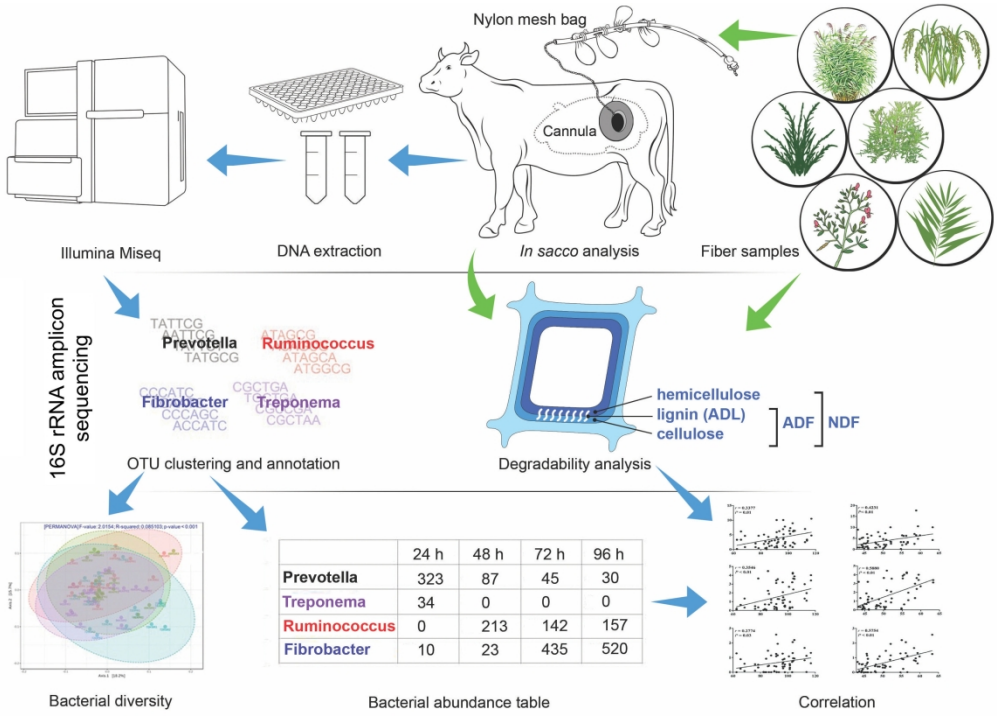
OXFORD UNIVERSITY PRESS | FEMS Microbiology Ecology

http://mc.manuscriptcentral.com/fems

Temporal changes in microbial communities attached to forages with different lignocellulosic compositions in the cattle rumen

Journal:	*FEMS Microbiology Ecology*
Manuscript ID	FEMSEC-19-03-0119.R8
Manuscript Type:	Research article
Date Submitted by the Author:	10-Apr-2020
Complete List of Authors:	Gharechahi, Javad; Agricultural Biotechnology Research Institute of Iran, Systems Biology Vahidi, Mohammad Farhad; Agricultural Biotechnology Research Institute of Iran, Systems Biology Ding, Xue-Zhi ; Chinese Academy of Agricultural Sciences Lanzhou Institute of Husbandry and Pharmaceutical Sciences, Key Laboratory of Yak Breeding Engineering Han, Jianlin; International Livestock Research Institute, Livestock Genetics Program Salekdeh, Ghasem Hosseini; Agricultural Biotechnology Research Institute of Iran, Systems Biology
Keywords:	microbiome, rumen, biomass degradation



	24 h	48 h	72 h	96 h
Prevotella	323	87	45	30
Treponema	34	0	0	0
Ruminococcus	0	213	142	157
Fibrobacter	10	23	435	520

Temporal changes in microbial communities attached to forages with different lignocellulosic compositions in the cattle rumen

Javad Gharechahi[1], Mohammad Farhad Vahidi[2], Xue-Zhi Ding[3], Jian-Lin Han[4,5], Ghasem Hosseini Salekdeh[2]

1. Human Genetics Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
2. Department of Systems Biology, Agricultural Biotechnology Research Institute of Iran, Agricultural Research, Education, and Extension Organization, Karaj, Iran
3. Key Laboratory of Yak Breeding Engineering, Lanzhou Institute of Husbandry and Pharmaceutical Sciences, Chinese Academy of Agricultural Sciences (CAAS), Lanzhou, China
4. CAAS-ILRI Joint Laboratory on Livestock and Forage Genetic Resources, Institute of Animal Science, Chinese Academy of Agricultural Sciences (CAAS), Beijing, China
5. Livestock Genetics Program, International Livestock Research Institute (ILRI), Nairobi, Kenya

To whom correspondence should be addressed

Ghasem Hosseini Salekdeh

E-mail: hsalekdeh@yahoo.com

Abstract

The attachment of rumen microbes to feed particles is critical to feed fermentation, degradation and digestion. However, the extent to which the physicochemical properties of feeds influence the colonization by rumen microbes is still unclear. We hypothesized that rumen microbial communities may have differential preferences for attachments to feeds with varying lignocellulose properties. To this end, the structure and composition of microbial communities attached to six common forages with different lignocellulosic compositions were analyzed following *in situ* rumen incubation in male Taleshi cattle. The results showed that differences in lignocellulosic compositions significantly affected the inter-sample diversity of forage-attached microbial communities in the first 24 hours (h) of rumen incubation, during which the highest dry matter degradation was achieved. However, extension of the incubation to 96 h resulted in the development of more uniform microbial communities across the forages. *Fibrobacteres* were significantly overrepresented in the bacterial communities attached to the forages with the highest neutral detergent fiber contents. *Ruminococcus* tended to attach to the forages with low acid detergent lignin contents. The extent of dry matter fermentation was significantly correlated with the populations of Fibrobacteraceae, unclassified Bacteroidales, Ruminococcaceae and Spirochaetacea. Our findings suggested that lignocellulosic compositions, more specifically the cellulose components, significantly affected the microbial attachment to and thus the final digestion of the forages.

Keywords: microbiome, microbiota, rumen, rumen incubation, 16S rRNA gene sequencing, rumen fermentation, biomass degradation

Introduction

The gastrointestinal tracts (GIT) of ruminant animals have evolved to allow the colonization by a diverse community of symbiotic microorganisms belonging to three taxonomic domains of life, i.e. Archaea, Bacteria and Eukarya. Particularly, as the first and the largest compartment of ruminant stomach, rumen is the main site of microbial colonization and fermentation. Without the aid of such microorganisms, the host ruminants cannot digest and convert plant lignocellulosic biomasses into energy and other essential metabolites (Jami and Mizrahi 2012; Mackie 2002). It is estimated that almost 70% of the energy requirement of ruminants is supplied by this microbial fermentation (Bergman 1990). In addition to their crucial role in animal nutrition and production, the GIT microorganisms remain vital to animal health, physiology and immunity against pathogenic microbes (Guarner and Malagelada 2003; Hungate 2013).

Bacteria are the major colonizers in the rumen and consequently make the greatest contribution to plant biomass fermentation, degradation and digestion. Early studies on rumen microbial communities relied largely on culture-based approaches, which were limited to the bacteria that can grow on culture medium. With the advances in next-generation sequencing technologies, culture-independent approaches have gained preference, enabling researchers to achieve a deeper insight into precise composition and structure of rumen bacterial communities. The latest high throughput amplicon-based 16S ribosomal RNA gene (rRNA) sequencing studies suggest that rumen bacterial communities of most ruminants are mainly affiliated to the phyla of Bacteroidetes, Firmicutes, Proteobacteria, Fibrobacteres, Spirocheaetes, Actinobacteria, Tenericutes and Verrucomicrobia, which collectively account for greater than 99% of the total rumen bacterial communities (Godoy-Vitorino *et al.* 2012; Jami *et al.* 2013; Jami and Mizrahi 2012; Zened *et al.* 2013). Microbial communities are dynamic in the rumen and depend on the host species and its diet, age, physiology and health status (Godoy-Vitorino *et al.* 2012; Jami *et al.* 2013; Jami and Mizrahi 2012; Kocherginskaya *et al.* 2001; Kong *et al.* 2010; Petri *et al.* 2012). Under normal physiological conditions, diet is the major driver to determine the composition and structure of rumen bacterial communities (Petri *et al.* 2012). Interestingly, the amount of dietary fiber is a key factor shaping the growth and multiplication of cellulolytic bacteria in the rumen. Changing dietary fiber content or substituting the fiber with easily fermentable carbohydrates has a profound impact on the community of rumen microbiota and may result in metabolic diseases, such as subacute ruminal acidosis (Khafipour *et al.* 2009; Petri *et al.* 2017).

Members of the rumen bacterial communities differ in their preferences for attachment to feed particles and rumen wall, therefore they are accordingly categorized into particle-attached (tightly attached to feed particles), liquid borne (freely available in liquid fraction) and epimural (attached to rumen epithelium) communities (De Mulder *et al.* 2017; Gharechahi *et al.* 2015; Kong *et al.* 2010; Sadet *et al.* 2007). The attachment of rumen microbial communities to feed particles is a key step in the process of rumen fermentation and digestion (McAllister *et al.* 1994) and it occurs shortly after feed entry into the rumen. Analysis of microbial communities associated with perennial ryegrass following *in situ* rumen incubation in Holstein–Friesian cows or steers shows that microbial attachment initiates within five minutes (min) of its rumen entry and stabilizes between 15 and 30 min of its rumen incubation (Edwards *et al.* 2007; Huws *et*

al. 2013). A recent analysis of temporal changes in bacterial community attached to wheat straw in Holstein cow rumen demonstrates that the first wave of microbial-based biomass degradation occurs within 30 min of its rumen entry. The community of bacteria attached to feed particles during this time retains even after 72 hours (h) of the rumen incubation (Jin *et al.* 2018). The degree of dry matter (DM) degradation differs among forages during the initial hours of their rumen entry (Cheng *et al.* 2017; Liu *et al.* 2016). The community composition of particle-attached microbes also varies among feeds and is likely influenced by the chemical compositions of feeds because cellulolytic bacteria such as *Fibrobacter*, *Ruminococcus*, *Butyrivibrio* and unclassified *Treponema* tend to attach to feeds with relatively high neutral detergent fiber (NDF, Cheng *et al*. 2017; Liu *et al*. 2016). The community of feed particle-attached bacteria also changes over the incubation time. For instance, rumen microbiota attachment to switchgrass in Friesian cow occurs at two successive stages: The first wave takes place immediately after its rumen entry (within the first 30 min) and is characterized by high abundances of Bacteroidia and Clostridia. The second wave, however, occurs after 16 h of its rumen incubation, during which the populations of Spirochaeta and Fibrobacteria become dominant (Piao *et al.* (2014).

There is limited knowledge on rumen bacteria diversity, their preference for attachment to and degradation of feeds with extreme values of cellulose and/or hemicellulose. Understanding the dynamics of bacteria attached to feeds at high level of lignification provides opportunities to improve the nutrient efficiency of low-quality forages through pre-treatments or manipulation of rumen microbial communities. We hypothesized that rumen bacterial communities differ in lignocellulose degrading capacities and thus show preference for attachment to feeds with different levels of lignification. We therefore aimed to evaluate whether the rumen microbes have any preference for attachment to forages with different cellulose and/or hemicellulose contents. We also explored the dynamic changes in microbial communities attached to the forages under prolonged incubation in the cattle rumen (e.g. up to 96 h with 24 h sampling intervals). Overall, our 16S rRNA genes-based diversity analysis revealed significant differences in the composition and structure of microbial communities attached to different forages.

Materials and methods

In situ rumen incubation and sample collection of the forages

All experimental procedures relevant to animals were approved by the Ethics Committee for Animal Experiments of the Animal Science Research Institute of Iran. Rumen cannulation was performed according to the American College of Veterinary Surgeons (ACVS) using a two-stage rumen cannulation technique (Martineau *et al.* 2015).

Six common lignocellulosic forages, including camelthorn (*Alhagi persarum*, AP; both stem and leaves), common reed (*Phragmites australis*, CR; stem and leaves), date palm (*Phoenix dactylifera*, DP; leaves), kochia (*Kochia scoparia*, KS; both stem and leaves), rice straw (*Oryza sativa*; cultivar Hashemi, RS; both stem and leaves) and salicornia (*Salicornia persica*, SC; both stem and leaves), were selected for *in situ* rumen incubation. Dried forage materials were cut into pieces of approximately 2 mm in length and an equal amount of the pieces (5 ± 0.05 g) was weighed into heat-sealed nylon bags (5 × 10 cm; 50 µm pore size). Three rumen-cannulated, purebred bulls (Taleshi

cattle, an Iranian local breed with a historical origin of *Bos taurus* mixed with *Bos indicus*) aged between 2.5 and 3 years were used for this study. Forty-eight heat-sealed bags, eight per forage, were simultaneously placed into each of the three rumens shortly after the cannulated bulls were offered the first meal in the morning. The bulls were housed together in a stable, fed on a mixed diet containing 70% wheat straw and 30% concentrate and provided free access to water. Two nylon bags from each of the six forages were retrieved from each rumen after 24, 48, 72 and 96 h of the incubation, washed thoroughly with distilled water three times to remove liquid borne and loosely attached microbes, which may not have a discriminating preference for attachment to forages with different lignocellulose properties, and finally squeezed by hands with sterile gloves to remove excess water. The bags were then transferred in liquid nitrogen to the laboratory where one replicate was stored at -70 °C for subsequent DNA extraction while the other was processed for physicochemical analysis.

Physicochemical analysis of the incubated forage materials

The forage lignocellulosic biomasses were analyzed for dry matter (DM) and the contents of neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) before and after the rumen incubation. DM was determined following 48 h air-drying of the samples in a fan-assisted oven maintained at 55 °C. The dried material was grounded to pass through a 1-mm sieve for the measurements of NDF, ADF and ADL according to the established procedures (Goering 1970; Van Soest *et al.* 1991). Cellulose content was estimated by subtracting ADL from ADF while hemicellulose content was measured by subtracting ADF from NDF.

Microbial cell recovery and metagenomic DNA extraction

Microbial cells firmly attached to the forages were subsequently stripped by incubating individual samples on ice in a dissociation buffer containing 0.1% (v/v) Tween 80, 1% (v/v) methanol and 1% (v/v) tertiary butanol (adjusted at pH 2), which has been particularly adapted for the dissociation of microbial cells from the rumen solid digesta. The samples were vigorously vortexed every 1 min and this step was repeated at least 5 times. The forage materials were sedimented by centrifugation at 500 × g and the liquid supernatant containing microbial cells was transferred to a new container. This step was repeated at least three times and the collected liquids were centrifuged at 12000 × g for 10 min to sediment the detached microbial cells. Metagenomic DNA from the detached cells was extracted using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for the isolation of DNA from stools for pathogen detection. The quality and quantity of the extracted DNA were evaluated by an agarose gel electrophoresis (0.8%) and a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

PCR amplification and Illumina sequencing of 16S rRNA gene

The V3-V4 hypervariable region of 16S rRNA gene was amplified using the universally conserved primer set S-D-Bact-0341-b-S-17 (5′-CCT ACG GGN GGC WGC AG-3′) and S-D-Bact-0785-a-A-21 (5′-GAC TAC HVG GGT ATC TAA TCC-3′), which generated a fragment of 464 bp suitable for paired-end sequencing using the Illumina MiSeq System (Illumina Inc. San Diego, CA, USA). PCR amplification was performed in triplicate in a 25 μL reaction

containing 12.5 µL 2× PCR master mix (Qiagen), 1 µL (10 pM) of each primer, 30 ng of microbial DNA and 5-9 µL of double-distilled water. The PCR condition consisted of an initial denaturation at 94 °C for 4 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. PCR products were purified and 2 µL of each reaction was used as a template for the second round of PCR, during which the Illumina adaptors and barcode sequences were incorporated to the 5'-end of the amplified products. The second PCR was also performed in triplicate under the same running condition except the number of cycles were limited to 15. The PCR amplicons were recovered using the Qiaquick® Gel Extraction Kit (Qiagen), quantified fluorometrically, pooled in equimolar quantities and paired-end sequenced (PE300) using the Illumina MiSeq System at Macrogen Inc. (Seoul, South Korea).

Sequence analysis

Quality filtered paired-end sequences were joined using the Flash with --max-overlap option set to 200 (Magoc and Salzberg 2011). Sequences failed to be assembled were discarded. The joined FASTQ files were processed using split_libraries_fastq.py script in the QIIME pipeline v1.9.1 (Caporaso *et al.* 2010b). This script demultiplexed sequences and filtered out sequences shorter than 200 bp or longer than 1000 bp, along with those containing ambiguous bases with a mean quality score < 20, having runs of six or more of the same nucleotides, carrying a missing quality score and including > two mismatches from the primer sequences. The multiplexed sequences were searched against the latest Ribosomal Database Project (RDP release 11.5, containing 3,356,809 16S rRNA sequences) to identify and discard chimeric sequences using the VSEARCH v2.8.3 operated under default setting (Rognes *et al.* 2016). Non-chimeric sequences were then used to pick operational taxonomic units (OTUs) using the pick_de_novo_otus.py script in the QIIME pipeline as described in details in our previous paper (Gharechahi *et al.* 2017). OTUs were defined at 97% identity using the Uclust (Edgar 2010). The most abundant sequence in each OTU cluster was selected as being a representative; and these sequences were then aligned against the Greengenes core set (gg_13_8; (DeSantis *et al.* 2006)) using the PyNAST aligner with a minimum sequence identity of 75% (Caporaso *et al.* 2010a). Taxonomies were assigned to each OTU using the Ribosomal Database Project naïve Bayesian classifier (Wang *et al.* 2007) by applying a minimum confidence value of 0.8. The OTU table was filtered for low abundant OTUs using the filter_otus_from_otu_table.py script with --min_count_fraction option set to 0.00001 (discarding OTUs represented by < 0.001% of the sequences) and then rarefied to the sequencing depth at 4660 reads corresponding to the number of reads in the sample with the smallest set of sequences. Rarefaction plots and alpha diversity indices, including Shannon, Simpson, Good's_coverage and Chao1, were calculated using the core_diversity_analyses.py script in the QIIME pipeline. Beta diversity indices, including weighted and unweighted Unifrac phylogenetic distance matrices, were constructed with the rarefied OTU table as input and visualized through the principal coordinate analysis (PCoA) plots in the MicrobiomeAnalyst web server (Dhariwal *et al.* 2017).

