Sciences, University of Arizona, Tucson, Arizona, United States of America

* zhangzhigang@mail.kiz.ac.cn (ZZ); tcslgy@126.com (GL)

Abstract

High throughput sequencing was used to examine the rumen microbiota of sika deer fed high (OLH) and low concentration (OLL) of tannin rich oak leaves. The results showed that Prevotella spp. were the most dominant bacteria. The most predominant methanogens were the members of the order Methanoplasmatales. The dominant rumen protozoa were Entodinium longinucleatum, Eudiplodinium maggii, and Epidinium caudatum, and the fungal communities were mostly represented by Piromyces spp. Moreover, the relative abundance of Pseudobutyrivibrio spp. (P=0.026), unidentified bacteria (P=0.028), and Prevotella spp. (P=0.022) was lower in the OLH group than in the OLL group. The concentration of propionate in the OLH group was greater than in the OLL group (P=0.006). Patterns of relationships showed that methanogens belonging to the order Methanoplasmatales were negatively correlated with Treponema spp., Ent. Longinucleatum, and acetate. Methanosphaera stadtmanae was positively correlated to propionate, while Methanobrevibacter ruminantium was negatively associated with Methanobrevibacter thaueri and Methanobrevibacter millerae. Tannins altered the rumen microbes and fermentation patterns. However, the response of the entire rumen microbiota and the relationship between rumen microorganisms and the fermentation parameters were not fully understood.

Introduction

Plant tannins (condensed and hydrolysable tannins) are ubiquitously distributed in forage trees, shrubs, legumes, and tree bark [1]. Previous studies reported that plant tannins had beneficial effects on protein metabolism, on fermentation patterns such as volatile fatty acids (VFAs), prevention of frothy bloat, and the potential to decrease methane emissions from ruminants [2–4].
The rumen is inhabited by a diverse consortium of microorganisms, including bacteria, archaea, protozoa, fungi, and viruses. Collectively referred to as the rumen microbiota, these microorganisms have a symbiotic relationship with the ruminant, and play a critical role in biomass degradation [5]. Therefore, the altered rumen metabolism is directly linked to the rumen microbiota [6,7]. However, there have been limited studies linking high-throughput sequence data of the rumen microbiota with fermentation patterns in response to feeding diets containing different concentrations of tannins.

A few studies have provided evidence for the relationship between the particular bacterial groups and fermentation products in the rumen. For example, Carberry et al. [8] found negative associations between Prevotella spp. and isobutyrate, and between Prevotella spp. and isovalerate concentrations in the rumen liquid based on the Spearman’s partial correlation. Kittelmann et al. [9] found that there was a positive correlation between the occurrence of the Methanobrevibacter ruminantium clade and bacteria in the family Fibrobacteraceae based on the Spearman’s rank correlations. Moreover, occurrence of the Methanobrevibacter gottschalkii clade was positively correlated with bacteria in the family Ruminococcaceae. In our previous study, the relationship, or interplay patterns between the bacterial community and the fermentation parameters in the rumen of sika deer fed tannin rich plants: oak leaves (Xylosma racemosum; tannin content, 100 mg/1 kg dried matter) based diets, were distinctly different than in the rumen of sika deer fed corn stover or corn silage based diets [10]. However, when the tannin rich plants were fed to sika deer, the interplay between the rumen microbiota, particularly the methanogens, and the fermentation patterns remained poorly understood. Therefore, using high throughput sequencing, we (i) investigated the rumen microbiota of sika deer fed two concentrations of oak leaves rich of tannins, (ii) compared rumen fermentations patterns under these two diets, and (iii) examined the interplay patterns between the rumen microbiota and metabolic phenotypes.

**Materials and Methods**

**Animals and sampling**

Four rumen-cannulated adult male sika deer (Cervus nippon), were used in the present study, and maintained at the research farm (44.04°N, 129.09°E) of the Institute of Special Animal and Plant Sciences, of the Chinese Academy of Agricultural Sciences, in Jilin Province. All animal were housed in individual pens, and all animal procedures were approved and authorized by the Chinese Academy of Agricultural Sciences Animal Care and Use Committee, and by the Institute of Special Animal and Plant Sciences Wild Animal and Plant Subcommittee.

Four sika deer received the different diets in a cross over study that were composed of the same concentrate (64.5% corn, 19.7% soybean meal, 12.8% corn distiller dried grains, and a 3% mixture of vitamins and mineral salts). The concentrate diet was mixed with either of the two different concentrations of oak leaves to form two different diets. The two different ratios of concentrate to oak leaves were 40:60 (OLH, tannin content: 60 mg/1 kg dried matter) and 60:40 (OLL, tannin content: 40 mg/1 kg dried matter). All sika deer were fed twice each day at 0800h and 1600h and had free access to water. After one week of adaption to the diets, sika deer received each diet for 28 days, and the rumen contents were obtained via rumen cannula immediately before the morning feeding at day 29 for 1 day. Rumen samples were stored at -80°C for later analysis.