Statistical analysis

Statistically significant differences in physicochemical data, including DM, NDF, ADF, ADL, cellulose and hemicellulose contents, were analyzed by one-way ANOVA using the general linear model (GLM) procedure in the SAS software v9.3 (SAS Institute Inc., Cary, NC, USA). Permutational multivariate analysis of variance (PERMANOVA) was performed using the adonis function of R-package vegan v2.5-5 to test for significant differences between community compositions of forage-attached microbial communities. In addition, permutation multivariate analysis of group dispersions (PERMDISP) based on the betadisper function of the vegan was used to test for the homogeneity of dispersions (variances). Differences in taxa abundances among forages and sampling intervals were estimated using analysis of composition of microbes (ANCOM) based on relative abundances of OTUs summarized at various taxonomic levels (Mandal *et al.* 2015). Means were compared by Duncan's Multiple Range test (DMRT) in PAST v3.26 given Bonferroni p-value cutoff < 0.05 (Hammer *et al*. 2001). Error correction was done based on the number of groupwise comparisons performed at each taxonomic level. The Pearson's correlation analysis was performed using the corr.test function of the psych package v1.8.12 and p-values were corrected using Bonferroni method based on the total number of correlations calculated for each variable separately. For all tests, p-values less than 0.05 were considered statistically significant.

Results

Physicochemical properties of the rumen-incubated forages

The six forages were analyzed for the contents of NDF, ADF, ADL, cellulose and hemicellulose before their rumen incubation (Figure 1). They showed different NDF contents ($p < 0.05$), being the highest in common reed (CR) but the lowest in both camelthorn (AP) and rice straw (RS). Most of them also contained different ADF contents ($p < 0.05$), being the highest in AP but the lowest in both RS and salicornia (SC). The contents of ADL also differed significantly between the forages ($p < 0.05$), with AP being the highest at 2 times that in date palm (DP), three times those in SC, CR and kochia (KS; the latter two with similar contents at $p > 0.05$) and 6 times the lowest value in RS (24.86% vs 4.06%). CR carried the highest cellulose followed by DP, RS, KS and SC while AP contained the lowest cellulose ($p < 0.05$). SC possessed the highest amount of hemicellulose followed by CR, KS, RS, DP and AP. Overall, all the six forages had different physicochemical properties in terms of their relative contents of hemicellulose, cellulose and lignin.

Lignocellulosic biomass degradation following the rumen incubation

The six forages were monitored for their changes in the contents of DM, NDF, ADF, ADL, cellulose and hemicellulose during the rumen incubation (Figure 1 and Figure S1). DM degradation was the fastest in AP but the slowest in CR (42% vs 34%, $p < 0.05$) during the first 24 h of the incubation. DM degradation was completed in AP at 24 h but continued in CR and KS until 48 h, and in DP, RS and SC up to 96 h of the incubation, with RS having the highest degraded DM (66%) followed by SC (56%) and DP (53%). AP, DP and KS demonstrated similar trends of significantly increased NDF, ADF, ADL and cellulose contents ($p < 0.05$), mirrored in their patterns of DM degradations, mostly within the first 24 h, but their hemicelluloses decreased continuously ($p < 0.05$ in most cases)

following the incubation. However, the patterns of these five fiber-related parameters were significantly segregated among CR, RS and SC during the incubation. Although ADF and ADL were significantly increased but celluloses were remarkably declined in CR and SC ($p < 0.05$), their NDF and hemicelluloses showed contrasting patterns, being significantly reduced in CR while steadily accumulated in SC ($p < 0.05$) throughout the incubation. ADF, cellulose and hemicellulose in RS maintained stable levels while its NDF and ADL were slightly increased ($p < 0.05$) along the incubation. It was apparent that the initial differences in fiber-related physicochemical properties significantly affected the rumen digestion of the six forages.

16S rRNA gene sequencing

The paired-end sequencing of PCR amplicons from the V3-V4 region of 16S rRNA gene resulted in 5,945,300 pairs of raw sequences (averaged at 82,573 sequences per sample) with an average length of 300 bp. Sequences were joined into 4,421,604 full-length amplicons at an average of 61,411 ± 38,275 per sample. The uneven sequencing depths across samples may be due to the true differences in microbial abundance of samples or the technical variations introduced during library preparation and sequencing. Although the numbers of sequences varied greatly among the forages and across the lengths of the rumen incubation, there was a general pattern of a steady increase from the lowest at 24 h (averaged at 44,100 per sample) to the highest at 72 h (averaged at 95,823 per sample).

Sequencing data analysis

Before processing the amplicon sequences for OTU picking, they were subjected to a single round of quality filtering, resulting in 3,602,510 high quality sequences, of which 994,147 (27%) were identified to be chimeric and thus discarded from further analyses. The qualified sequences (2,493,285) were clustered at 97% similarity level into 110,804 OTUs, of which 106,982 (96.4%, representing 202,403 sequences) were labeled as low abundant features (e.g. those representing reads with frequencies less than 0.001% of the total sequences) and therefore filtered out from the OTU table. Finally, 3,822 clean OTUs representing 2,290,882 sequences were subjected to further downstream analyses. To assess whether our sequencing effort provided sufficient sequencing depths to describe the diversity of forage-attached microbiotaes, rarefaction curves describing the numbers of observed OTUs, species richness (Chao1), Shannon and Simpson diversity at various sequencing depths were generated for all samples (Figure S2a-d). Rarefaction analysis based on observed species and species richness revealed incomplete sampling of microbiota attached to the forages and thus indicated that highly diverse microbial communities attached to the forages with different lignocellulose properties(Figure S2a and b). However, Shannon (Figure S2c) and Simpson indices (Figure S2d) also showedreached plateaus, indicating that the majority of the diversity was explored that forage-attached microbiota had not been evenly sampled.

Microbial diversity analysis

Differences in alpha diversity indices of forage-attached microbiota were mostly limited to the first 24 h of the rumen incubation, during which the maximal differences in microbial attachment occurred (Figure S3). At this time, CR and

RS (two forages with the highest initial cellulose contents) had the lowest while AP and KS (two forages with the lowest initial cellulose contents) had the highest average observed species and species richness. CR and RS also showed the lowest average Shannon index, indicating their limited microbial diversity ($p < 0.05$). Diversity indices in almost all forages were not affected by the incubation length (Figure S4) except that CR had very low alpha diversity measures at 24 h of its incubation ($p < 0.05$). All samples showed a high Good's coverage (> 0.91, data not shown) at all sampling intervals, indicating that our sequencing effort figured out > 90% of the microbial diversity attached to the forages. However, the uneven sequencing depths did not allow us to fully explore the diversity of forage-attached microbiota. Considering potential random errors due to limited numbers of experimental animals and replicates in this study, such differences in alpha diversity indices among the forages were not robust enough to be interpreted with any biological significance.

Beta diversity analysis also showed limited variations among the forages as well as across the lengths of the rumen incubation. Weighted Unifrac dissimilarity matrix, which considered taxa relative abundances and their phylogenetic distances, explained 21% and 26% of the variations among the forages and across the lengths of the incubation, respectively. PERMANOVA revealed that at least some forages (e.g. CR and SC) had different microbial communities (Figure 2a, $p < 0.001$) while testing for homogeneity of group dispersions (sample distance from group centroid) identified no significant difference between group dispersions (Figure 2b, PERMDISP $p > 0.1$). Nevertheless, differences in microbial communities across the incubation lengths (Figure 2c, PERMANOVA $p < 0.001$) appeared to be mainly affected by within-group dispersions, either by the forage types or inter-animal variations, particularly during the first 24 h (Figure 2d, PERMDISP $p < 0.05$). At this time, CR-attached microbiota was well-separated from those attached to other forages. The entire microbial community structure showed a clear shift among the forages during the incubation because differences in the microbiota were apparent at 24 h but disappeared in later sampling intervals (Figure 2c and d). This finding suggested the existence of a strong preference of rumen microbiota for attachment to the forages of different digestibility while the rate and extent of such preference were quickly compromised after the initial hours of rumen digestion (24 h).

Forage-attached microbial community

A total of 18 bacterial phyla and one archaeal phylum were identified from the forage-attached microbial communities colonized in the Taleshi cattle rumen. The communities were dominated by phyla Firmicutes (45%) and Bacteroidetes (41%) followed with Fibrobacteres (5%), Spirochaetes (3%) and Proteobacteria (2%). Variations in the abundances of major bacterial phyla attached to the forages have been depicted in Figure 3a. The ANCOM analysis followed by Duncan's post hoc test revealed differential abundances of Bacteroidetes, Fibrobacteres, Lentisphaerae and Spirochaetes among the forages (Figure 3b, ANCOM $p < 0.05$). Bacteroidetes were significantly overrepresented in AP and CR compared with DP and SC (DMRT Bonferroni $p < 0.001$). Interestingly, fiber-utilizing bacteria belonging to Fibrobacteres were observed in more than 9% of the reads of CR and SC (two forages with the highest initial NDF contents) but in less than 2% of the reads of AP and KS ($p < 0.05$) and also in DP and RS (Figure 3a). CR contained

more Lentisphaerae compared with other forages ($p < 0.001$) while SC carried more Spirochaetes relative to AP ($p < 0.02$).

At the family level (Figure S5), the forage-attached microbes were affiliated to 76 families, of which nine showed differential abundances among the forages, including Bacteroidaceae, Clostridiaceae, Fibrobacteraceae, Victivallaceae, Christensenellaceae, Lachnospiraceae, Spirochaetaceae, Oxalobacteraceae and RFP12 (ANCOM $p < 0.05$). Interestingly, Victivallaceae were significantly overrepresented in CR compared with other forages ($p < 0.05$), while Bacteroidaceae was significantly enriched in CR and RS compared with AP, DP, KS and SC. Oxalobacteraceae was more abundant in KS and SC than in AP, CR, DP and RS ($p < 0.05$). Members of Fibrobacteraceae also more frequently appeared in CR and SC compared with AP, KS and RS with the lowest initial NDF contents ($p < 0.02$), while those of Clostridiaceae and Lachnospiraceae were underrepresented in CR compared with other forages ($p < 0.05$).

Particle-attached microbiota were affiliated to 119 genera (taxonomic level 6), of which 12 displayed differential abundances among the forages (ANCOM $p < 0.05$, Figure 4) most of which were among high abundant members of rumen community which are known to play key roles in plant lignocellulose degradation, including *Ruminococcus*, *Fibrobacter*, *Prevotella*, *Treponema*, *Lachnospira*, *Succinivibrio*, *Pseudobutyrivibrio*, *Butyrivibrio*, *Oxalobacter*, *Clostridium*, BF311 and *Succiniclasticum*. *Ruminococcus* and members of BF311 were more dominant in RS than in AP, KS and SC ($p < 0.001$). *Fibrobacter* were more highly represented in CR and SC. Species of *Lachnospira* were present in 0.3% of the reads of AP and KS ($p < 0.05$) but only 0.11% of CR and 0.09% of SC. *Succinivibrio* were overrepresented in AP and SC compared with CR ($p < 0.05$). Species of *Prevotella* were more abundant in CR (> 21% of the reads) than in DP and SC ($p < 0.008$). Compared to other forages, CR carried less *Butyrivibrio* and *Pseudobutyrivibrio* species ($p < 0.007$).

Changes in forage-attached microbes during the rumen incubation

In order to examine whether the community composition of forage-attached microbes changed during the rumen incubation, the relative abundances of the microbes were tracked at 24 h intervals (Figure 5). The abundances of eight out of the 119 bacterial genera showed significant differences among the incubation lengths (Bonferroni corrected $p < 0.05$). Interestingly, the proportions of cellulolytic bacteria belonging to unclassified Ruminococcaceae linearly increased with the incubation lengths in AP, KS and SC. The proportion of *Fibrobacter* sharply dropped after the first 24 h and reached to an average of 4% between 48 and 96 h of the incubation in CR. Members of *Pseudobutyribibrio* linearly decreased in AP and KS while those of *Butyrivibrio* increased in CR with the incubation lengths. Members of *Clostridium* increased in SC but those of *Prevotella* and unclassified Paraprevotellaceae decreased with the incubation lengths, which were consistent with findings of Cheng *et al.* (2017).

Relationship between lignocellulose degradation and forage-attached microbial communities

The Pearson's correlation between the initial physicochemical properties of the forages and the composition of the forage-attached microbiota during initial hours (24 h) of the rumen incubation was performed to determine whether rumen microbes preferred specific forages for attachment. Only correlations with p-values (Bonferroni-corrected) less than 0.05 were considered to have significant biological terms. This analysis demonstrated that the prevalence of the family Fibrobacteraceae ($r = 0.77$, $p = 0.03$) was positively correlated with NDF contents of the forages. At the genus level, the abundance of *Fibrobacter* ($r = 0.77$, $p = 0.05$) was positively but an unclassified Erysipelotrichaceae genus p-75-a5 ($r = -0.79$, $p = 0.03$) was negatively correlated with NDF contents in the forages. When hemicellulose contents were considered, a negative correlation with members of the family Pirellulaceae ($r = -0.79$, $p = 0.02$) was detected.

We also correlated microbial profiles with physicochemical properties of the forages during the rumen incubation. This analysis revealed that the prevalence of the families Fibrobacteraceae ($r= 0.83$, $p = 0.03$ in CR), Anaeroplasmataceae ($r = 0.82$, $p = 0.04$ in CR), Prevotellaceae ($r = 0.83$, $r = 0.04$ in KS) and Paraprevotellaceae ($r = 0.85$, $p = 0.02$ in SC) were positively but Ruminococcaceae ($r = < -0.85$, $p < 0.01$ in CR and SC) and unclassified Bacteroidales ($r = -0.84$, $p = 0.05$ in RS) were negatively corelated with DM contents of the forages. Particularly, members of Ruminococcaceae were positively correlated with ADF, ADL and hemicellulose contents in CR, DP and SC ($r > 0.8$ and $p < 0.04$), the forages with the highest initial NDF contents (Figure 1). The population of Lachnospiraceae was also positively correlated ($r = 0.83$, and $p = 0.04$) with cellulose content in CR.

To ascertain whether there was any relationship between the rumen microbiota and lignocellulose degradation, an additional Pearson's correlation analysis was performed between DM loss and the relative abundance of forage-attached microbial communities during the rumen incubation. Interestingly, DM degradation was positively correlated with the prevalence of the families Fibrobacteraceae ($r > 0.76$, $p < 0.001$ in CR) and Spirochaetaceae ($r = 0.91$, $p = 0.0004$ in KS) but was negatively corelated with species belonging to unclassified Bacteroidales ($r < -0.82$, $p < 0.05$ in CR and DP), Ruminococcaceae ($r = -0.83$, $p = 0.04$ in CR), Mogibacteriaceae ($r < -0.82$, $p < 0.05$ in CR and RS) and Erysiopelotrichaceae ($r = -0.87$, $p = 0.01$ in CR). The correlations of cellulose and hemicellulose degradations with the abundances of forage-attached microbes also showed similar patterns.

Discussion

In this study, we investigated the relationship between biomass degradation of and microbial attachment to six common lignocellulosic forages varying in their physicochemical properties, including percentages of NDF, ADF, ADL and the contents of cellulose and hemicellulose. Forages containing the highest cellulose contents [(common reed (CR) vs. camelthorn (AP)] were degraded to a limited extent during the initial hours of their rumen incubation. The total DM degradation was mainly determined by NDF contents of the forages, because as rice straw (RS) with the lowest initial NDF (77.4%) had the fastest (66%) while CR with the highest initial NDF (87.2%) had the lowest (42%) DM loss over 96 h of the incubation. Variability in DM degradation of feeds reflected the differences in lignocellulose composition of their cell walls (Bruno-Soares *et al.* 2000; Jančík *et al.* 2010). The rate and extent of DM fermentation in the rumen determines the nutrition efficiency of feeds to ruminants (Jančík *et al.* 2010).

The 16S rRNA gene sequencing data allowed taxonomic identification and quantification of the rumen microbiota tightly attached to the forages. Rarefaction analysis based on the indices reflecting species richness and species relative abundance, e.g. Shannon and Simpson (Kim *et al.* 2017), indicated that the majority of the diversity of rumen microbiota attached to the forages was already sampled ~~not sufficiently and evenly sampled~~. Alpha diversity analysis also showed a limited biologically significant difference among the forages, which could likely be attributed to a high microbial heterogeneity among animals included in the study. The changes in diversity measures were largely restricted to the initial hours of rumen incubation when maximal DM degradation occurred. The differences in species richness and evenness were linked to cellulose contents of the forages, where CR and RS with the highest cellulose contents displayed limited species diversity. This result was in agreement with the data on rice straw and alfalfa hay being fed to Holstein cows, in which more bacteria attached to alfalfa with lower NDF (Liu *et al.* 2016). These findings suggested that only a limited fraction of rumen microbiota was capable of attachment to feeds with high lignocellulose contents.

Analysis of community structure of forage-attached microbiota by Unifrac dissimilarity matrix revealed significant differences among the forages and across the length of rumen incubation. Particularly, CR-attached microbiota was well separated from those of other forages at the first 24 h of the incubation. In addition, microbiota attached to CR and SC, the two forages with the highest initial NDF contents, were also distantly clustered while those of other forages did not show any apparent separation, indicating the similarity of their communities. These variations could likely be determined by the nature of the forages, e.g., their chemical compositions. Unifrac diversity measure also showed that extension of the incubation time contributed to the increased similarity across the samples, as reflected by the overlapped clustering in the PCoA plots for most samples collected at 72 and 96 h of the incubation. This could be explained by the fact that initial DM degradation resulted in the reduction of digestible components but the accumulation of indigestible residues which had quite similar properties across the forages, thus favoring the attachment of structurally similar communities of rumen microbiota. Huws *et al.* (2013) also observed similar changes in the microbial communities attached to perennial ryegrass following its rumen incubation.

Members of Firmicutes, Bacteroidetes, Fibrobacteres, Spirochaetes and Proteobacteria were dominant in all samples, accounting for greater than 96% of the bacterial communities attached to the forages, consistent with the findings for rice straw and alfalfa hay (Liu *et al.* 2016). The abundance of these bacterial phyla varied among the forages and across the incubation lengths. Particularly, species of Bacteroidetes were more abundantly attached to the forages with high ADF contents (AP and CR). Bacteroidetes were among highly abundant members of the rumen microbiota being best recognized for their saccharolytic activities. The presence of a high number of pectinolytic and cellulolytic enzymes in their genomes clustered with other lignocellulose degrading enzymes into polysaccharide utilization loci (PUL) suggested that they are also actively involved in pectin, hemicellulose and cellulose degradation (Gharechahi and Salekdeh 2018; Lapebie *et al.* 2019; Naas *et al.* 2014). Within this phylum, members of the families Bacteroidaceae, Prevotellaceae and Paraprevotellaceae displayed significant differences among the forages. Particularly, Bacteroidaceae were significantly overrepresented in the forages with the lowest initial ADL contents (CR and RS). At the genus-level, this differential abundance was only affiliated to the BF311, an uncultured and

unknown rumen bacterium. Prevotellaceae were particularly positively correlated with DM degradation, suggesting their active role in lignocellulose degradation in the rumen. *Prevotella* spp. are abundant members of the rumen microbiome that have a high genetic diversity and thus the ability to thrive on a wide range of substrates, including cellulose, hemicellulose, pectin, proteins and peptides (Dodd *et al.* 2010; Golder *et al.* 2014). They are known for their xylanolytic properties in the rumen and thus play an important role in fiber degradation (Dodd *et al.* 2010).

Fibrobacteres were predominantly represented in microbiota attached to the forages with the highest NDF contents [e.g. CR and Salicornia (SC)]. They are known to produce a battery of cellulolytic enzymes capable of degrading cellulose as a sole carbon source (Flint *et al.* 2008; Suen *et al.* 2011). The association of forage NDF content with the prevalence of *Fibrobacter* is also recently observed in the cow rumen microbiota (Liu *et al.* 2016). In contrast to Fibrobacteres, the genera *Clostridium*, *Shuttleworthia* and *Ruminococcus* tended to attach the forages with limited NDF and cellulose contents. This is consistent with previous findings on the preference of *Ruminococcus* species for attachment to high quality sugar-rich hays (Klevenhusen *et al.* 2017). Ruminococcaceae showed a strong negative correlation with total DM contents in the forages as well. Shinkai *et al.* (2010) also reported that members of Fibrobacteres, including *F. succinogenes*, abundantly attached to less digestible fibers while those of Ruminococcaceae, specifically *R. flavefaciens,* preferred easily digestible fibers and thus were infrequently detected in the stem parts of hays. The rate and extent of fiber degradation by *F. succinogenes* are also greater than *R. albus* and *R. flavefaciens* (Kobayashi *et al.* 2008). Early microscopy analysis indicated that *R. albus* is less commonly attached to plant cell walls while *F. succinogenes* forms extensive microcapsula enveloping the cell walls (Chesson *et al.* 1986). The community of unclassified Ruminococcaceae tended to linearly expand in AP, KS and SC following their rumen incubation. This finding suggested that the degradation of surface accessible fibers turned forage residues into favored substrates for the attachment by species of Ruminococcaceae. A declined abundance of species of *Ruminococcus* has been reported in the cow rumen when the starch content of diet was increased (Zened *et al.* 2013). *Ruminococcus* spp. have been equipped with specialized mechanisms for fiber adhesion and degradation, in which multiple carbohydrate degrading enzymes assemble into multienzyme cellulosome complexes capable of attachment to and degradation of polysaccharides in various plant cell walls (Bayer *et al.* 2004; Doi and Kosugi 2004).

Recently, Huws *et al.* (2016) reported that microbial colonization of perennial ryegrass occurred at two successive stages: The primary (the first 1-2 h after its rumen entry) and the secondary phases (4-8 h). It was the secondary phase, during which the maximal DM degradation was achieved and the proportion of *Succinivibrio* spp. decreased while those of *Pseudobutyrivibrio*, *Roseburia* and *Ruminococcus* spp. increased. Piao *et al.* (2014) also observed that the populations of *Pseudobutyrivibrio* and *Ruminococcus* spp. increased during their secondary colonization to switchgrass in the Friesian cow rumen. The microbial communities attached to feeds during the primary phase were believed to utilize soluble and easily accessible nutrients while those colonized feeds during the secondary phase were considered to be the true lignocellulose degraders (Huws *et al.* 2016; Liu *et al.* 2016). In this study, an increased prevalence of *Butyrivibrio* was noted in CR. *Butyrivibrio* spp. are known for their proteolytic, hemicellulolytic and biohydrogenating activities (Krause *et al.* 2003). Liu *et al.* (2016) reported a strong positive correlation between the

abundance of *Butyrivibrio* spp. and the crude protein contents in the rice straw and alfalfa hay, suggesting their preference for the attachment to proteinaceous components of the feeds.

In summary, our results demonstrated that physicochemical compositions of the forages, more specifically their cellulose contents, were among the major factors influencing microbial attachments and thus lignocellulose degradation in the cattle rumen. No taxonomic lineage was found to be specific of a particular forage, suggesting that most rumen microbes had an inherent tendency for attachment to lignocellulosic substrates. However, differential attachments to the forages by rumen microbiota suggested that the physicochemical properties were the key factors influencing the rate of microbial attachment. Our results also revealed that members of the rumen microbiota competed for their attachments to the forages with different lignocellulose compositions mostly during the initial hours of the rumen incubation. However, after the degradation of easily digestible components in the forages, relatively uniform microbial communities developed on their surface.

It should be noted that the bacterial community composition detected in each forage at each sampling interval may not necessarily reflect the actual abundance of microbes due to an inherent bias in sample collection, processing and storage as well as DNA extraction and PCR amplification, which may cause over- or under-representation of some taxa. For example, a recent comparative analysis of various DNA extraction methods used in rumen microbiome analyses argued that DNA extraction method can have an adverse effect on the community composition of rumen samples and may associate with an increased or decreased abundance of some specific taxa (Henderson *et al.* 2013). Particularly, the inclusion of a physical lysis step using bead-beating method increased the efficacy of DNA extraction from Gram-positive bacteria (Knudsen *et al.* 2016). The extent to which our DNA extraction method, in which the physical lysis step was not included, affected community composition of the forage-attached microbiota was unknown. In addition, the high number of PCR cycles may increase the number of PCR artifacts and bias the microbial compositions.

Acknowledgements

This research was funded by the Agricultural Biotechnology Research Institute of Iran (ABRII), the international cooperation and exchange program of the National Natural Science Foundation of China (No. 31461143020), and the Chinese government contribution to CAAS-ILRI Joint Laboratory on Livestock and Forage Genetic Resources in Beijing. The paper contributes to the CGIAR Research Program on Livestock.

Figure legends

Figure 1. Differences among the six forages in dry matter (DM) and percentages of neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), cellulose and hemicellulose before (0 h) and after 24, 48, 72, and 96 h of the rumen incubation. The solid circle and error bar in each boxplot show mean and standard deviation, respectively. Statistically significant differences were determined using one-way ANOVA. Means were compared

using Duncan's multiple range test and are labelled with different letters at each sampling interval for $p < 0.05$. AP, camelthorn; CR, common reed; DP, date palm; KS, Kochia; RS, rice straw; and SC, Salicornia.

Figure 2. Beta diversity analysis of rumen microbial communities attached to the six forages during the rumen incubation. PCoA plots show the distribution of samples based on weighted Unifrac distance matrix, in which samples are grouped according to the forages (a) and the sampling intervals (b). Significant differences were tested using PERMANOVA with a p-value cutoff 0.01. The percentage of variation explained by each principle coordinate is indicated next to the corresponding axis. The homogeneity of dispersions was also tested for this diversity measure to examine variance differences between the forages (c) and the sampling intervals (d). Significant differences were determined using the betadisper function of R package vegan v2.5-5 at 999 permutations. P-values < 0.05 were considered statistically significant. For each treatment, data in triplicate representing three separate bags, were included. AP, camelthorn; CR, common reed; DP, date palm; KS, Kochia; RS, rice straw; and SC, Salicornia.

Figure 3. (a) The proportions of reads (mean ± SD) assigned to the major bacterial phyla ($n = 7$) detected in the forage-attached microbial communities. The relative abundances were calculated based on the proportion of reads assigned to each phylum in the rarefied OTU table at an even sequencing depth of 4660 reads. (b) Box plots show relative abundance of phyla differentially attached to the forages. Statistically significant differences were calculated based on the analysis of composition of microbes (ANCOM) using $p < 0.05$ following Duncan's multiple range test for comparison of the means. Center lines indicate the median value, boxes the interquartile range and red squares the mean. Means with different letters differ at Bonferroni corrected $p < 0.05$. AP, camelthorn; CR, common reed; DP, date palm; KS, Kochia; RS, rice straw; and SC, Salicornia.

Figure 4. The log of relative abundances of genera differentially attached to the six forages following the rumen incubation. Differential abundances were statistically tested using ANCOM with a p-value cutoff < 0.05. Means were compared using Duncan's multiple range test only accepting Bonferroni corrected p-values < 0.05. Boxplots labeled with different letters show statistically significant differences. Center line represents median value. AP, camelthorn; CR, common reed; DP, date palm; KS, Kochia; RS, rice straw; and SC, Salicornia.

Figure 5. The average relative abundances of taxa (genus level) differentially represented in microbial communities attached to the six forages during the rumen incubation (up to 96 h with 24 h intervals). Statistically significant differences were calculated using ANCOM and means were compared using Duncan's multiple range test. Error bars represent standard deviations and their lengths are adjusted at 95% confidence interval. Means with different letters are statistically significant at Bonferroni corrected $p < 0.05$. No taxa were found to be differentially represented in DP. AP, camelthorn; CR, common reed; KS, Kochia; RS, rice straw; and SC, Salicornia. "Un" refers to unclassified.

Supplementary materials

Figure S1.

Changes in DM contents and percentages of NDF, ADF, ADL, cellulose and hemicellulose in six forages during the rumen incubation. The solid circle in each boxplot shows mean and error bars represent standard deviation.

Statistically significant differences were determined using one-way ANOVA. Means were compared using Duncan's multiple range test and labelled with different letters for $p < 0.05$.

Figure S2.

Alpha diversity analysis through rarefaction plots. (a) Rarefaction curves showing the increase in number of observed OTUs; (b) Species richness (number of observed OTUs + number of unobserved OTUs, Chao1); (c) Shannon; and (d) Simpson diversity indices on Y-axis as a function of the number of reads sampled on X-axis.

Figure S3.

Alpha diversity indices of rumen microbiota attached to the six forages with different lignocellulosic compositions at 24, 48, 72 and 96 hours of the rumen incubation. Alpha diversity indices were measured based on OTUs present at an even sequencing depth of 4,660 reads (corresponding to the sequencing depth of the sample with the lowest number of reads) in all samples. Statistically significant differences were determined using one-way ANOVA and means were compared by Duncan's multiple range test. * p value < 0.05, ** p value < 0.01 and *** p value < 0.001.

Figure S4.

Alpha diversity analysis of rumen microbial communities attached to the six forages during the rumen incubation. The OTU table for each forage was sampled at an even sequencing depth (n = 4660) and diversity measures were calculated using plot_richness function of R package phyloseq and visualized using geom_boxplot function of R package ggplot2. Statistically significant differences were calculated using one-way ANOVA at p-value cutoff of 0.05. Differences between means were determined using Duncan's multiple range test in PAST v3.26. * p value < 0.05, ** p value < 0.01 and *** p value < 0.001.

Figure S5.

The proportions of reads affiliated to OTUs at the taxonomic level of family in the six forages during the rumen incubation. Data for each sampling interval is the mean of three biological replicates corresponding to three bags retrieved one each from the three cannulated bulls. For each forage, the OTU table is rarefied to the sequencing depth of the sample with the lowest number of reads.

References

Bayer EA, Belaich JP, Shoham Y *et al.* The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. *Annu Rev Microbiol* 2004;**58**: 521-54.

Bergman EN. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol Rev* 1990;**70**: 567-90.

Bruno-Soares AM, Abreu JMF, Guedes CVM *et al.* Chemical composition, DM and NDF degradation kinetics in rumen of seven legume straws. *Animal Feed Science and Technology* 2000;**83**: 75-80.

Caporaso JG, Bittinger K, Bushman FD *et al.* PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 2010a;**26**: 266-7.

Caporaso JG, Kuczynski J, Stombaugh J *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010b;**7**: 335-6.

Cheng Y, Wang Y, Li Y *et al.* Progressive Colonization of Bacteria and Degradation of Rice Straw in the Rumen by Illumina Sequencing. *Front Microbiol* 2017;**8**: 2165.

Chesson A, Stewart CS, Dalgarno K *et al.* Degradation of isolated grass mesophyll, epidermis and fibre cell walls in the rumen and by cellulolytic rumen bacteria in axenic culture. *Journal of Applied Bacteriology* 1986;**60**: 327-36.

De Mulder T, Goossens K, Peiren N *et al.* Exploring the methanogen and bacterial communities of rumen environments: solid adherent, fluid and epimural. *FEMS Microbiol Ecol* 2017;**93**.

DeSantis TZ, Hugenholtz P, Larsen N *et al.* Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 2006;**72**: 5069-72.

Dhariwal A, Chong J, Habib S *et al.* MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res* 2017;**45**: W180-W88.

Dodd D, Moon YH, Swaminathan K *et al.* Transcriptomic analyses of xylan degradation by Prevotella bryantii and insights into energy acquisition by xylanolytic bacteroidetes. *J Biol Chem* 2010;**285**: 30261-73.

Doi RH, Kosugi A. Cellulosomes: plant-cell-wall-degrading enzyme complexes. *Nat Rev Microbiol* 2004;**2**: 541-51.

Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010;**26**: 2460-1.

Edwards JE, Huws SA, Kim EJ *et al.* Characterization of the dynamics of initial bacterial colonization of nonconserved forage in the bovine rumen. *FEMS Microbiol Ecol* 2007;**62**: 323-35.

Flint HJ, Bayer EA, Rincon MT *et al.* Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat Rev Microbiol* 2008;**6**: 121-31.

Gharechahi J, Kharazian ZA, Sarikhan S *et al.* The dynamics of the bacterial communities developed in maize silage. *Microb Biotechnol* 2017;**10**: 1663-76.

Gharechahi J, Salekdeh GH. A metagenomic analysis of the camel rumen's microbiome identifies the major microbes responsible for lignocellulose degradation and fermentation. *Biotechnol Biofuels* 2018;**11**: 216.

Gharechahi J, Zahiri HS, Noghabi KA *et al.* In-depth diversity analysis of the bacterial community resident in the camel rumen. *Syst Appl Microbiol* 2015;**38**: 67-76.

Godoy-Vitorino F, Goldfarb KC, Karaoz U *et al.* Comparative analyses of foregut and hindgut bacterial communities in hoatzins and cows. *ISME J* 2012;**6**: 531-41.

Goering HK. Forage fiber analyses (apparatus, reagents, procedures, and some applications). In: Van Soest PJ (ed.). Washington, D.C. :: Agricultural Research Service, U.S. Dept. of Agriculture, 1970.

Golder HM, Denman SE, McSweeney C *et al.* Ruminal bacterial community shifts in grain-, sugar-, and histidine-challenged dairy heifers. *J Dairy Sci* 2014;**97**: 5131-50.

Guarner F, Malagelada JR. Gut flora in health and disease. *Lancet* 2003;**361**: 512-9.

Hammer Ø, Harper DA, Ryan PD. PAST: paleontological statistics software package for education and data analysis. 2001.

Henderson G, Cox F, Kittelmann S *et al.* Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. *PLoS One* 2013;**8**: e74787.

Hungate RE. *The rumen and its microbes*: Elsevier, 2013.

Huws SA, Edwards JE, Creevey CJ *et al.* Temporal dynamics of the metabolically active rumen bacteria colonizing fresh perennial ryegrass. *FEMS Microbiol Ecol* 2016;**92**.

Huws SA, Mayorga OL, Theodorou MK *et al.* Successional colonization of perennial ryegrass by rumen bacteria. *Lett Appl Microbiol* 2013;**56**: 186-96.

Jami E, Israel A, Kotser A *et al.* Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J* 2013;**7**: 1069-79.