**DNA extraction**

Total genomic DNA of microorganism was extracted from the whole ruminal contents containing solid and liquid fractions of each animal using the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer’s instructions.
Amplification of target genes and high-throughput sequencing

The bacterial 16S rRNA gene was amplified using primers 27F [11] and 519R [12], the methanogenic 16S rRNA gene was amplified using primers Met86F [13] and 519R [12], the partial 18S rRNA gene of protozoa was amplified using primers GIC1080F and GIC1578R [14], and the internal transcribed spacer region of fungi was amplified using the primers 1737F and 2043R [15]. Each specific primer pair contained the appropriate Illumina adapter sequence, and a 8 bp barcode. The resulting amplicons were purified using QIAquick PCR Purification Kit (QIAGEN, Valencia, CA). The purified amplicons were quantified using QuantiFluor-P Fluorometer (Promega, CA), pooled in equimolar concentrations, and sequenced on Illumina PE MiSeq 300 platform generating paired 300-basepair reads.

Bioinformatics analysis

The read pairs were extracted and concatenated according to the barcodes for each paired read from each sample generating contigs. Contigs with an average quality <20 over a 10 bp sliding window were culled. The retained contigs were processed and analyzed using QIIME ver. 1.7.0 [16].

Contigs were examined for quality control using the following criteria: the minimum sequence length was 400 nt; the maximum sequence length was 500 nt; minimum quality score for a single nucleotide was 25; the maximum number of errors in the barcode was 0; the maximum length of homopolymer run was 6 (default parameter, this does not affect the interpretations of the data); the number of mismatches in the primer was 0; ambiguous and unassigned characters were excluded. The remaining sequences were clustered into operational taxonomic units (OTUs) using Usearch61 according to the sequence identity of 97% at species level, for the 16S rRNA (bacteria and methanogens), and the ITS region (fungi) [9,17], and the sequence identity of 95% at species level for the 18S rRNA (protozoa) genes. Representative sequences of OTUs were aligned to the Greengenes database for bacteria and methanogen 16S rRNA genes [18], to the Silva database for protozoal 18S rRNA genes [19], and to the Unite ITS database for fungi [20]. Potential chimera sequences were removed using Chimera Slayer [21]. The remaining representative OTUs were screened using Basic Local Alignment Search Tool [22].

OTUs that were found in at least 4 samples (animals) for each community (bacteria, methanogens, protozoa and fungi) were retained for the further analysis. Alpha-diversity from all samples including Shannon-Wiener and Simpson indices were also calculated from QIIME [16]. The sequences were deposited in the Sequence Read Archive under accession number SRP050105.

Measurement of metabolic phenotypes in the rumen of sika deer fed two diets

Rumen fluid was centrifuged at 15,000 g for 10 min at 4°C, and 0.2 ml of 2-ethylbutyric acid (internal standard, 2 g/L) in meta phosphoric acid (25% w/v) was added to 1 ml of clarified rumen fluid. The concentrations of VFAs in the rumen were determined by gas chromatography with a flame ionization detector and a DB-FFAP column (30 m×0.25 μm ×0.25 μm, Agilent Technologies 6890GC, USA). The carrier gas was N2 at a flow rate of 2.2 ml/min. The analysis was a gradient oven temperature of 80–170°C with an incremental rate of 10°C/min for optimal separation and a detector temperature of 250°C.
Relationships between rumen microbiota and fermentation parameters

The interactive analysis among bacteria, methanogens, protozoa, fungi and metabolic phenotypes was performed in R package (V3.1.0, http://www.r-project.org/). Spearman’s rank correlations and P-values were calculated and plotted using the packages hmisc (http://cran.r-project.org/web/packages/Hmisc), and corrplot (http://cran.r-project.org/web/packages/corrplot). Moreover, the interrelations were constructed using the method as described by Zhang et al. [23]. Statistic P-values were corrected using the false discovery rate method of the p.adjust package in R. Correlations have an absolute Pearson’s correlation above 0.7 with an false discovery rate-corrected significance level under 0.05. These correlations were transformed into links, and were then visualized using Cytoscape 2.8.2 [24].

Statistical analysis

Statistical analysis was performed using the SigmaPlot 12.0 (Systat Software, Inc.) and R software packages. All variations in different groups were checked for normal distribution using the Shapiro-Wilk test (significance value of $P<0.05$). When normally distributed, multiple samples comparisons were performed using one-way analysis of variance (parametric), and using Kruskal-Wallis analysis on ranks (non-parametric) for un-normal distribution with the significant value of $P<0.05$. All parameters were expressed as the mean and standard error of each group.

Results

Summary of high-throughput sequencing data

A total of 783,651 high-quality sequences were generated. Based on the 97% sequence identity, 249,393 bacterial sequences were assigned to 1,249 OTUs, 282,503 methanogen sequences were assigned to 76 OTUs, 138,597 rumen ciliate sequences were assigned to 9 OTUs, and 39,349 fungal sequences were assigned to 5 OTUs. The results of Good’s coverage [25] showed that 98%-100% of the microbial species were sampled for the four groups of microorganisms, indicating that the sampling effort had sufficient sequence coverage for each microbial group (Table 1).