Jami E, Mizrahi I. Composition and similarity of bovine rumen microbiota across individual animals. *PLoS One* 2012;**7**: e33306.
Jančík F, Koukolová V, Homolka P. Ruminal degradability of dry matter and neutral detergent fibre of grasses. *Czech J Anim Sci* 2010;**55**: 359-71.
Jin W, Wang Y, Li Y *et al.* Temporal changes of the bacterial community colonizing wheat straw in the cow rumen. *Anaerobe* 2018;**50**: 1-8.
Khafipour E, Li S, Plaizier JC *et al.* Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. *Appl Environ Microbiol* 2009;**75**: 7115-24.
Kim BR, Shin J, Guevarra R *et al.* Deciphering Diversity Indices for a Better Understanding of Microbial Communities. *J Microbiol Biotechnol* 2017;**27**: 2089-93.
Klevenhusen F, Petri RM, Kleefisch MT *et al.* Changes in fibre-adherent and fluid-associated microbial communities and fermentation profiles in the rumen of cattle fed diets differing in hay quality and concentrate amount. *FEMS Microbiol Ecol* 2017;**93**.
Knudsen BE, Bergmark L, Munk P *et al.* Impact of Sample Type and DNA Isolation Procedure on Genomic Inference of Microbiome Composition. *mSystems* 2016;**1**.
Kobayashi Y, Shinkai T, Koike S. Ecological and physiological characterization shows that Fibrobacter succinogenes is important in rumen fiber digestion - review. *Folia Microbiol (Praha)* 2008;**53**: 195-200.
Kocherginskaya SA, Aminov RI, White BA. Analysis of the Rumen Bacterial Diversity under two Different Diet Conditions using Denaturing Gradient Gel Electrophoresis, Random Sequencing, and Statistical Ecology Approaches. *Anaerobe* 2001;**7**: 119-34.
Kong Y, Teather R, Forster R. Composition, spatial distribution, and diversity of the bacterial communities in the rumen of cows fed different forages. *FEMS Microbiol Ecol* 2010;**74**: 612-22.
Krause DO, Denman SE, Mackie RI *et al.* Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiol Rev* 2003;**27**: 663-93.
Lapebie P, Lombard V, Drula E *et al.* Bacteroidetes use thousands of enzyme combinations to break down glycans. *Nat Commun* 2019;**10**: 2043.
Liu J, Zhang M, Xue C *et al.* Characterization and comparison of the temporal dynamics of ruminal bacterial microbiota colonizing rice straw and alfalfa hay within ruminants. *J Dairy Sci* 2016;**99**: 9668-81.
Mackie RI. Mutualistic fermentative digestion in the gastrointestinal tract: diversity and evolution. *Integr Comp Biol* 2002;**42**: 319-26.
Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 2011;**27**: 2957-63.
Mandal S, Van Treuren W, White RA *et al.* Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb Ecol Health Dis* 2015;**26**: 27663.
Martineau R, Proulx JG, Cortes C *et al.* Two-Stage Rumen Cannulation Technique in Dairy Cows. *Vet Surg* 2015;**44**: 551-6.
McAllister TA, Bae HD, Jones GA *et al.* Microbial attachment and feed digestion in the rumen. *J Anim Sci* 1994;**72**: 3004-18.
Naas AE, Mackenzie AK, Mravec J *et al.* Do rumen Bacteroidetes utilize an alternative mechanism for cellulose degradation? *mBio* 2014;**5**: e01401-14.
Petri RM, Forster RJ, Yang W *et al.* Characterization of rumen bacterial diversity and fermentation parameters in concentrate fed cattle with and without forage. *J Appl Microbiol* 2012;**112**: 1152-62.
Petri RM, Pourazad P, Khiaosa-Ard R *et al.* Temporal dynamics of in-situ fiber-adherent bacterial community under ruminal acidotic conditions determined by 16S rRNA gene profiling. *PLoS One* 2017;**12**: e0182271.
Piao H, Lachman M, Malfatti S *et al.* Temporal dynamics of fibrolytic and methanogenic rumen microorganisms during in situ incubation of switchgrass determined by 16S rRNA gene profiling. *Front Microbiol* 2014;**5**: 307.
Rognes T, Flouri T, Nichols B *et al.* VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 2016;**4**: e2584.
Sadet S, Martin C, Meunier B *et al.* PCR-DGGE analysis reveals a distinct diversity in the bacterial population attached to the rumen epithelium. *Animal* 2007;**1**: 939-44.
Shinkai T, Ueki T, Kobayashi Y. Detection and identification of rumen bacteria constituting a fibrolytic consortium dominated by Fibrobacter succinogenes. *Anim Sci J* 2010;**81**: 72-9.
Suen G, Weimer PJ, Stevenson DM *et al.* The complete genome sequence of Fibrobacter succinogenes S85 reveals a cellulolytic and metabolic specialist. *PLoS One* 2011;**6**: e18814.
Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci* 1991;**74**: 3583-97.

Wang Q, Garrity GM, Tiedje JM *et al.* Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007;**73**: 5261-7.

Zened A, Combes S, Cauquil L *et al.* Microbial ecology of the rumen evaluated by 454 GS FLX pyrosequencing is affected by starch and oil supplementation of diets. *FEMS Microbiol Ecol* 2013;**83**: 504-14.

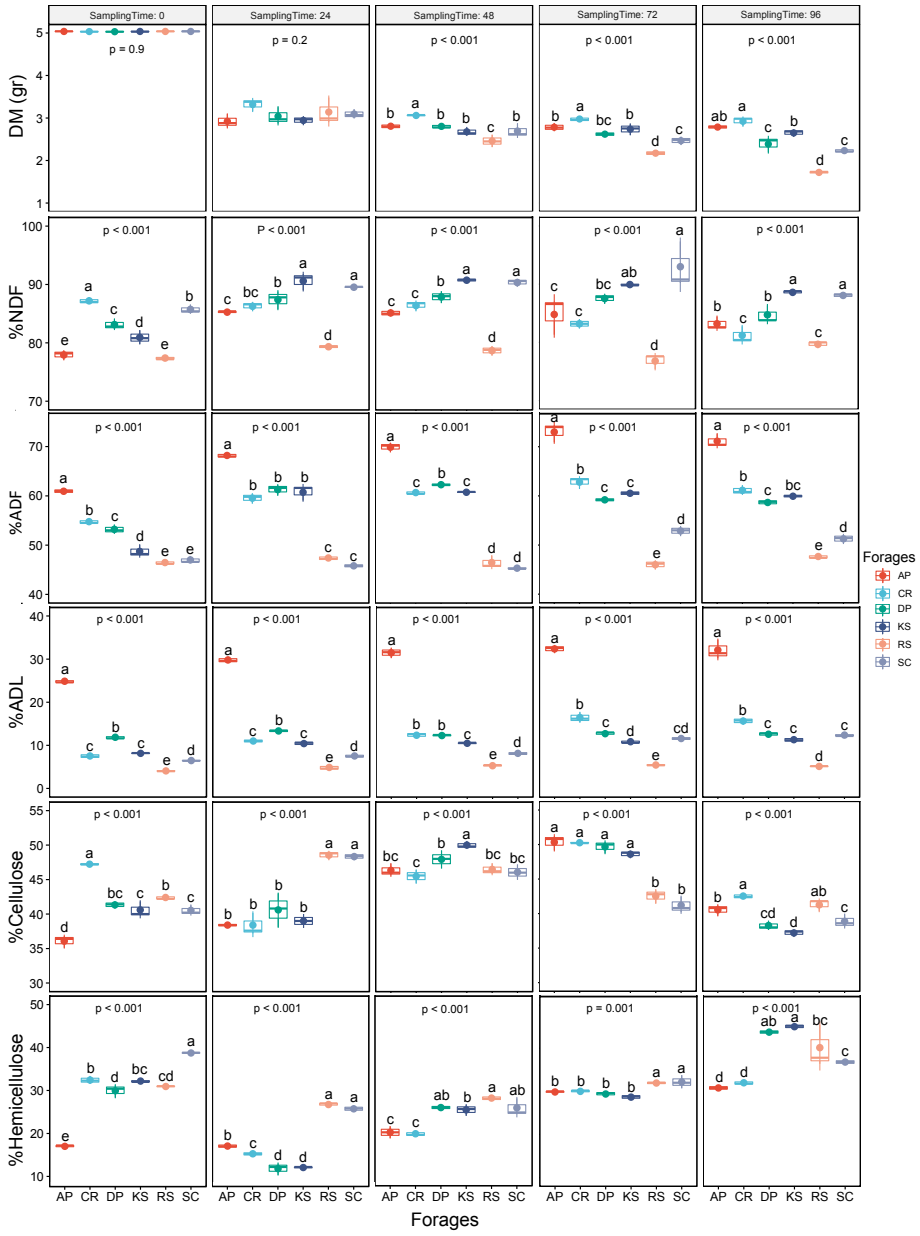

SamplingTime: 0
SamplingTime: 24
SamplingTime: 48
SamplingTime: 72
SamplingTime: 96
DM (gr)
%NDF
%ADF
%ADL
%Cellulose
%Hemicellulose
Forages
AP
CR
DP
KS
RS
SC

Figure 1.

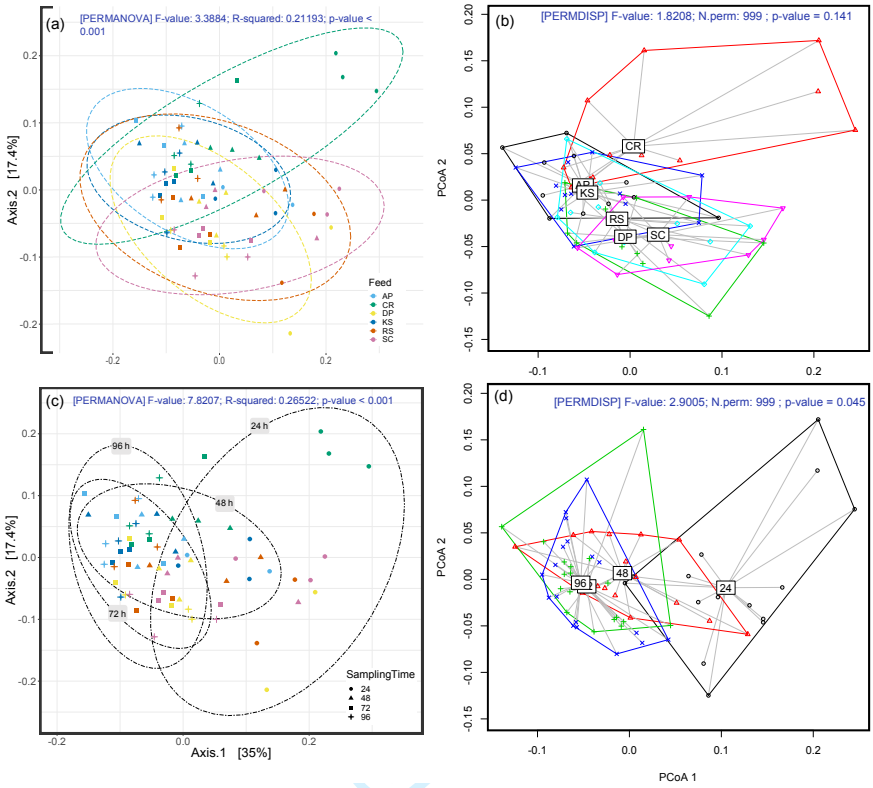


Figure 2.

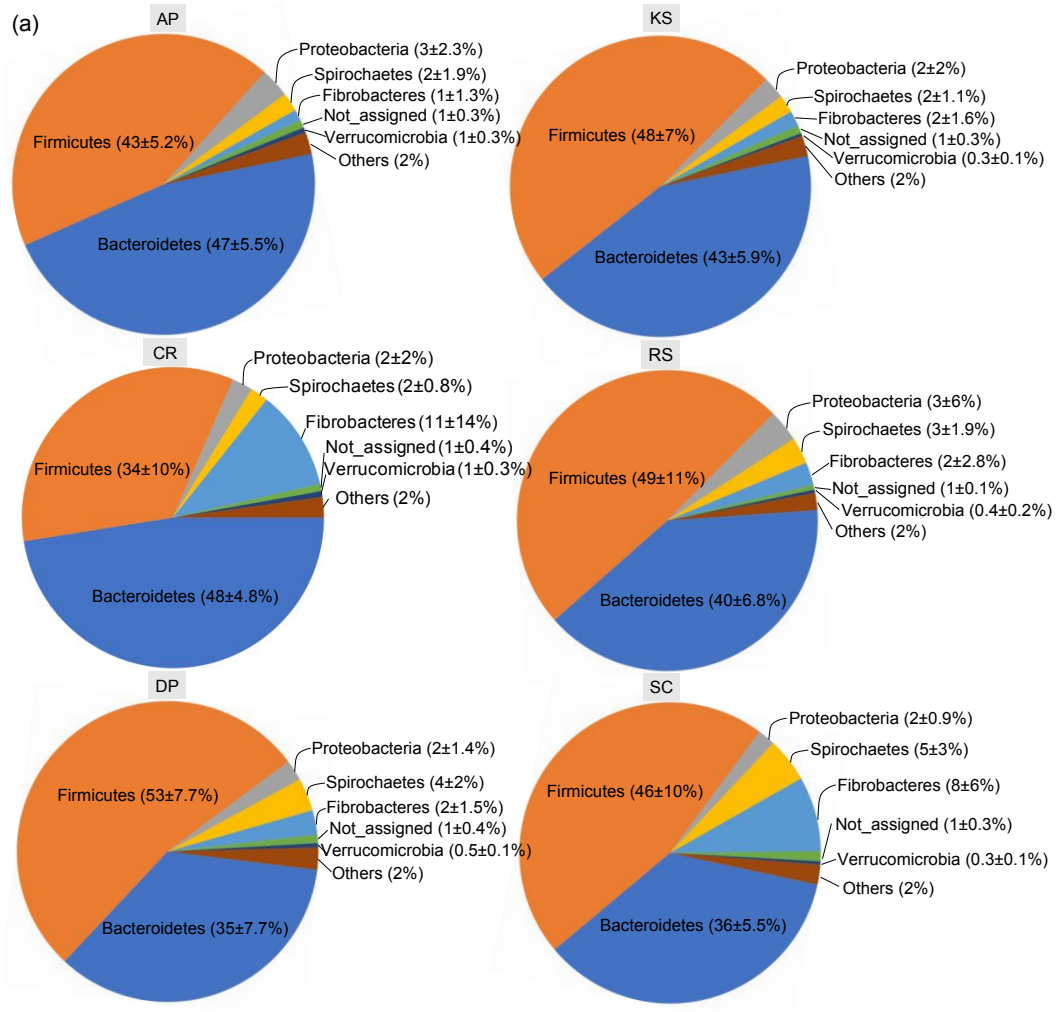

(a)
AP
Proteobacteria (3±2.3%)
Spirochaetes (2±1.9%)
Fibrobacteres (1±1.3%)
Not_assigned (1±0.3%)
Verrucomicrobia (1±0.3%)
Others (2%)
Firmicutes (43±5.2%)
Bacteroidetes (47±5.5%)
KS
Proteobacteria (2±2%)
Spirochaetes (2±1.1%)
Fibrobacteres (2±1.6%)
Not_assigned (1±0.3%)
Verrucomicrobia (0.3±0.1%)
Others (2%)
Firmicutes (48±7%)
Bacteroidetes (43±5.9%)
CR
Proteobacteria (2±2%)
Spirochaetes (2±0.8%)
Fibrobacteres (11±14%)
Not_assigned (1±0.4%)
Verrucomicrobia (1±0.3%)
Others (2%)
Firmicutes (34±10%)
Bacteroidetes (48±4.8%)
RS
Proteobacteria (3±6%)
Spirochaetes (3±1.9%)
Fibrobacteres (2±2.8%)
Not_assigned (1±0.1%)
Verrucomicrobia (0.4±0.2%)
Others (2%)
Firmicutes (49±11%)
Bacteroidetes (40±6.8%)
DP
Proteobacteria (2±1.4%)
Spirochaetes (4±2%)
Fibrobacteres (2±1.5%)
Not_assigned (1±0.4%)
Verrucomicrobia (0.5±0.1%)
Others (2%)
Firmicutes (53±7.7%)
Bacteroidetes (35±7.7%)
SC
Proteobacteria (2±0.9%)
Spirochaetes (5±3%)
Fibrobacteres (8±6%)
Not_assigned (1±0.3%)
Verrucomicrobia (0.3±0.1%)
Others (2%)
Firmicutes (46±10%)
Bacteroidetes (36±5.5%)
(b)
p__Bacteroidetes
p__Fibrobacteres
p__Lentisphaerae
p__Spirochaetes
Relative abundance
AP CR DP KS RS SC
Grouping factor
forages

Figure 3.

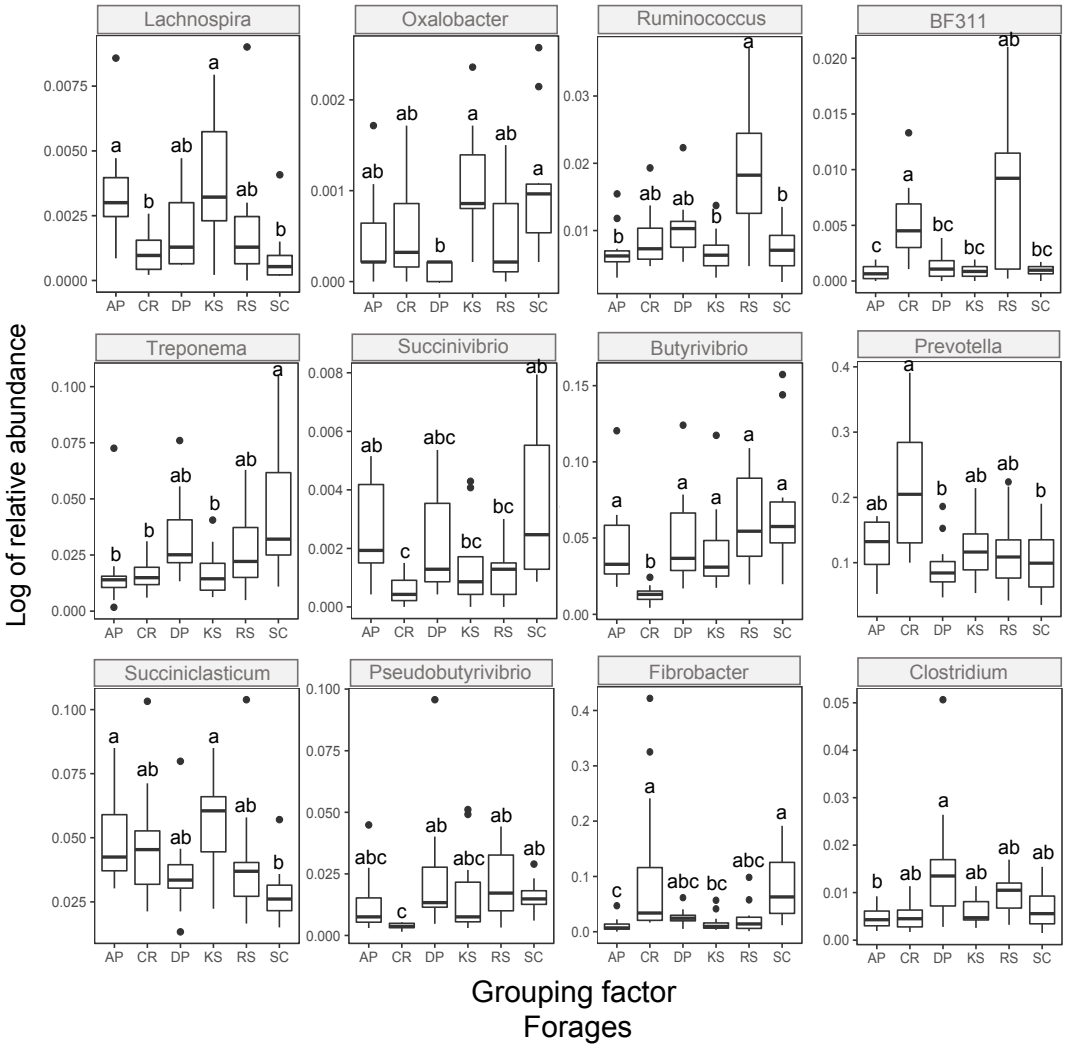

Lachnospira
Oxalobacter
Ruminococcus
BF311
Treponema
Succinivibrio
Butyrivibrio
Prevotella
Succiniclasticum
Pseudobutyrivibrio
Fibrobacter
Clostridium
AP CR DP KS RS SC
Log of relative abundance
Grouping factor
Forages

Figure 4

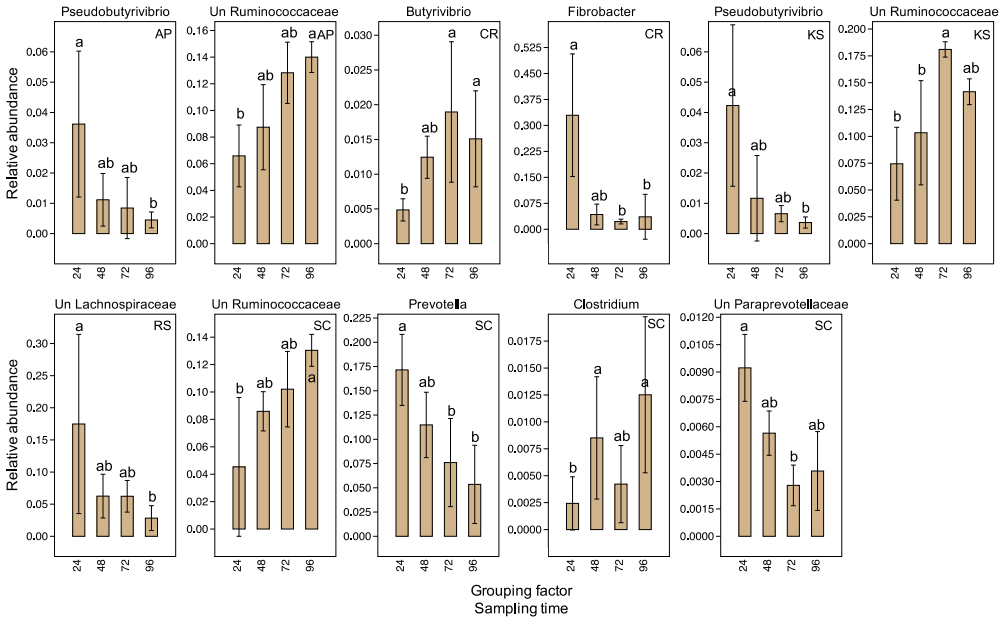


Figure 5.

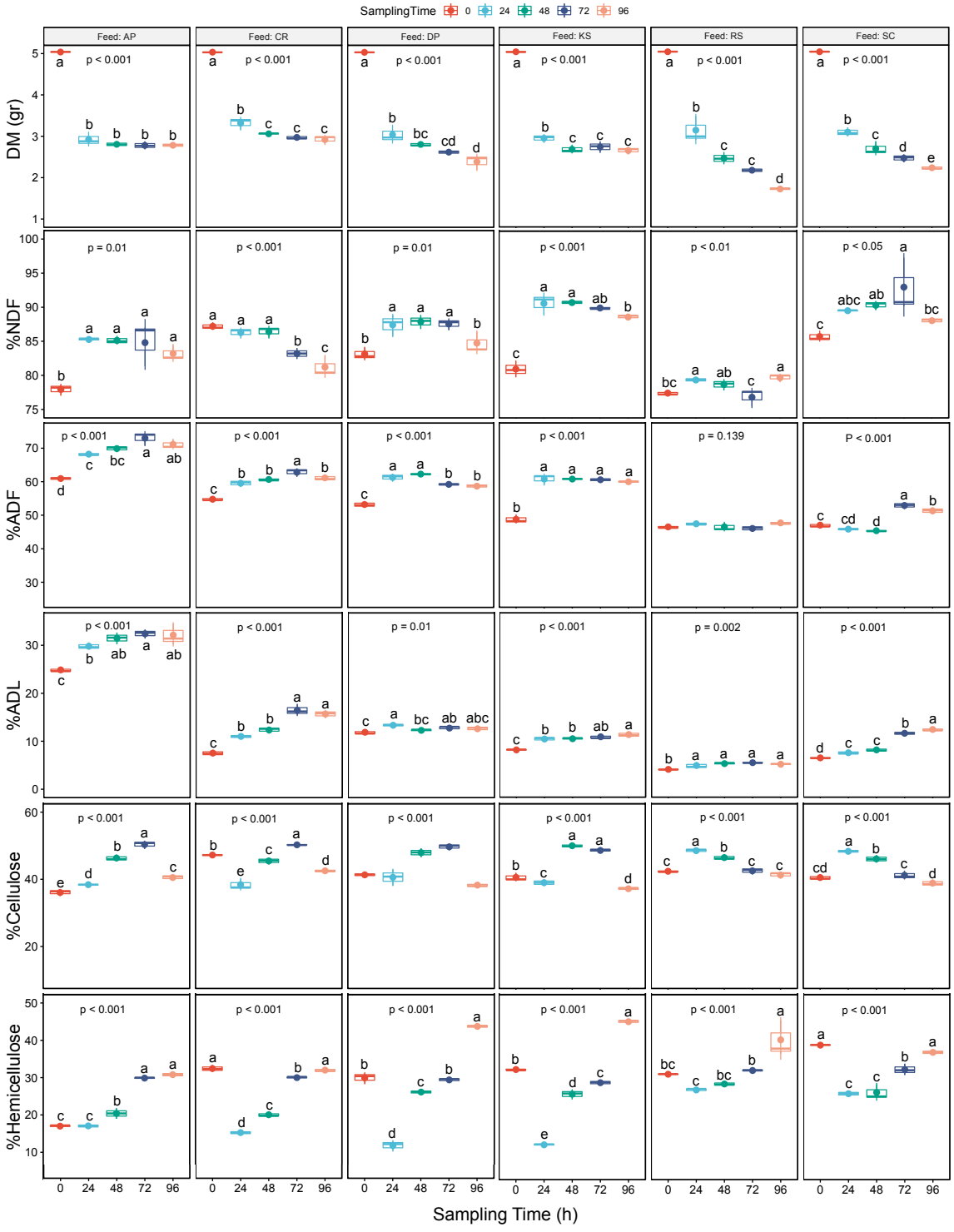

SamplingTime 0 24 48 72 96
Feed: AP
Feed: CR
Feed: DP
Feed: KS
Feed: RS
Feed: SC
DM (gr)
%NDF
%ADF
%ADL
%Cellulose
%Hemicellulose
Sampling Time (h)

Figure S1.

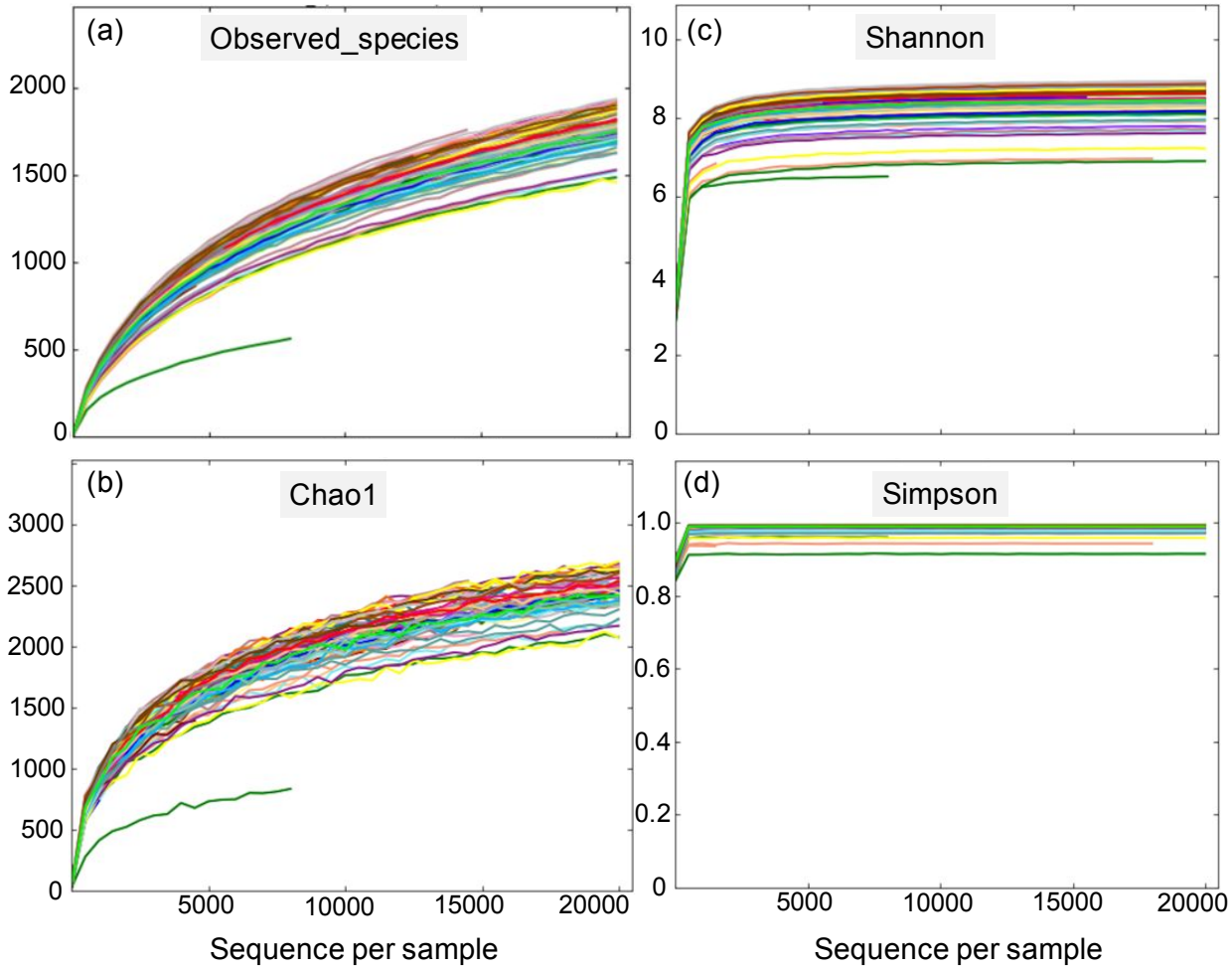

(a)
Observed_species
(c)
Shannon
(b)
Chao1
(d)
Simpson
Sequence per sample
Sequence per sample

Figure S2.

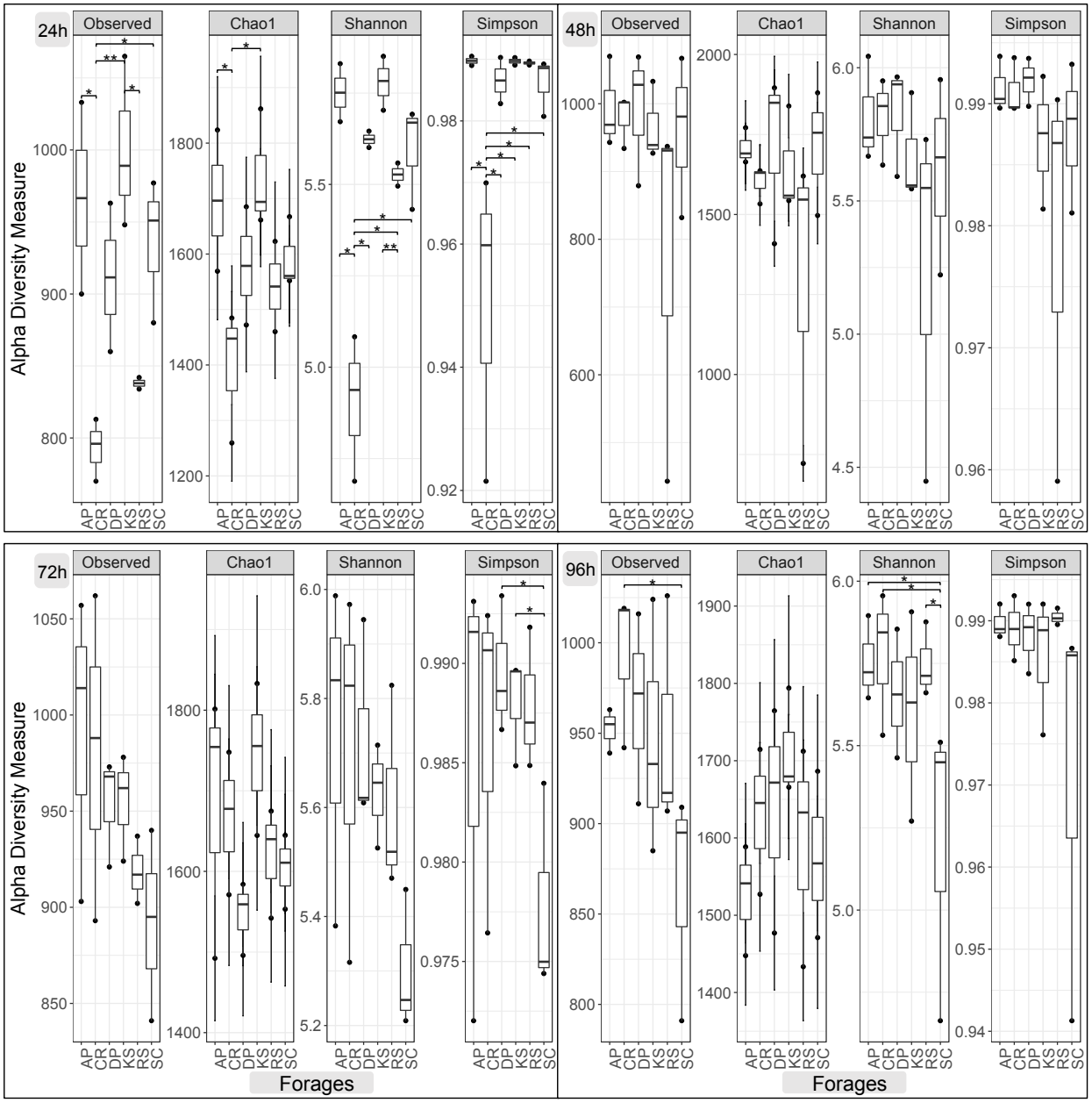

24h
48h
72h
96h
Observed
Chao1
Shannon
Simpson
Alpha Diversity Measure
Forages
AP
CR
DP
KS
RS
SC

Figure S3.

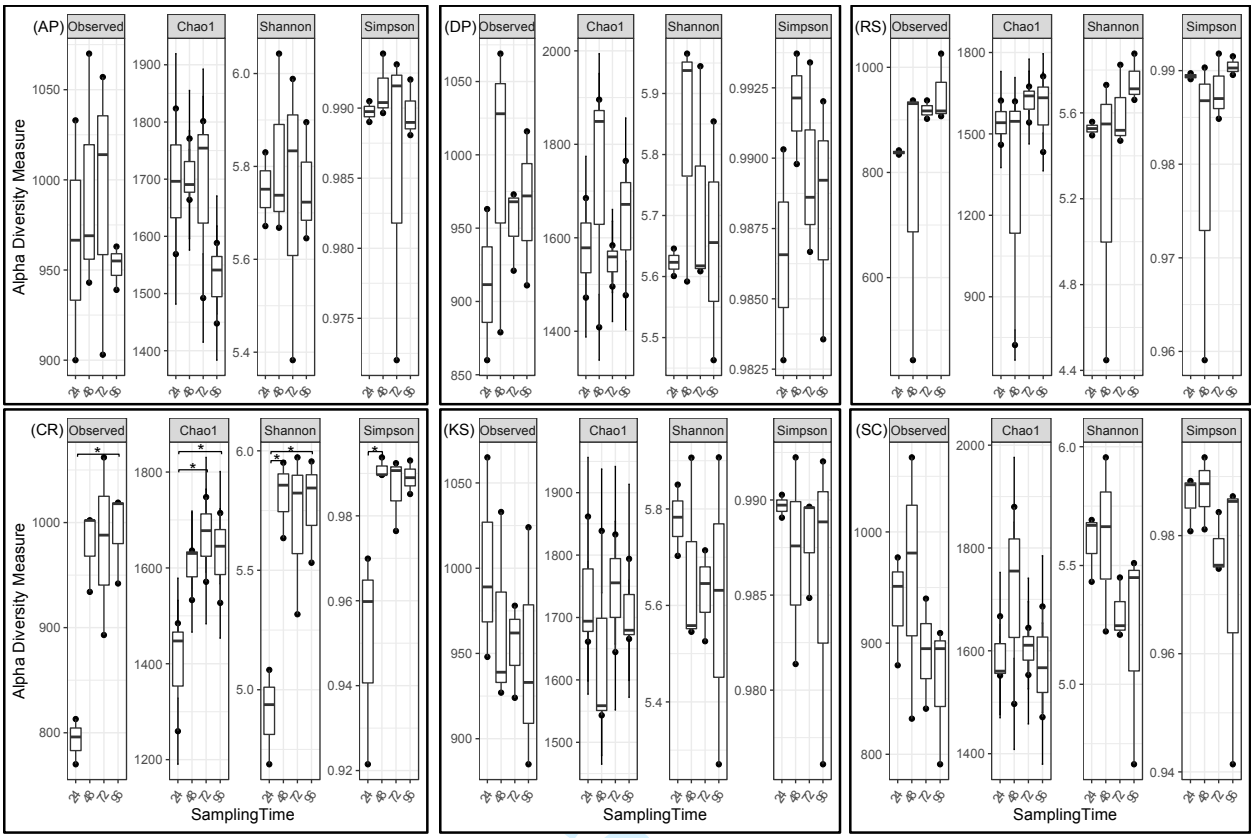


Figure S4.