Microbial communities in the OLH and OLL groups

Overall, 1,249 bacterial OTUs represented 18 phyla and 94 genera. On average, the top three bacterial phyla in the two diet groups were Bacteroidetes (Mean±S.E: 42.7±2.1%), Firmicutes (28.5±5.6%) and Proteobacteria (14.1±2.6%) (Fig 1A). However, the distribution of Firmicutes

| Taxa     | Groups | Seq/sample | OTUs/sample | Good's Coverage | Shannon | Simpson |
|----------|--------|------------|-------------|-----------------|---------|---------|
| Bacteria | OLH    | 30,113     | 1,004       | 0.99            | 7.44    | 0.96    |
|         | OLL    | 32,234     | 999         | 0.99            | 7.69    | 0.98    |
| Methanogen | OLH | 31,319     | 76          | 0.99            | 3.51    | 0.85    |
|         | OLL    | 39,306     | 76          | 0.99            | 3.18    | 0.79    |
| Protozoa | OLH    | 19,666     | 7           | 1.00            | 1.68    | 0.62    |
|         | OLL    | 14,938     | 6           | 1.00            | 1.23    | 0.42    |
| Fungi    | OLH    | 5,662      | 5           | 1.00            | 0.70    | 0.27    |
|         | OLL    | 4,176      | 4           | 1.00            | 0.73    | 0.29    |

OLH: High group; OLL: Low group

doi:10.1371/journal.pone.0123481.t001
in the OLH group (24.0±3.1%) was lower than in the OLL group (33.0±1.8%) \((P = 0.039)\). Moreover, the relative abundance of bacteria belonging to the phyla Proteobacteria and Tenericutes tended to increase in the OLH group (17.8±4.3% and 9.2±4.3%, respectively) as compared to the OLL group (10.3±2.3% and 3.2±0.9%, respectively).

At the genus level, *Prevotella* spp. was the most dominant bacteria, accounting for 15.3±0.3% and 18.0±1.6% in the OLH and OLL groups, respectively, followed by unidentified bacteria within the order Bacteroidales (OLH: 13.5±2.1%; OLL: 13.6±2.0%) and *Paludibacter* spp. (OLH: 8.2±2.0%; OLL: 7.6±1.3%) \((Fig\ 1B)\). The relative abundance of *Pseudobutyrivibrio* spp. \((P = 0.026)\), unidentified bacteria \((P = 0.028)\), and *Prevotella* spp. \((P = 0.022)\) belonging to the family Paraprevotellaceae were lower in the OLH group \(1.4±0.2\%, 0.9±0.1\% and 0.09±0.04\%, respectively\) than in the OLL group \(2.4±0.2\%, 1.7±0.2\% and 0.3±0.04\%, respectively\). Moreover, the proportion of *Ruminobacter* spp. \(8.4±2.4\%) , *Anaeroplasma* spp. \(8.0±4.8\%\) and unidentified bacteria within the family Succinivibrionaceae \(7.9±2.3\%) tended to increase in the OLH group in comparison to the OLL group \(3.8±1.7\%, 2.3±0.7\% and 4.4±1.3\%, respectively\), but *Ruminococcus* spp. was decreased \(OLH: 5.6±0.9\%; OLL: 11.1±2.8\%\).

For the methanogens, all 76 OTUs in the OLH and OLL groups were assigned to two orders: Methanobacteriales \(OLH: 36.2±1.9\%; OLL: 36.7±2.3\%) and Methanoplasmatales \(OLH: 63.8±2.2\%; OLL: 63.3±2.6\%). In order to examine methanogen composition at the species level, these OTU sequences were examined against the NCBI nr database using Basic Local Alignment Search Tool \([22]\). *Methanobrevibacter* spp. accounted for 33.6±2.0% \(OLH\) and 34.3±2.7% \(OLL\), and *Methanosphaera* spp. accounted for 2.6±0.1% \(OLH\) and 2.4±0.6% \(OLL\). Moreover, *Methanobrevibacter ruminantium* \(OLH: 17.6±5.6\%; OLL: 19.2±6.8\%) and *Methanobrevibacter*...
thaueri (OLH: 10.1±6.1%; OLL: 8.7±4.7%) were the most prevalent phylotypes, followed by Methanobrevibacter millerae (OLH: 5.1±1.2%; OLL: 5.7±0.6%). All OTUs within the order Methanoplasmatales were similar to Candidatus Methanomethylophilus alvus with the 98%-100% sequence identity (Fig 2).

For the protozoa, Entodinium longinucleatum accounted for 40.6±7.2% of the rumen protozoa in the OLH group, followed by Eudiplodinium maggii (30.0±5.9%), and Epidinium caudatum (19.5±8.8%). While, Ep. caudatum accounted for 38.3±2.6% in the OLL group, followed by Ent. longinucleatum (37.1±8.5%) and Eud. maggii (19.0±3.6%) (Fig 3).