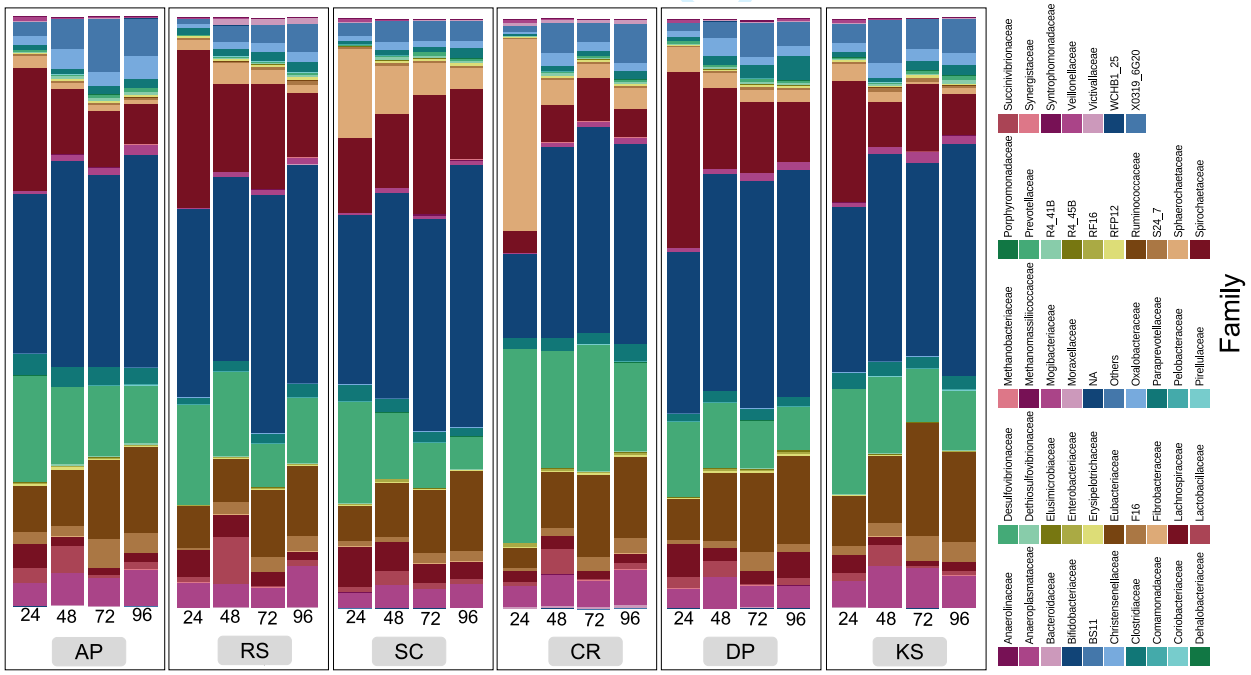


Figure S5.

Temporal changes in microbial communities attached to forages with different lignocellulosic compositions in the cattle rumen

Javad Gharechahi[1], Mohammad Farhad Vahidi[2], Xue-Zhi Ding[3], Jian-Lin Han[4,5], Ghasem Hosseini Salekdeh[2]

1. Human Genetics Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
2. Department of Systems Biology, Agricultural Biotechnology Research Institute of Iran, Agricultural Research, Education, and Extension Organization, Karaj, Iran
3. Key Laboratory of Yak Breeding Engineering, Lanzhou Institute of Husbandry and Pharmaceutical Sciences, Chinese Academy of Agricultural Sciences (CAAS), Lanzhou, China
4. CAAS-ILRI Joint Laboratory on Livestock and Forage Genetic Resources, Institute of Animal Science, Chinese Academy of Agricultural Sciences (CAAS), Beijing, China
5. Livestock Genetics Program, International Livestock Research Institute (ILRI), Nairobi, Kenya

To whom correspondence should be addressed

Ghasem Hosseini Salekdeh

E-mail: hsalekdeh@yahoo.com

Abstract

The attachment of rumen microbes to feed particles is critical to feed fermentation, degradation and digestion. However, the extent to which the physicochemical properties of feeds influence the colonization by rumen microbes is still unclear. We hypothesized that rumen microbial communities may have differential preferences for attachments to feeds with varying lignocellulose properties. To this end, the structure and composition of microbial communities attached to six common forages with different lignocellulosic compositions were analyzed following *in situ* rumen incubation in male Taleshi cattle. The results showed that differences in lignocellulosic compositions significantly affected the inter-sample diversity of forage-attached microbial communities in the first 24 hours (h) of rumen incubation, during which the highest dry matter degradation was achieved. However, extension of the incubation to 96 h resulted in the development of more uniform microbial communities across the forages. *Fibrobacteres* were significantly overrepresented in the bacterial communities attached to the forages with the highest neutral detergent fiber contents. *Ruminococcus* tended to attach to the forages with low acid detergent lignin contents. The extent of dry matter fermentation was significantly correlated with the populations of Fibrobacteraceae, unclassified Bacteroidales, Ruminococcaceae and Spirochaetacea. Our findings suggested that lignocellulosic compositions, more specifically the cellulose components, significantly affected the microbial attachment to and thus the final digestion of the forages.

Keywords: microbiome, microbiota, rumen, rumen incubation, 16S rRNA gene sequencing, rumen fermentation, biomass degradation

Introduction

The gastrointestinal tracts (GIT) of ruminant animals have evolved to allow the colonization by a diverse community of symbiotic microorganisms belonging to three taxonomic domains of life, i.e. Archaea, Bacteria and Eukarya. Particularly, as the first and the largest compartment of ruminant stomach, rumen is the main site of microbial colonization and fermentation. Without the aid of such microorganisms, the host ruminants cannot digest and convert plant lignocellulosic biomasses into energy and other essential metabolites (Jami and Mizrahi 2012; Mackie 2002). It is estimated that almost 70% of the energy requirement of ruminants is supplied by this microbial fermentation (Bergman 1990). In addition to their crucial role in animal nutrition and production, the GIT microorganisms remain vital to animal health, physiology and immunity against pathogenic microbes (Guarner and Malagelada 2003; Hungate 2013).

Bacteria are the major colonizers in the rumen and consequently make the greatest contribution to plant biomass fermentation, degradation and digestion. Early studies on rumen microbial communities relied largely on culture-based approaches, which were limited to the bacteria that can grow on culture medium. With the advances in next-generation sequencing technologies, culture-independent approaches have gained preference, enabling researchers to achieve a deeper insight into precise composition and structure of rumen bacterial communities. The latest high throughput amplicon-based 16S ribosomal RNA gene (rRNA) sequencing studies suggest that rumen bacterial communities of most ruminants are mainly affiliated to the phyla of Bacteroidetes, Firmicutes, Proteobacteria, Fibrobacteres, Spirocheaetes, Actinobacteria, Tenericutes and Verrucomicrobia, which collectively account for greater than 99% of the total rumen bacterial communities (Godoy-Vitorino *et al.* 2012; Jami *et al.* 2013; Jami and Mizrahi 2012; Zened *et al.* 2013). Microbial communities are dynamic in the rumen and depend on the host species and its diet, age, physiology and health status (Godoy-Vitorino *et al.* 2012; Jami *et al.* 2013; Jami and Mizrahi 2012; Kocherginskaya *et al.* 2001; Kong *et al.* 2010; Petri *et al.* 2012). Under normal physiological conditions, diet is the major driver to determine the composition and structure of rumen bacterial communities (Petri *et al.* 2012). Interestingly, the amount of dietary fiber is a key factor shaping the growth and multiplication of cellulolytic bacteria in the rumen. Changing dietary fiber content or substituting the fiber with easily fermentable carbohydrates has a profound impact on the community of rumen microbiota and may result in metabolic diseases, such as subacute ruminal acidosis (Khafipour *et al.* 2009; Petri *et al.* 2017).

Members of the rumen bacterial communities differ in their preferences for attachment to feed particles and rumen wall, therefore they are accordingly categorized into particle-attached (tightly attached to feed particles), liquid borne (freely available in liquid fraction) and epimural (attached to rumen epithelium) communities (De Mulder *et al.* 2017; Gharechahi *et al.* 2015; Kong *et al.* 2010; Sadet *et al.* 2007). The attachment of rumen microbial communities to feed particles is a key step in the process of rumen fermentation and digestion (McAllister *et al.* 1994) and it occurs shortly after feed entry into the rumen. Analysis of microbial communities associated with perennial ryegrass following *in situ* rumen incubation in Holstein–Friesian cows or steers shows that microbial attachment initiates within five minutes (min) of its rumen entry and stabilizes between 15 and 30 min of its rumen incubation (Edwards *et al.* 2007; Huws *et*

al. 2013). A recent analysis of temporal changes in bacterial community attached to wheat straw in Holstein cow rumen demonstrates that the first wave of microbial-based biomass degradation occurs within 30 min of its rumen entry. The community of bacteria attached to feed particles during this time retains even after 72 hours (h) of the rumen incubation (Jin *et al.* 2018). The degree of dry matter (DM) degradation differs among forages during the initial hours of their rumen entry (Cheng *et al.* 2017; Liu *et al.* 2016). The community composition of particle-attached microbes also varies among feeds and is likely influenced by the chemical compositions of feeds because cellulolytic bacteria such as *Fibrobacter*, *Ruminococcus*, *Butyrivibrio* and unclassified *Treponema* tend to attach to feeds with relatively high neutral detergent fiber (NDF, Cheng *et al*. 2017; Liu *et al*. 2016). The community of feed particle-attached bacteria also changes over the incubation time. For instance, rumen microbiota attachment to switchgrass in Friesian cow occurs at two successive stages: The first wave takes place immediately after its rumen entry (within the first 30 min) and is characterized by high abundances of Bacteroidia and Clostridia. The second wave, however, occurs after 16 h of its rumen incubation, during which the populations of Spirochaeta and Fibrobacteria become dominant (Piao *et al.* (2014).

There is limited knowledge on rumen bacteria diversity, their preference for attachment to and degradation of feeds with extreme values of cellulose and/or hemicellulose. Understanding the dynamics of bacteria attached to feeds at high level of lignification provides opportunities to improve the nutrient efficiency of low-quality forages through pre-treatments or manipulation of rumen microbial communities. We hypothesized that rumen bacterial communities differ in lignocellulose degrading capacities and thus show preference for attachment to feeds with different levels of lignification. We therefore aimed to evaluate whether the rumen microbes have any preference for attachment to forages with different cellulose and/or hemicellulose contents. We also explored the dynamic changes in microbial communities attached to the forages under prolonged incubation in the cattle rumen (e.g. up to 96 h with 24 h sampling intervals). Overall, our 16S rRNA genes-based diversity analysis revealed significant differences in the composition and structure of microbial communities attached to different forages.

Materials and methods

In situ rumen incubation and sample collection of the forages

All experimental procedures relevant to animals were approved by the Ethics Committee for Animal Experiments of the Animal Science Research Institute of Iran. Rumen cannulation was performed according to the American College of Veterinary Surgeons (ACVS) using a two-stage rumen cannulation technique (Martineau *et al.* 2015).

Six common lignocellulosic forages, including camelthorn (*Alhagi persarum*, AP; both stem and leaves), common reed (*Phragmites australis*, CR; stem and leaves), date palm (*Phoenix dactylifera*, DP; leaves), kochia (*Kochia scoparia*, KS; both stem and leaves), rice straw (*Oryza sativa*; cultivar Hashemi, RS; both stem and leaves) and salicornia (*Salicornia persica*, SC; both stem and leaves), were selected for *in situ* rumen incubation. Dried forage materials were cut into pieces of approximately 2 mm in length and an equal amount of the pieces (5 ± 0.05 g) was weighed into heat-sealed nylon bags (5 × 10 cm; 50 µm pore size). Three rumen-cannulated, purebred bulls (Taleshi

cattle, an Iranian local breed with a historical origin of *Bos taurus* mixed with *Bos indicus*) aged between 2.5 and 3 years were used for this study. Forty-eight heat-sealed bags, eight per forage, were simultaneously placed into each of the three rumens shortly after the cannulated bulls were offered the first meal in the morning. The bulls were housed together in a stable, fed on a mixed diet containing 70% wheat straw and 30% concentrate and provided free access to water. Two nylon bags from each of the six forages were retrieved from each rumen after 24, 48, 72 and 96 h of the incubation, washed thoroughly with distilled water three times to remove liquid borne and loosely attached microbes, which may not have a discriminating preference for attachment to forages with different lignocellulose properties, and finally squeezed by hands with sterile gloves to remove excess water. The bags were then transferred in liquid nitrogen to the laboratory where one replicate was stored at -70 °C for subsequent DNA extraction while the other was processed for physicochemical analysis.

Physicochemical analysis of the incubated forage materials

The forage lignocellulosic biomasses were analyzed for dry matter (DM) and the contents of neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) before and after the rumen incubation. DM was determined following 48 h air-drying of the samples in a fan-assisted oven maintained at 55 °C. The dried material was grounded to pass through a 1-mm sieve for the measurements of NDF, ADF and ADL according to the established procedures (Goering 1970; Van Soest *et al.* 1991). Cellulose content was estimated by subtracting ADL from ADF while hemicellulose content was measured by subtracting ADF from NDF.

Microbial cell recovery and metagenomic DNA extraction

Microbial cells firmly attached to the forages were subsequently stripped by incubating individual samples on ice in a dissociation buffer containing 0.1% (v/v) Tween 80, 1% (v/v) methanol and 1% (v/v) tertiary butanol (adjusted at pH 2), which has been particularly adapted for the dissociation of microbial cells from the rumen solid digesta. The samples were vigorously vortexed every 1 min and this step was repeated at least 5 times. The forage materials were sedimented by centrifugation at 500 × g and the liquid supernatant containing microbial cells was transferred to a new container. This step was repeated at least three times and the collected liquids were centrifuged at 12000 × g for 10 min to sediment the detached microbial cells. Metagenomic DNA from the detached cells was extracted using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for the isolation of DNA from stools for pathogen detection. The quality and quantity of the extracted DNA were evaluated by an agarose gel electrophoresis (0.8%) and a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

PCR amplification and Illumina sequencing of 16S rRNA gene

The V3-V4 hypervariable region of 16S rRNA gene was amplified using the universally conserved primer set S-D-Bact-0341-b-S-17 (5′-CCT ACG GGN GGC WGC AG-3′) and S-D-Bact-0785-a-A-21 (5′-GAC TAC HVG GGT ATC TAA TCC-3′), which generated a fragment of 464 bp suitable for paired-end sequencing using the Illumina MiSeq System (Illumina Inc. San Diego, CA, USA). PCR amplification was performed in triplicate in a 25 μL reaction

containing 12.5 µL 2× PCR master mix (Qiagen), 1 µL (10 pM) of each primer, 30 ng of microbial DNA and 5-9 µL of double-distilled water. The PCR condition consisted of an initial denaturation at 94 °C for 4 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. PCR products were purified and 2 µL of each reaction was used as a template for the second round of PCR, during which the Illumina adaptors and barcode sequences were incorporated to the 5'-end of the amplified products. The second PCR was also performed in triplicate under the same running condition except the number of cycles were limited to 15. The PCR amplicons were recovered using the Qiaquick® Gel Extraction Kit (Qiagen), quantified fluorometrically, pooled in equimolar quantities and paired-end sequenced (PE300) using the Illumina MiSeq System at Macrogen Inc. (Seoul, South Korea).

Sequence analysis

Quality filtered paired-end sequences were joined using the Flash with --max-overlap option set to 200 (Magoc and Salzberg 2011). Sequences failed to be assembled were discarded. The joined FASTQ files were processed using split_libraries_fastq.py script in the QIIME pipeline v1.9.1 (Caporaso *et al.* 2010b). This script demultiplexed sequences and filtered out sequences shorter than 200 bp or longer than 1000 bp, along with those containing ambiguous bases with a mean quality score < 20, having runs of six or more of the same nucleotides, carrying a missing quality score and including > two mismatches from the primer sequences. The multiplexed sequences were searched against the latest Ribosomal Database Project (RDP release 11.5, containing 3,356,809 16S rRNA sequences) to identify and discard chimeric sequences using the VSEARCH v2.8.3 operated under default setting (Rognes *et al.* 2016). Non-chimeric sequences were then used to pick operational taxonomic units (OTUs) using the pick_de_novo_otus.py script in the QIIME pipeline as described in details in our previous paper (Gharechahi *et al.* 2017). OTUs were defined at 97% identity using the Uclust (Edgar 2010). The most abundant sequence in each OTU cluster was selected as being a representative; and these sequences were then aligned against the Greengenes core set (gg_13_8; (DeSantis *et al.* 2006)) using the PyNAST aligner with a minimum sequence identity of 75% (Caporaso *et al.* 2010a). Taxonomies were assigned to each OTU using the Ribosomal Database Project naïve Bayesian classifier (Wang *et al.* 2007) by applying a minimum confidence value of 0.8. The OTU table was filtered for low abundant OTUs using the filter_otus_from_otu_table.py script with --min_count_fraction option set to 0.00001 (discarding OTUs represented by < 0.001% of the sequences) and then rarefied to the sequencing depth at 4660 reads corresponding to the number of reads in the sample with the smallest set of sequences. Rarefaction plots and alpha diversity indices, including Shannon, Simpson, Good's_coverage and Chao1, were calculated using the core_diversity_analyses.py script in the QIIME pipeline. Beta diversity indices, including weighted and unweighted Unifrac phylogenetic distance matrices, were constructed with the rarefied OTU table as input and visualized through the principal coordinate analysis (PCoA) plots in the MicrobiomeAnalyst web server (Dhariwal *et al.* 2017).

Statistical analysis

Statistically significant differences in physicochemical data, including DM, NDF, ADF, ADL, cellulose and hemicellulose contents, were analyzed by one-way ANOVA using the general linear model (GLM) procedure in the SAS software v9.3 (SAS Institute Inc., Cary, NC, USA). Permutational multivariate analysis of variance (PERMANOVA) was performed using the adonis function of R-package vegan v2.5-5 to test for significant differences between community compositions of forage-attached microbial communities. In addition, permutation multivariate analysis of group dispersions (PERMDISP) based on the betadisper function of the vegan was used to test for the homogeneity of dispersions (variances). Differences in taxa abundances among forages and sampling intervals were estimated using analysis of composition of microbes (ANCOM) based on relative abundances of OTUs summarized at various taxonomic levels (Mandal *et al.* 2015). Means were compared by Duncan's Multiple Range test (DMRT) in PAST v3.26 given Bonferroni p-value cutoff < 0.05 (Hammer *et al*. 2001). Error correction was done based on the number of groupwise comparisons performed at each taxonomic level. The Pearson's correlation analysis was performed using the corr.test function of the psych package v1.8.12 and p-values were corrected using Bonferroni method based on the total number of correlations calculated for each variable separately. For all tests, p-values less than 0.05 were considered statistically significant.