For the fungi, Piromyces sp. A-BRL-3 was the most abundant in the OLH (84.0±6.3%) and OLL (88.1±4.9%) groups, followed by Piromyces sp. I-GRL-10 (OLH: 16.0±4.8%; OLL: 11.9±2.9%) (Fig 4).

### Composition of VFAs in the rumen of sika deer from the OLH and OLL groups

As shown in Table 2, the concentration of propionate, in the OLH group (10.14±0.79 mmol/L) was greater than in the OLL group (7.44±0.47 mmol/L, \( P = 0.006 \)), and the ratio of acetate to propionate in the OLH group (3.91±0.18) was remarkably decreased as compared to the OLL group (4.85±0.16, \( P = 0.003 \)).

### Relationships between rumen microbiota and metabolic phenotypes

The results (Fig 5A and 5B) showed that Methanobrevibacter gottschalkii was positively correlated to unidentified bacteria belonging to the family Succinivibrionaceae (\( R = 0.88; P = 2.7 \times 10^{-15} \)), Piromyces sp. A-BRL-3 (\( R = 0.97; P = 2.9 \times 10^{-25} \)), Mbr. thaueri (\( R = 0.96; P = 1.9 \times 10^{-10} \)) and Mbr. millerae (\( R = 0.79; P = 1.3 \times 10^{-10} \)). However, Mbr. gottschalkii was
negatively associated with *Paludibacter* spp. (R = -0.90; P = 5.6×10^{-22}) and *Piromyces* sp. I-GRL-10 (R = -0.97; P = 3.5×10^{-36}). *Methanobrevibacter ruminantium* was negatively associated with *Mbr. thaueri* (R = -0.82; P = 8.1×10^{-12}), *Mbr. millerae* (R = -0.82; P = 4.0×10^{-12}) and *Mbr. smithii* (R = -0.58; P = 3.3×10^{-5}). Moreover, *Fibrobacter* spp. had a positive interaction with *Mbr. smithii* (R = 0.69; P = 1.2×10^{-7}).

*Epidinium caudatum* was negatively (R = -0.79; P = 0.02) associated with butyrate concentrations, whereas *Eud. maggi* was positively (R = 0.80; P = 0.01) associated with butyrate concentrations. Methanogens belonging to the order Methanoplasmatales were negatively correlated to acetate concentration (R = -0.65; P = 1.2×10^{-6}), *Treponema* spp. (R = -0.93; P = 1.6×10^{-23}), and *Ent. longinucleatum* (R = -0.73; P = 1.1×10^{-8}), whereas *Methanosphaera stadtmannae* positively interacted with propionate concentrations (R = 0.73; P = 1.3×10^{-8}). *Prevotella* spp. negatively interacted with *Ruminobacter* spp. (R = -0.72; P = 2.4×10^{-8}), *Eud. maggi* (R = -0.88; P = 3.6×10^{-15}), propionate concentrations (R = -0.71; P = 6.0×10^{-8}), isobutyrate (R = -0.85; P = 7.7×10^{-14}) and isovalerate concentrations (R = -0.81; P = 2.1×10^{-13}). However, unidentified bacteria within the family Veillonellaceae showed positive relationships with isovalerate (R = 0.91; P = 8.3×10^{-25}) and isobutyrate (R = 0.89; P = 2.2×10^{-16}) concentrations, respectively. Valerate concentrations were negatively related to *Succinivibrio* spp. (R = -0.85; P = 1.6×10^{-15}), *Prevotella* spp. belonging to the family Paraprevotellaceae (R = -0.86; P = 4.4×10^{-14}) and ratio of acetate to propionate (R = -0.86; P = 1.3×10^{-13}), but positively related to *Ruminobacter* spp. (R = 0.70; P = 1.0×10^{-7}), and bacteria belonging to the family Succinivibrionaceae (R = 0.90; P = 8.7×10^{-29}).

**Discussion**

Using high-throughput sequencing, the present study showed the responses of rumen microbiota of sika deer fed tannin rich plants, and revealed the interplay patterns between rumen...
microbiota and metabolic parameters. These results will help us to understand the beneficial effects of tannins on ruminants and to use tannin rich plants in ruminants farming.

The results revealed that *Prevotella* spp. were the dominant bacteria in the rumen of sika deer fed oak leaves, consistent with the results of other cervids [26–29]. *Prevotella* spp. made up a large part of the genetic and metabolic diversity in rumen microbial communities [30]. Recent studies also showed that species of *Prevotella* provided the ability to adapt to various diets [31]. The current study demonstrated that *Prevotella* spp. played important roles in the fermentation of oak leaves.

![Fig 4. The composition of rumen fungi in the two groups. OLH = High group, OLL = Low group. doi:10.1371/journal.pone.0123481.g004](image)

| VFAs(mmol/L) | OLH Mean ± S.E | OLL Mean ± S.E | P value |
|--------------|----------------|----------------|---------|
| Acetate      | 39.67±1.77     | 35.98±2.34     | 0.778   |
| Propionate   | 10.14±0.79     | 7.44±0.47      | 0.006   |
| Isobutyrate  | 0.53±0.02      | 0.47±0.045     | 0.264   |
| Butyrate     | 5.16±0.21      | 4.60±0.45      | 0.27    |
| Isovalerate  | 0.69±0.022     | 0.57±0.078     | 0.161   |
| Valerate     | 0.52±0.027     | 0.49±0.016     | 0.258   |
| TVFAs        | 56.70±2.43     | 49.53±3.20     | 0.085   |
| Acetate/Propionate | 3.91±0.18 | 4.85±0.16     | 0.003   |

VFAs, volatile fatty acids; TVFAs, total volatile fatty acids; S.E: Standard Error; OLH: High group; OLL: Low group
The distribution of Firmicutes (e.g. *Ruminococcus* spp.) in the OLH group was decreased compared to that in the OLL group ($P = 0.039$) indicating that some species belonging to the phylum Firmicutes may be sensitive to tannins [32]. However, bacteria belonging to the family Succinivibrionaceae, which could ferment glucose and other carbohydrates and produce succinate and acetate, were increased in the OLH group. Additionally, the ratio of Firmicutes to Bacteroidetes in the OLH group (0.59) tended to decrease as compared to the OLL group (0.74). Bacteroidetes utilize the succinate pathway via methylmalonyl-CoA to generate propionate [33]. Consequently, the concentrations of propionate in the OLH group were increased as compared to the OLL group. These results suggested that the altered bacterial community composition linked with the production of different VFAs.

The rumens of sika deer in both diets were dominated by methanogens belonging to the order Methanoplasmatales, followed by *Methanobrevibacter* spp. Similarly, Min et al. [34] also...
found that *Methanobrevibacter* spp. was decreased with the supplementation of tannins in feces of goat. Additionally, previous studies showed that methanogens belonging to the order Methanoplasmatales were more abundant in the free-living community within the rumen, and *Methanobrevibacter* spp. were predominant in the protozoa-associated methanogen communities [35]. As tannins are known to decrease the density of protozoa [36], perhaps, the dominance of methanogens belonging to the order Methanoplasmatales may be explained by the presence of tannins in the diets.

The protozoal communities were mainly comprised of *Ent. longinucleatum*, *Eud. maggii*, and *Ep. caudatum*, representing the B-type rumen ciliate community [37]. In agreement with the previous studies, *Epidinium* spp. and *Eudiplodinium* spp. were also found in the rumen of sika deer [38]. Meanwhile, the diversity of fungal communities in the rumen of sika deer were also consistent with the results of Kittelmann et al. [9], who found the *Piromyces* sp. accounted for 20%.

The interactive relationship also showed the response of rumen microbiota and metabolic phenotypes in the rumen of sika deer fed tannin rich plants. Similar to the findings by Kang et al. [39], the present study showed that *Prevotella* spp. and unidentified bacteria within the family Veillonellaceae appeared in the co-occurrence network, indicating a potential metabolic link between the two groups of bacteria in the rumen of sika deer fed oak leaves. In agreement with our findings, Carberry et al. [8] also observed the negative associations between *Prevotella* spp. and isobutyrate, and isovalerate. The concentrations of isobutyrate and isovalerate were positively correlated with unidentified bacteria belonging to the family Veillonellaceae, suggesting that they may be the important producer of branched VFAs when tannins were fed to sika deer. In contrast, Mao et al. [40] found that *Anaerovibrio* spp., *Desulfovibrio* spp., *Leucobacter* spp. and *Moryella* spp. were positively correlated with isobutyrate and isovalerate in the feces of dairy cows. This difference may be caused by the distinct bacterial communities between rumen and fecal pellets [41].

The study also found that *Ep. caudatum* was negatively correlated with butyrate, whereas *Eud. maggii* was positively correlated with butyrate. This was interesting, given that butyrate is an important end product of rumen ciliate metabolism [42]. Michałowski et al. [43] found that *Eud. maggii* promoted butyrate production rate or concentration compared to defaunated animals. These results indicated that the composition of protozoa at the species level in the rumen may affect the energy supply to the host, as butyrate contributed to approximately 70% of the daily metabolic energy of ruminants [44].

Interestingly, methanogens belonging to the order Methanoplasmatales were negatively related to *Treponema* spp. A recent study showed that these methylo trophic methanogens mainly used hydrogen to reduce methanol and methylamines to methane [45]. However, *Treponema* spp. can utilize hydrogen to reduce carbon dioxide to acetate [46]. These findings suggested that the concentration and partial pressure of hydrogen in the rumen of sika deer may be one of the key factors affecting methanogenesis. Similar to recent findings [9,47], the present study also found a negative correlation between *Mbr. gottschalkii*, *Mbr. thaueri*, and *Mbr. millerae* with *Mbr. ruminantium*, indicating that the activity of different methanogens may play more important roles in methane production, rather than the density of methanogens [48]. Moreover, this may be due to the fact that these methanogens presumably compete for hydrogen as substrate [49,50]. The genome analysis also partially supported this notion. *Methanobrevibacter smithii* PS encodes a methyl coenzyme reductase II (*mcrII*), an isoenzyme of the methyl CoM reductase I (*mcrI*) enzyme, which was differentially regulated during growth to mediate methane formation at high partial pressure of hydrogen, while *Methanobrevibacter ruminantium* M1 contains only the *mcrI* system for the final methyl-CoM reduction step in methanogenesis [51,52]. In addition, *Piromyces* sp. A-BRL-3 and *Piromyces* sp. I-GRL-10 displayed positive and negative relationships with *Mbr. gottschalkii*, respectively. This may be related to the considerable variation...
in the fibrolytic ability the genus *Piromyces*, and in turn, resulted in the different substrates for methanogenesis [53]. Overall, the interactive analysis provided us novel insights into elucidating the symbiotic relationships between rumen microbiota and metabolism.