Results

Physicochemical properties of the rumen-incubated forages

The six forages were analyzed for the contents of NDF, ADF, ADL, cellulose and hemicellulose before their rumen incubation (Figure 1). They showed different NDF contents ($p < 0.05$), being the highest in common reed (CR) but the lowest in both camelthorn (AP) and rice straw (RS). Most of them also contained different ADF contents ($p < 0.05$), being the highest in AP but the lowest in both RS and salicornia (SC). The contents of ADL also differed significantly between the forages ($p < 0.05$), with AP being the highest at 2 times that in date palm (DP), three times those in SC, CR and kochia (KS; the latter two with similar contents at $p > 0.05$) and 6 times the lowest value in RS (24.86% vs 4.06%). CR carried the highest cellulose followed by DP, RS, KS and SC while AP contained the lowest cellulose ($p < 0.05$). SC possessed the highest amount of hemicellulose followed by CR, KS, RS, DP and AP. Overall, all the six forages had different physicochemical properties in terms of their relative contents of hemicellulose, cellulose and lignin.

Lignocellulosic biomass degradation following the rumen incubation

The six forages were monitored for their changes in the contents of DM, NDF, ADF, ADL, cellulose and hemicellulose during the rumen incubation (Figure 1 and Figure S1). DM degradation was the fastest in AP but the slowest in CR (42% vs 34%, $p < 0.05$) during the first 24 h of the incubation. DM degradation was completed in AP at 24 h but continued in CR and KS until 48 h, and in DP, RS and SC up to 96 h of the incubation, with RS having the highest degraded DM (66%) followed by SC (56%) and DP (53%). AP, DP and KS demonstrated similar trends of significantly increased NDF, ADF, ADL and cellulose contents ($p < 0.05$), mirrored in their patterns of DM degradations, mostly within the first 24 h, but their hemicelluloses decreased continuously ($p < 0.05$ in most cases)

following the incubation. However, the patterns of these five fiber-related parameters were significantly segregated among CR, RS and SC during the incubation. Although ADF and ADL were significantly increased but celluloses were remarkably declined in CR and SC ($p < 0.05$), their NDF and hemicelluloses showed contrasting patterns, being significantly reduced in CR while steadily accumulated in SC ($p < 0.05$) throughout the incubation. ADF, cellulose and hemicellulose in RS maintained stable levels while its NDF and ADL were slightly increased ($p < 0.05$) along the incubation. It was apparent that the initial differences in fiber-related physicochemical properties significantly affected the rumen digestion of the six forages.

16S rRNA gene sequencing

The paired-end sequencing of PCR amplicons from the V3-V4 region of 16S rRNA gene resulted in 5,945,300 pairs of raw sequences (averaged at 82,573 sequences per sample) with an average length of 300 bp. Sequences were joined into 4,421,604 full-length amplicons at an average of 61,411 ± 38,275 per sample. The uneven sequencing depths across samples may be due to the true differences in microbial abundance of samples or the technical variations introduced during library preparation and sequencing. Although the numbers of sequences varied greatly among the forages and across the lengths of the rumen incubation, there was a general pattern of a steady increase from the lowest at 24 h (averaged at 44,100 per sample) to the highest at 72 h (averaged at 95,823 per sample).

Sequencing data analysis

Before processing the amplicon sequences for OTU picking, they were subjected to a single round of quality filtering, resulting in 3,602,510 high quality sequences, of which 994,147 (27%) were identified to be chimeric and thus discarded from further analyses. The qualified sequences (2,493,285) were clustered at 97% similarity level into 110,804 OTUs, of which 106,982 (96.4%, representing 202,403 sequences) were labeled as low abundant features (e.g. those representing reads with frequencies less than 0.001% of the total sequences) and therefore filtered out from the OTU table. Finally, 3,822 clean OTUs representing 2,290,882 sequences were subjected to further downstream analyses. To assess whether our sequencing effort provided sufficient sequencing depths to describe the diversity of forage-attached microbiota, rarefaction curves describing the numbers of observed OTUs, species richness (Chao1), Shannon and Simpson diversity at various sequencing depths were generated for all samples (Figure S2a-d). Rarefaction analysis based on observed species and species richness revealed incomplete sampling of microbiota and thus indicated that highly diverse microbial communities attached to the forages (Figure S2a and b). However, Shannon (Figure S2c) and Simpson indices (Figure S2d) reached plateaus, indicating that the majority of the diversity was explored.

Microbial diversity analysis

Differences in alpha diversity indices of forage-attached microbiota were mostly limited to the first 24 h of the rumen incubation, during which the maximal differences in microbial attachment occurred (Figure S3). At this time, CR and RS (two forages with the highest initial cellulose contents) had the lowest while AP and KS (two forages with the

lowest initial cellulose contents) had the highest average observed species and species richness. CR and RS also showed the lowest average Shannon index, indicating their limited microbial diversity ($p < 0.05$). Diversity indices in almost all forages were not affected by the incubation length (Figure S4) except that CR had very low alpha diversity measures at 24 h of its incubation ($p < 0.05$). All samples showed a high Good's coverage (> 0.91, data not shown) at all sampling intervals, indicating that our sequencing effort figured out > 90% of the microbial diversity attached to the forages. However, the uneven sequencing depths did not allow us to fully explore the diversity of forage-attached microbiota. Considering potential random errors due to limited numbers of experimental animals and replicates in this study, such differences in alpha diversity indices among the forages were not robust enough to be interpreted with any biological significance.

Beta diversity analysis also showed limited variations among the forages as well as across the lengths of the rumen incubation. Weighted Unifrac dissimilarity matrix, which considered taxa relative abundances and their phylogenetic distances, explained 21% and 26% of the variations among the forages and across the lengths of the incubation, respectively. PERMANOVA revealed that at least some forages (e.g. CR and SC) had different microbial communities (Figure 2a, $p < 0.001$) while testing for homogeneity of group dispersions (sample distance from group centroid) identified no significant difference between group dispersions (Figure 2b, PERMDISP $p > 0.1$). Nevertheless, differences in microbial communities across the incubation lengths (Figure 2c, PERMANOVA $p < 0.001$) appeared to be mainly affected by within-group dispersions, either by the forage types or inter-animal variations, particularly during the first 24 h (Figure 2d, PERMDISP $p < 0.05$). At this time, CR-attached microbiota was well-separated from those attached to other forages. The entire microbial community structure showed a clear shift among the forages during the incubation because differences in the microbiota were apparent at 24 h but disappeared in later sampling intervals (Figure 2c and d). This finding suggested the existence of a strong preference of rumen microbiota for attachment to the forages of different digestibility while the rate and extent of such preference were quickly compromised after the initial hours of rumen digestion (24 h).

Forage-attached microbial community

A total of 18 bacterial phyla and one archaeal phylum were identified from the forage-attached microbial communities colonized in the Taleshi cattle rumen. The communities were dominated by phyla Firmicutes (45%) and Bacteroidetes (41%) followed with Fibrobacteres (5%), Spirochaetes (3%) and Proteobacteria (2%). Variations in the abundances of major bacterial phyla attached to the forages have been depicted in Figure 3a. The ANCOM analysis followed by Duncan's post hoc test revealed differential abundances of Bacteroidetes, Fibrobacteres, Lentisphaerae and Spirochaetes among the forages (Figure 3b, ANCOM $p < 0.05$). Bacteroidetes were significantly overrepresented in AP and CR compared with DP and SC (DMRT Bonferroni $p < 0.001$). Interestingly, fiber-utilizing bacteria belonging to Fibrobacteres were observed in more than 9% of the reads of CR and SC (two forages with the highest initial NDF contents) but in less than 2% of the reads of AP and KS ($p < 0.05$) and also in DP and RS (Figure 3a). CR contained more Lentisphaerae compared with other forages ($p < 0.001$) while SC carried more Spirochaetes relative to AP ($p < 0.02$).

At the family level (Figure S5), the forage-attached microbes were affiliated to 76 families, of which nine showed differential abundances among the forages, including Bacteroidaceae, Clostridiaceae, Fibrobacteraceae, Victivallaceae, Christensenellaceae, Lachnospiraceae, Spirochaetaceae, Oxalobacteraceae and RFP12 (ANCOM $p < 0.05$). Interestingly, Victivallaceae were significantly overrepresented in CR compared with other forages ($p < 0.05$), while Bacteroidaceae was significantly enriched in CR and RS compared with AP, DP, KS and SC. Oxalobacteraceae was more abundant in KS and SC than in AP, CR, DP and RS ($p < 0.05$). Members of Fibrobacteraceae also more frequently appeared in CR and SC compared with AP, KS and RS with the lowest initial NDF contents ($p < 0.02$), while those of Clostridiaceae and Lachnospiraceae were underrepresented in CR compared with other forages ($p < 0.05$).

Particle-attached microbiota were affiliated to 119 genera (taxonomic level 6), of which 12 displayed differential abundances among the forages (ANCOM $p < 0.05$, Figure 4) most of which were among high abundant members of rumen community which are known to play key roles in plant lignocellulose degradation, including *Ruminococcus*, *Fibrobacter*, *Prevotella*, *Treponema*, *Lachnospira*, *Succinivibrio*, *Pseudobutyrivibrio*, *Butyrivibrio*, *Oxalobacter*, *Clostridium*, BF311 and *Succiniclasticum*. *Ruminococcus* and members of BF311 were more dominant in RS than in AP, KS and SC ($p < 0.001$). *Fibrobacter* were more highly represented in CR and SC. Species of *Lachnospira* were present in 0.3% of the reads of AP and KS ($p < 0.05$) but only 0.11% of CR and 0.09% of SC. *Succinivibrio* were overrepresented in AP and SC compared with CR ($p < 0.05$). Species of *Prevotella* were more abundant in CR (> 21% of the reads) than in DP and SC ($p < 0.008$). Compared to other forages, CR carried less *Butyrivibrio* and *Pseudobutyrivibrio* species ($p < 0.007$).

Changes in forage-attached microbes during the rumen incubation

In order to examine whether the community composition of forage-attached microbes changed during the rumen incubation, the relative abundances of the microbes were tracked at 24 h intervals (Figure 5). The abundances of eight out of the 119 bacterial genera showed significant differences among the incubation lengths (Bonferroni corrected $p < 0.05$). Interestingly, the proportions of cellulolytic bacteria belonging to unclassified Ruminococcaceae linearly increased with the incubation lengths in AP, KS and SC. The proportion of *Fibrobacter* sharply dropped after the first 24 h and reached to an average of 4% between 48 and 96 h of the incubation in CR. Members of *Pseudobutyribibrio* linearly decreased in AP and KS while those of *Butyrivibrio* increased in CR with the incubation lengths. Members of *Clostridium* increased in SC but those of *Prevotella* and unclassified Paraprevotellaceae decreased with the incubation lengths, which were consistent with findings of Cheng *et al.* (2017).

Relationship between lignocellulose degradation and forage-attached microbial communities

The Pearson's correlation between the initial physicochemical properties of the forages and the composition of the forage-attached microbiota during initial hours (24 h) of the rumen incubation was performed to determine whether rumen microbes preferred specific forages for attachment. Only correlations with p-values (Bonferroni-corrected) less than 0.05 were considered to have significant biological terms. This analysis demonstrated that the prevalence of the

family Fibrobacteraceae (r = 0.77, p = 0.03) was positively correlated with NDF contents of the forages. At the genus level, the abundance of *Fibrobacter* (r = 0.77, p = 0.05) was positively but an unclassified Erysipelotrichaceae genus p-75-a5 (r = -0.79, p = 0.03) was negatively correlated with NDF contents in the forages. When hemicellulose contents were considered, a negative correlation with members of the family Pirellulaceae (r = -0.79, p = 0.02) was detected.

We also correlated microbial profiles with physicochemical properties of the forages during the rumen incubation. This analysis revealed that the prevalence of the families Fibrobacteraceae (r= 0.83, p = 0.03 in CR), Anaeroplasmataceae (r = 0.82, p = 0.04 in CR), Prevotellaceae (r = 0.83, r = 0.04 in KS) and Paraprevotellaceae (r = 0.85, p = 0.02 in SC) were positively but Ruminococcaceae (r = < -0.85, p < 0.01 in CR and SC) and unclassified Bacteroidales (r = -0.84, p = 0.05 in RS) were negatively corelated with DM contents of the forages. Particularly, members of Ruminococcaceae were positively correlated with ADF, ADL and hemicellulose contents in CR, DP and SC (r > 0.8 and p < 0.04), the forages with the highest initial NDF contents (Figure 1). The population of Lachnospiraceae was also positively correlated (r = 0.83, and p = 0.04) with cellulose content in CR.

To ascertain whether there was any relationship between the rumen microbiota and lignocellulose degradation, an additional Pearson's correlation analysis was performed between DM loss and the relative abundance of forage-attached microbial communities during the rumen incubation. Interestingly, DM degradation was positively correlated with the prevalence of the families Fibrobacteraceae (r > 0.76, p < 0.001 in CR) and Spirochaetaceae (r = 0.91, p = 0.0004 in KS) but was negatively corelated with species belonging to unclassified Bacteroidales (r < -0.82, p < 0.05 in CR and DP), Ruminococcaceae (r = -0.83, p = 0.04 in CR), Mogibacteriaceae (r < -0.82, p < 0.05 in CR and RS) and Erysiopelotrichaceae (r = -0.87, p = 0.01 in CR). The correlations of cellulose and hemicellulose degradations with the abundances of forage-attached microbes also showed similar patterns.

Discussion

In this study, we investigated the relationship between biomass degradation of and microbial attachment to six common lignocellulosic forages varying in their physicochemical properties, including percentages of NDF, ADF, ADL and the contents of cellulose and hemicellulose. Forages containing the highest cellulose contents [(common reed (CR) vs. camelthorn (AP)] were degraded to a limited extent during the initial hours of their rumen incubation. The total DM degradation was mainly determined by NDF contents of the forages, because as rice straw (RS) with the lowest initial NDF (77.4%) had the fastest (66%) while CR with the highest initial NDF (87.2%) had the lowest (42%) DM loss over 96 h of the incubation. Variability in DM degradation of feeds reflected the differences in lignocellulose composition of their cell walls (Bruno-Soares *et al.* 2000; Jančík *et al.* 2010). The rate and extent of DM fermentation in the rumen determines the nutrition efficiency of feeds to ruminants (Jančík *et al.* 2010).

The 16S rRNA gene sequencing data allowed taxonomic identification and quantification of the rumen microbiota tightly attached to the forages. Rarefaction analysis based on the indices reflecting species richness and species relative abundance, e.g. Shannon and Simpson (Kim *et al.* 2017), indicated that the majority of the diversity of rumen microbiota attached to the forages was already sampled. Alpha diversity analysis also showed a limited biologically

significant difference among the forages, which could likely be attributed to a high microbial heterogeneity among animals included in the study. The changes in diversity measures were largely restricted to the initial hours of rumen incubation when maximal DM degradation occurred. The differences in species richness and evenness were linked to cellulose contents of the forages, where CR and RS with the highest cellulose contents displayed limited species diversity. This result was in agreement with the data on rice straw and alfalfa hay being fed to Holstein cows, in which more bacteria attached to alfalfa with lower NDF (Liu *et al.* 2016). These findings suggested that only a limited fraction of rumen microbiota was capable of attachment to feeds with high lignocellulose contents.

Analysis of community structure of forage-attached microbiota by Unifrac dissimilarity matrix revealed significant differences among the forages and across the length of rumen incubation. Particularly, CR-attached microbiota was well separated from those of other forages at the first 24 h of the incubation. In addition, microbiota attached to CR and SC, the two forages with the highest initial NDF contents, were also distantly clustered while those of other forages did not show any apparent separation, indicating the similarity of their communities. These variations could likely be determined by the nature of the forages, e.g., their chemical compositions. Unifrac diversity measure also showed that extension of the incubation time contributed to the increased similarity across the samples, as reflected by the overlapped clustering in the PCoA plots for most samples collected at 72 and 96 h of the incubation. This could be explained by the fact that initial DM degradation resulted in the reduction of digestible components but the accumulation of indigestible residues which had quite similar properties across the forages, thus favoring the attachment of structurally similar communities of rumen microbiota. Huws *et al.* (2013) also observed similar changes in the microbial communities attached to perennial ryegrass following its rumen incubation.

Members of Firmicutes, Bacteroidetes, Fibrobacteres, Spirochaetes and Proteobacteria were dominant in all samples, accounting for greater than 96% of the bacterial communities attached to the forages, consistent with the findings for rice straw and alfalfa hay (Liu *et al.* 2016). The abundance of these bacterial phyla varied among the forages and across the incubation lengths. Particularly, species of Bacteroidetes were more abundantly attached to the forages with high ADF contents (AP and CR). Bacteroidetes were among highly abundant members of the rumen microbiota being best recognized for their saccharolytic activities. The presence of a high number of pectinolytic and cellulolytic enzymes in their genomes clustered with other lignocellulose degrading enzymes into polysaccharide utilization loci (PUL) suggested that they are also actively involved in pectin, hemicellulose and cellulose degradation (Gharechahi and Salekdeh 2018; Lapebie *et al.* 2019; Naas *et al.* 2014). Within this phylum, members of the families Bacteroidaceae, Prevotellaceae and Paraprevotellaceae displayed significant differences among the forages. Particularly, Bacteroidaceae were significantly overrepresented in the forages with the lowest initial ADL contents (CR and RS). At the genus-level, this differential abundance was only affiliated to the BF311, an uncultured and unknown rumen bacterium. Prevotellaceae were particularly positively correlated with DM degradation, suggesting their active role in lignocellulose degradation in the rumen. *Prevotella* spp. are abundant members of the rumen microbiome that have a high genetic diversity and thus the ability to thrive on a wide range of substrates, including cellulose, hemicellulose, pectin, proteins and peptides (Dodd *et al.* 2010; Golder *et al.* 2014). They are known for their xylanolytic properties in the rumen and thus play an important role in fiber degradation (Dodd *et al.* 2010).