**Conclusions**

In conclusion, this study demonstrated that the microbiota and fermentation in the rumen of sika deer fed tannin rich plants were altered. Different groups of methanogens were interactive with distinct rumen microbiota and fermentation products, suggesting the need to consider the different methanogen communities when developing strategies for mitigating methane emissions in ruminants. Such results would help us to understand the underlying mechanisms of decreased methane emission for ruminants by tannins, and improve strategies aiming to use tanniferous plants to reduce enteric methane emissions.

**Acknowledgments**

The author thanks Dr. Kittelmann for her great help in the co-occurrence analysis. The authors also would like to thank Kunming Biological Diversity Regional Center of Large Apparatus and Equipments, Kunming Institute of Zoology, Chinese Academy of Sciences for their super technical assistances.

**Author Contributions**

Conceived and designed the experiments: GL HL FY. Performed the experiments: ZL ZF. Analyzed the data: ZL ZZ. Contributed reagents/materials/analysis tools: ZZ. Wrote the paper: ZL ADGW.

**References**

1. McSweeney CS, Palmer B, McNeill DM, Krause DO. Microbial interactions with tannins: nutritional consequences for ruminants. Anim Feed Sci Technol. 2001; 91(1–2): 83–93.
2. Cieslak A, Szumacher-Strabel M, Stockmal A, Oleszek W. Plant components with specific activities against rumen methanogens. animal. 2013; 7(Supplements2): 253–65.
3. Jayanegara A, Leiber F, Kreuzer M. Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from *in vivo* and *in vitro* experiments. J Anim Physiol Anim Nutr (Berl). 2012; 96(3): 365–75.
4. Min BR, Barry TN, Attwood GT, McNabb WC. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. Anim Feed Sci Technol. 2003; 106(1–4): 3–19.
5. Russell JB, Rychlik JL. Factors that alter rumen microbial ecology. Science. 2001; 292(5519): 1119–22. PMID: 11352069
6. Tan HY, Sieo CC, Abdullah N, Liang JB, Huang XD, Ho YW. Effects of condensed tannins from *Leucaena* on methane production, rumen fermentation and populations of methanogens and protozoa *in vitro*. Anim Feed Sci Technol. 2011; 169(3–4): 185–93.
7. Ross EM, Moate PJ, Maret L, Cocks BG, Hayes BJ. Investigating the effect of two methane-mitigating diets on the rumen microbiome using massively parallel sequencing. J Dairy Sci. 2013; 96(9): 6030–46. doi: 10.3168/jds.2013-6766 PMID: 23871375
8. Carberry CA, Kenny DA, Han S, McCabe MS, Waters SM. Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. Appl Environ Microbiol. 2012; 78(14): 4949–58. doi: 10.1128/AEM.07759-11 PMID: 22562991
9. Kittelmann S, Seedorf H, Walters WA, Clemente JC, Knight R, Gordon JI, et al. Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. PLoS ONE. 2013; 8(2): e47879. doi: 10.1371/journal.pone.0047879 PMID: 23408926
10. Li ZP, Wright ADG, Liu HL, Bao K, Zhang TT, Wang KY, et al. Bacterial community composition and fermentation patterns in the rumen of sika deer (Cervus nippon) fed three different diets. Microb Ecol. 2015; 69(2): 307–318. doi: 10.1007/s00248-014-0497-z PMID: 25252928

11. Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E GM, editor. Nucleic acid techniques in bacteria systematics. New York: Wiley Press; 1991. p. 115–75.

12. Turner S, Pryer KM, Miao VPW, Palmer JD. Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. J Eukaryot Microbiol. 1999; 46(4): 327–38. PMID: 10461381

13. Wright ADG, Pimm C. Improved strategy for presumptive identification of methanogens using 16S ribo-printing. J Microbiol Methods. 2003; 55(2): 337–49. PMID: 14529955

14. Ishaq SL, Wright ADG. Design and validation of four new primers for next-generation sequencing to target the 16S rRNA genes of gastrointestinal ciliate protozoa. Appl Environ Microbiol. 2014; 80(17): 5515–21. doi: 10.1128/AEM.01644-14 PMID: 24973070

15. Bellmermain E, Carlsen T, Brochmann C, Coissac E, Taberlet P, Kauserud H. ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. BMC Microbiol. 2010; 10(1): 189.

16. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Meth. 2010; 7(5): 335–6.

17. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010; 26(19): 2460–1. doi: 10.1093/bioinformatics/btp461 PMID: 20709691

18. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006; 72(7): 5069–72. PMID: 16820507

19. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013; 41(Database issue): D590–6. doi: 10.1093/nar/gks1219 PMID: 23193283

20. Abarenkov K, Henrik Nilsson R, Larsson K-H, Alexander IJ, Eberhardt U, Erland S, et al. The UNITE database for molecular identification of fungi—recent updates and future perspectives. New Phytol. 2010; 186(2): 281–5. doi: 10.1111/j.1469-8137.2009.03160.x PMID: 20409185

21. Altschul SF, Madden TL, Schaffer AA, Zhang JW, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997; 25(13): 3389–402. PMID: 9254694

22. Zhang ZG, Geng JW, Tang XD, Fan H, Xu JC, Wen XJ, et al. Spatial heterogeneity and co-occurrence patterns of human mucosal-associated intestinal microbiota. ISME J. 2014; 8(4): 881–93. doi: 10.1038/ismej.2013.185 PMID: 24132077

23. Smoot ME, Ono K, Ruscheinski J, Wang PL, Ideker T. Cytoscape 2.8: new features for data integration and network visualization. Bioinformatics. 2011; 27(3): 431–2. doi: 10.1093/bioinformatics/btq675 PMID: 21149340

24. Balme BM, Ryan JA, Lim J, Brierley CL, Hodgkinson JS, et al. The food and nutrient intakes of the Hadza hunter-gatherers. Nat Commun. 2014; 5. doi: 10.1038/ncomms4654

25. Schnorr SL, Candela M, Rampelli S, Centanni M, Consolandi C, Basaglia G, et al. Gut microbiome of the Hadza hunter-gatherers. Nat Commun. 2014; 5. doi: 10.1038/ncomms4654

26. Ishaq SL, Wright ADG. High-throughput DNA sequencing of the ruminal bacteria from Moose (Alces alces) in Vermont, Alaska, and Norway. Microb Ecol. 2014; 68(2): 185–95. doi: 10.1007/s00248-014-0399-0 PMID: 24595908

27. Gruninger RJ, Sensen CW, McAllister TA, Forster RJ. Diversity of rumen bacteria in Canadian cervids. PLoS ONE. 2014; 9(4): e98682. doi: 10.1371/journal.pone.0098682 PMID: 24586961

28. Li ZP, Liu HL, Li GY, Bao K, Wang KY, Xu C, et al. Molecular diversity of rumen bacterial communities from tannin-rich and fiber-rich forage fed domestic Sika deer (Cervus nippon) in China. BMC Microbiol. 2013; 13(1): 151.

29. Sundset MA, Praesteng KE, Cann IK, Mathiesen SD, Mackie RI. Novel rumen bacterial diversity in two geographically separated sub-species of reindeer. Microb Ecol. 2007; 54(3): 424–38. PMID: 17479904

30. Bekele AZ, Koike S, Kobayashi Y. Genetic diversity and diet specificity of ruminal Prevotella revealed by 16S rRNA gene-based analysis. FEMS Microbiol Lett. 2010; 305(1): 49–57. doi: 10.1111/j.1574-6968.2010.01911.x PMID: 20188525

31. Mau BE, Gabriel SS, Darling AE, Roop N, Subramanian S, et al. The Genomic Encyclopedia of Bacteria and Archaea database project: improved data processing and web-based tools. Nucleic Acids Res. 2013; 41(Database issue): D58–67. doi: 10.1093/nar/gkt1342 PMID: 23502976

32. Ishaq SL, Wright ADG. Design and validation of four new primers for next-generation sequencing to target the 16S rRNA genes of gastrointestinal ciliate protozoa. Appl Environ Microbiol. 2014; 80(17): 5515–21. doi: 10.1128/AEM.01644-14 PMID: 24973070

33. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Meth. 2010; 7(5): 335–6.

34. Balme BM, Ryan JA, Lim J, Brierley CL, Hodgkinson JS, et al. The food and nutrient intakes of the Hadza hunter-gatherers. Nat Commun. 2014; 5. doi: 10.1038/ncomms4654

35. Smoot ME, Ono K, Ruscheinski J, Wang PL, Ideker T. Cytoscape 2.8: new features for data integration and network visualization. Bioinformatics. 2011; 27(3): 431–2. doi: 10.1093/bioinformatics/btq675 PMID: 21149340

36. GOOD IJ. The population frequencies of species and the estimation of population parameters. Biometrika. 1953; 40(3–4): 237–64.

37. Ishaq SL, Wright ADG. High-throughput DNA sequencing of the ruminal bacteria from Moose (Alces alces) in Vermont, Alaska, and Norway. Microb Ecol. 2014; 68(2): 185–95. doi: 10.1007/s00248-014-0399-0 PMID: 24595908

38. Gruninger RJ, Sensen CW, McAllister TA, Forster RJ. Diversity of rumen bacteria in Canadian cervids. PLoS ONE. 2014; 9(4): e98682. doi: 10.1371/journal.pone.0098682 PMID: 24586961

39. Li ZP, Liu HL, Li GY, Bao K, Wang KY, Xu C, et al. Molecular diversity of rumen bacterial communities from tannin-rich and fiber-rich forage fed domestic Sika deer (Cervus nippon) in China. BMC Microbiol. 2013; 13(1): 151.

40. Sundset MA, Praesteng KE, Cann IK, Mathiesen SD, Mackie RI. Novel rumen bacterial diversity in two geographically separated sub-species of reindeer. Microb Ecol. 2007; 54(3): 424–38. PMID: 17479904

41. Bekele AZ, Koike S, Kobayashi Y. Genetic diversity and diet specificity of ruminal Prevotella revealed by 16S rRNA gene-based analysis. FEMS Microbiol Lett. 2010; 305(1): 49–57. doi: 10.1111/j.1574-6968.2010.01911.x PMID: 20188525

42. Schnorr SL, Candela M, Rampelli S, Centanni M, Consolandi C, Basaglia G, et al. Gut microbiome of the Hadza hunter-gatherers. Nat Commun. 2014; 5. doi: 10.1038/ncomms4654
32. Min BR, Attwood GT, McNabb WC, Molan AL, Barry TN. The effect of condensed tannins from *Lotus corniculatus* on the proteolytic activities and growth of rumen bacteria. Anim Feed Sci Technol. 2005; 121(1–2): 45–58.

33. Reichardt N, Duncan SH, Young P, Belenguer A, McWilliam Leitch C, Scott KP, et al. Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. ISME J. 2014; 8(6): 1323–35. doi: 10.1038/ismej.2014.14 PMID: 24553467

34. Min BR, Solaiman S, Shange R, Eun JS. Gastrointestinal bacterial and methanogenic archaea diversity and methanogen communities in the bovine rumen differ according to comparative analysis of 16S rRNA and mcrA genes. Microbiology. 2012; 158(Pt 7): 1808–17. doi: 10.1099/mic.0.057984-0 PMID: 22539164

35. Tymensen LD, Beauchemin KA, McAllister TA. Structures of free-living and protozoa-associated methanogen communities in the bovine rumen differ according to comparative analysis of 16S rDNA. Appl Environ Microbiol. 2011; 77(16): 5682–7. doi: 10.1128/AEM.05130-11 PMID: 21705541

36. Williams AG, Coleman GS. The Rumen Protozoa. New York: Springer-Verlag; 1992.

37. Michaelowski T, Beizaecki G, Kwiatkowska E, Pajak JJ. The effect of selected rumen fauna on fibrolytic enzyme activities, bacterial mass, fibre disappearance and fermentation pattern in sheep. J Anim Feed Sci. 2003; 12(1): 45–64.

38. Poulsen M, Schwab C, Jensen BB, Engberg RM, Spang A, Canibe N, et al. Methylo trophic methanogenic Thermoplasmata implicated in reduced methane emissions from bovine rumen. Nat Commun. 2013; 4: 1428. doi: 10.1038/ncomms2432 PMID: 23385573

39. Leadbetter JR, Schmidt TM, Graber JR, Breznak JA. Acetogenesis from H2 plus CO2 by spirochetes and other fermenters in intestinal microflora of autistic children. PLOS ONE. 2013; 8(7): e68322. doi: 10.1371/journal.pone.0068322 PMID: 23844187

40. King EE, Smith RP, St-Pierre B, Wright ADG. Differences in the rumen methanogen populations of lactating Jersey and Holstein dairy cows under the same diet regimen. Appl Environ Microbiol. 2011; 77(16): 5682–7. doi: 10.1128/AEM.05130-11 PMID: 21705541

41. Rea S, Bowman JP, Popovski S, Pimm C, Wright ADG. *Methanobrevibacter smithii* sp. nov. and *Methanobrevibacter smithii* sp. nov. from the ovine and bovine rumen that can utilize formate for growth. Int J Syst Evol Microbiol. 2007; 57(3): 450–6. PMID: 17329767

42. Samuel BS, Hansen EE, Manchester JK, Coulthol PM, Henri ssat B, Fulton R, et al. Genomic and metabolic adaptations of *Methanobrevibacter smithii* to the human gut. Proceedings of the National Academy of Sciences. 2007; 104(25): 10643–8. PMID: 17563350

43. Leahy SC, Kelly WJ, Altermann E, Ronimus RS, Yeoman CJ, Pacheco DM, et al. The genome sequence of the rumen methanogen *Methanobrevibacter ruminantium* reveals new possibilities for controlling ruminant methane emissions. PLOS ONE. 2010; 5(1): e9296. doi: 10.1371/journal.pone.0008926 PMID: 20126622
53. Paul SS, Deb SM, Punia BS, Singh D, Kumar R. Fibrolytic potential of anaerobic fungi (*Piromyces* sp.) isolated from wild cattle and blue bulls in pure culture and effect of their addition on in vitro fermentation of wheat straw and methane emission by rumen fluid of buffaloes. J Sci Food Agric. 2010; 90(7): 1218–26. doi: 10.1002/jsfa.3952 PMID: 20394004