Fibrobacteres were predominantly represented in microbiota attached to the forages with the highest NDF contents [e.g. CR and Salicornia (SC)]. They are known to produce a battery of cellulolytic enzymes capable of degrading cellulose as a sole carbon source (Flint *et al.* 2008; Suen *et al.* 2011). The association of forage NDF content with the prevalence of *Fibrobacter* is also recently observed in the cow rumen microbiota (Liu *et al.* 2016). In contrast to Fibrobacteres, the genera *Clostridium*, *Shuttleworthia* and *Ruminococcus* tended to attach the forages with limited NDF and cellulose contents. This is consistent with previous findings on the preference of *Ruminococcus* species for attachment to high quality sugar-rich hays (Klevenhusen *et al.* 2017). Ruminococcaceae showed a strong negative correlation with total DM contents in the forages as well. Shinkai *et al.* (2010) also reported that members of Fibrobacteres, including *F. succinogenes*, abundantly attached to less digestible fibers while those of Ruminococcaceae, specifically *R. flavefaciens,* preferred easily digestible fibers and thus were infrequently detected in the stem parts of hays. The rate and extent of fiber degradation by *F. succinogenes* are also greater than *R. albus* and *R. flavefaciens* (Kobayashi *et al.* 2008). Early microscopy analysis indicated that *R. albus* is less commonly attached to plant cell walls while *F. succinogenes* forms extensive microcapsula enveloping the cell walls (Chesson *et al.* 1986). The community of unclassified Ruminococcaceae tended to linearly expand in AP, KS and SC following their rumen incubation. This finding suggested that the degradation of surface accessible fibers turned forage residues into favored substrates for the attachment by species of Ruminococcaceae. A declined abundance of species of *Ruminococcus* has been reported in the cow rumen when the starch content of diet was increased (Zened *et al.* 2013). *Ruminococcus* spp. have been equipped with specialized mechanisms for fiber adhesion and degradation, in which multiple carbohydrate degrading enzymes assemble into multienzyme cellulosome complexes capable of attachment to and degradation of polysaccharides in various plant cell walls (Bayer *et al.* 2004; Doi and Kosugi 2004).

Recently, Huws *et al.* (2016) reported that microbial colonization of perennial ryegrass occurred at two successive stages: The primary (the first 1-2 h after its rumen entry) and the secondary phases (4-8 h). It was the secondary phase, during which the maximal DM degradation was achieved and the proportion of *Succinivibrio* spp. decreased while those of *Pseudobutyrivibrio*, *Roseburia* and *Ruminococcus* spp. increased. Piao *et al.* (2014) also observed that the populations of *Pseudobutyrivibrio* and *Ruminococcus* spp. increased during their secondary colonization to switchgrass in the Friesian cow rumen. The microbial communities attached to feeds during the primary phase were believed to utilize soluble and easily accessible nutrients while those colonized feeds during the secondary phase were considered to be the true lignocellulose degraders (Huws *et al.* 2016; Liu *et al.* 2016). In this study, an increased prevalence of *Butyrivibrio* was noted in CR. *Butyrivibrio* spp. are known for their proteolytic, hemicellulolytic and biohydrogenating activities (Krause *et al.* 2003). Liu *et al.* (2016) reported a strong positive correlation between the abundance of *Butyrivibrio* spp. and the crude protein contents in the rice straw and alfalfa hay, suggesting their preference for the attachment to proteinaceous components of the feeds.

In summary, our results demonstrated that physicochemical compositions of the forages, more specifically their cellulose contents, were among the major factors influencing microbial attachments and thus lignocellulose degradation in the cattle rumen. No taxonomic lineage was found to be specific of a particular forage, suggesting that most rumen microbes had an inherent tendency for attachment to lignocellulosic substrates. However, differential

attachments to the forages by rumen microbiota suggested that the physicochemical properties were the key factors influencing the rate of microbial attachment. Our results also revealed that members of the rumen microbiota competed for their attachments to the forages with different lignocellulose compositions mostly during the initial hours of the rumen incubation. However, after the degradation of easily digestible components in the forages, relatively uniform microbial communities developed on their surface.

It should be noted that the bacterial community composition detected in each forage at each sampling interval may not necessarily reflect the actual abundance of microbes due to an inherent bias in sample collection, processing and storage as well as DNA extraction and PCR amplification, which may cause over- or under-representation of some taxa. For example, a recent comparative analysis of various DNA extraction methods used in rumen microbiome analyses argued that DNA extraction method can have an adverse effect on the community composition of rumen samples and may associate with an increased or decreased abundance of some specific taxa (Henderson *et al.* 2013). Particularly, the inclusion of a physical lysis step using bead-beating method increased the efficacy of DNA extraction from Gram-positive bacteria (Knudsen *et al.* 2016). The extent to which our DNA extraction method, in which the physical lysis step was not included, affected community composition of the forage-attached microbiota was unknown. In addition, the high number of PCR cycles may increase the number of PCR artifacts and bias the microbial compositions.

Acknowledgements

This research was funded by the Agricultural Biotechnology Research Institute of Iran (ABRII), the international cooperation and exchange program of the National Natural Science Foundation of China (No. 31461143020), and the Chinese government contribution to CAAS-ILRI Joint Laboratory on Livestock and Forage Genetic Resources in Beijing. The paper contributes to the CGIAR Research Program on Livestock.

Figure legends

Figure 1. Differences among the six forages in dry matter (DM) and percentages of neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), cellulose and hemicellulose before (0 h) and after 24, 48, 72, and 96 h of the rumen incubation. The solid circle and error bar in each boxplot show mean and standard deviation, respectively. Statistically significant differences were determined using one-way ANOVA. Means were compared using Duncan's multiple range test and are labelled with different letters at each sampling interval for $p < 0.05$. AP, camelthorn; CR, common reed; DP, date palm; KS, Kochia; RS, rice straw; and SC, Salicornia.

Figure 2. Beta diversity analysis of rumen microbial communities attached to the six forages during the rumen incubation. PCoA plots show the distribution of samples based on weighted Unifrac distance matrix, in which samples are grouped according to the forages (a) and the sampling intervals (b). Significant differences were tested using PERMANOVA with a p-value cutoff 0.01. The percentage of variation explained by each principle coordinate is indicated next to the corresponding axis. The homogeneity of dispersions was also tested for this diversity measure to

examine variance differences between the forages (c) and the sampling intervals (d). Significant differences were determined using the betadisper function of R package vegan v2.5-5 at 999 permutations. P-values < 0.05 were considered statistically significant. For each treatment, data in triplicate representing three separate bags, were included. AP, camelthorn; CR, common reed; DP, date palm; KS, Kochia; RS, rice straw; and SC, Salicornia.

Figure 3. (a) The proportions of reads (mean ± SD) assigned to the major bacterial phyla (n = 7) detected in the forage-attached microbial communities. The relative abundances were calculated based on the proportion of reads assigned to each phylum in the rarefied OTU table at an even sequencing depth of 4660 reads. (b) Box plots show relative abundance of phyla differentially attached to the forages. Statistically significant differences were calculated based on the analysis of composition of microbes (ANCOM) using $p < 0.05$ following Duncan's multiple range test for comparison of the means. Center lines indicate the median value, boxes the interquartile range and red squares the mean. Means with different letters differ at Bonferroni corrected $p < 0.05$. AP, camelthorn; CR, common reed; DP, date palm; KS, Kochia; RS, rice straw; and SC, Salicornia.

Figure 4. The log of relative abundances of genera differentially attached to the six forages following the rumen incubation. Differential abundances were statistically tested using ANCOM with a p-value cutoff < 0.05. Means were compared using Duncan's multiple range test only accepting Bonferroni corrected p-values < 0.05. Boxplots labeled with different letters show statistically significant differences. Center line represents median value. AP, camelthorn; CR, common reed; DP, date palm; KS, Kochia; RS, rice straw; and SC, Salicornia.

Figure 5. The average relative abundances of taxa (genus level) differentially represented in microbial communities attached to the six forages during the rumen incubation (up to 96 h with 24 h intervals). Statistically significant differences were calculated using ANCOM and means were compared using Duncan's multiple range test. Error bars represent standard deviations and their lengths are adjusted at 95% confidence interval. Means with different letters are statistically significant at Bonferroni corrected $p < 0.05$. No taxa were found to be differentially represented in DP. AP, camelthorn; CR, common reed; KS, Kochia; RS, rice straw; and SC, Salicornia. "Un" refers to unclassified.

Supplementary materials

Figure S1.

Changes in DM contents and percentages of NDF, ADF, ADL, cellulose and hemicellulose in six forages during the rumen incubation. The solid circle in each boxplot shows mean and error bars represent standard deviation. Statistically significant differences were determined using one-way ANOVA. Means were compared using Duncan's multiple range test and labelled with different letters for $p < 0.05$.

Figure S2.

Alpha diversity analysis through rarefaction plots. (a) Rarefaction curves showing the increase in number of observed OTUs; (b) Species richness (number of observed OTUs + number of unobserved OTUs, Chao1); (c) Shannon; and (d) Simpson diversity indices on Y-axis as a function of the number of reads sampled on X-axis.

Figure S3.

Alpha diversity indices of rumen microbiota attached to the six forages with different lignocellulosic compositions at 24, 48, 72 and 96 hours of the rumen incubation. Alpha diversity indices were measured based on OTUs present at an even sequencing depth of 4,660 reads (corresponding to the sequencing depth of the sample with the lowest number of reads) in all samples. Statistically significant differences were determined using one-way ANOVA and means were compared by Duncan's multiple range test. * p value < 0.05, ** p value < 0.01 and *** p value < 0.001.

Figure S4.

Alpha diversity analysis of rumen microbial communities attached to the six forages during the rumen incubation. The OTU table for each forage was sampled at an even sequencing depth (n = 4660) and diversity measures were calculated using plot_richness function of R package phyloseq and visualized using geom_boxplot function of R package ggplot2. Statistically significant differences were calculated using one-way ANOVA at p-value cutoff of 0.05. Differences between means were determined using Duncan's multiple range test in PAST v3.26. * p value < 0.05, ** p value < 0.01 and *** p value < 0.001.

Figure S5.

The proportions of reads affiliated to OTUs at the taxonomic level of family in the six forages during the rumen incubation. Data for each sampling interval is the mean of three biological replicates corresponding to three bags retrieved one each from the three cannulated bulls. For each forage, the OTU table is rarefied to the sequencing depth of the sample with the lowest number of reads.

References

Bayer EA, Belaich JP, Shoham Y *et al.* The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. *Annu Rev Microbiol* 2004;**58**: 521-54.

Bergman EN. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol Rev* 1990;**70**: 567-90.

Bruno-Soares AM, Abreu JMF, Guedes CVM *et al.* Chemical composition, DM and NDF degradation kinetics in rumen of seven legume straws. *Animal Feed Science and Technology* 2000;**83**: 75-80.

Caporaso JG, Bittinger K, Bushman FD *et al.* PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 2010a;**26**: 266-7.

Caporaso JG, Kuczynski J, Stombaugh J *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010b;**7**: 335-6.

Cheng Y, Wang Y, Li Y *et al.* Progressive Colonization of Bacteria and Degradation of Rice Straw in the Rumen by Illumina Sequencing. *Front Microbiol* 2017;**8**: 2165.

Chesson A, Stewart CS, Dalgarno K *et al.* Degradation of isolated grass mesophyll, epidermis and fibre cell walls in the rumen and by cellulolytic rumen bacteria in axenic culture. *Journal of Applied Bacteriology* 1986;**60**: 327-36.

De Mulder T, Goossens K, Peiren N *et al.* Exploring the methanogen and bacterial communities of rumen environments: solid adherent, fluid and epimural. *FEMS Microbiol Ecol* 2017;**93**.

DeSantis TZ, Hugenholtz P, Larsen N *et al.* Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 2006;**72**: 5069-72.

Dhariwal A, Chong J, Habib S *et al.* MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res* 2017;**45**: W180-W88.

Dodd D, Moon YH, Swaminathan K *et al.* Transcriptomic analyses of xylan degradation by Prevotella bryantii and insights into energy acquisition by xylanolytic bacteroidetes. *J Biol Chem* 2010;**285**: 30261-73.

Doi RH, Kosugi A. Cellulosomes: plant-cell-wall-degrading enzyme complexes. *Nat Rev Microbiol* 2004;**2**: 541-51.

Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010;**26**: 2460-1.

Edwards JE, Huws SA, Kim EJ *et al.* Characterization of the dynamics of initial bacterial colonization of nonconserved forage in the bovine rumen. *FEMS Microbiol Ecol* 2007;**62**: 323-35.

Flint HJ, Bayer EA, Rincon MT *et al.* Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat Rev Microbiol* 2008;**6**: 121-31.

Gharechahi J, Kharazian ZA, Sarikhan S *et al.* The dynamics of the bacterial communities developed in maize silage. *Microb Biotechnol* 2017;**10**: 1663-76.

Gharechahi J, Salekdeh GH. A metagenomic analysis of the camel rumen's microbiome identifies the major microbes responsible for lignocellulose degradation and fermentation. *Biotechnol Biofuels* 2018;**11**: 216.

Gharechahi J, Zahiri HS, Noghabi KA *et al.* In-depth diversity analysis of the bacterial community resident in the camel rumen. *Syst Appl Microbiol* 2015;**38**: 67-76.

Godoy-Vitorino F, Goldfarb KC, Karaoz U *et al.* Comparative analyses of foregut and hindgut bacterial communities in hoatzins and cows. *ISME J* 2012;**6**: 531-41.

Goering HK. Forage fiber analyses (apparatus, reagents, procedures, and some applications). In: Van Soest PJ (ed.). Washington, D.C. :: Agricultural Research Service, U.S. Dept. of Agriculture, 1970.

Golder HM, Denman SE, McSweeney C *et al.* Ruminal bacterial community shifts in grain-, sugar-, and histidine-challenged dairy heifers. *J Dairy Sci* 2014;**97**: 5131-50.

Guarner F, Malagelada JR. Gut flora in health and disease. *Lancet* 2003;**361**: 512-9.

Hammer Ø, Harper DA, Ryan PD. PAST: paleontological statistics software package for education and data analysis. 2001.

Henderson G, Cox F, Kittelmann S *et al.* Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. *PLoS One* 2013;**8**: e74787.

Hungate RE. *The rumen and its microbes*: Elsevier, 2013.

Huws SA, Edwards JE, Creevey CJ *et al.* Temporal dynamics of the metabolically active rumen bacteria colonizing fresh perennial ryegrass. *FEMS Microbiol Ecol* 2016;**92**.

Huws SA, Mayorga OL, Theodorou MK *et al.* Successional colonization of perennial ryegrass by rumen bacteria. *Lett Appl Microbiol* 2013;**56**: 186-96.

Jami E, Israel A, Kotser A *et al.* Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J* 2013;**7**: 1069-79.

Jami E, Mizrahi I. Composition and similarity of bovine rumen microbiota across individual animals. *PLoS One* 2012;**7**: e33306.
Jančík F, Koukolová V, Homolka P. Ruminal degradability of dry matter and neutral detergent fibre of grasses. *Czech J Anim Sci* 2010;**55**: 359-71.
Jin W, Wang Y, Li Y *et al.* Temporal changes of the bacterial community colonizing wheat straw in the cow rumen. *Anaerobe* 2018;**50**: 1-8.
Khafipour E, Li S, Plaizier JC *et al.* Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. *Appl Environ Microbiol* 2009;**75**: 7115-24.
Kim BR, Shin J, Guevarra R *et al.* Deciphering Diversity Indices for a Better Understanding of Microbial Communities. *J Microbiol Biotechnol* 2017;**27**: 2089-93.
Klevenhusen F, Petri RM, Kleefisch MT *et al.* Changes in fibre-adherent and fluid-associated microbial communities and fermentation profiles in the rumen of cattle fed diets differing in hay quality and concentrate amount. *FEMS Microbiol Ecol* 2017;**93**.
Knudsen BE, Bergmark L, Munk P *et al.* Impact of Sample Type and DNA Isolation Procedure on Genomic Inference of Microbiome Composition. *mSystems* 2016;**1**.
Kobayashi Y, Shinkai T, Koike S. Ecological and physiological characterization shows that Fibrobacter succinogenes is important in rumen fiber digestion - review. *Folia Microbiol (Praha)* 2008;**53**: 195-200.
Kocherginskaya SA, Aminov RI, White BA. Analysis of the Rumen Bacterial Diversity under two Different Diet Conditions using Denaturing Gradient Gel Electrophoresis, Random Sequencing, and Statistical Ecology Approaches. *Anaerobe* 2001;**7**: 119-34.
Kong Y, Teather R, Forster R. Composition, spatial distribution, and diversity of the bacterial communities in the rumen of cows fed different forages. *FEMS Microbiol Ecol* 2010;**74**: 612-22.
Krause DO, Denman SE, Mackie RI *et al.* Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiol Rev* 2003;**27**: 663-93.
Lapebie P, Lombard V, Drula E *et al.* Bacteroidetes use thousands of enzyme combinations to break down glycans. *Nat Commun* 2019;**10**: 2043.
Liu J, Zhang M, Xue C *et al.* Characterization and comparison of the temporal dynamics of ruminal bacterial microbiota colonizing rice straw and alfalfa hay within ruminants. *J Dairy Sci* 2016;**99**: 9668-81.
Mackie RI. Mutualistic fermentative digestion in the gastrointestinal tract: diversity and evolution. *Integr Comp Biol* 2002;**42**: 319-26.
Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 2011;**27**: 2957-63.
Mandal S, Van Treuren W, White RA *et al.* Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb Ecol Health Dis* 2015;**26**: 27663.
Martineau R, Proulx JG, Cortes C *et al.* Two-Stage Rumen Cannulation Technique in Dairy Cows. *Vet Surg* 2015;**44**: 551-6.
McAllister TA, Bae HD, Jones GA *et al.* Microbial attachment and feed digestion in the rumen. *J Anim Sci* 1994;**72**: 3004-18.
Naas AE, Mackenzie AK, Mravec J *et al.* Do rumen Bacteroidetes utilize an alternative mechanism for cellulose degradation? *mBio* 2014;**5**: e01401-14.
Petri RM, Forster RJ, Yang W *et al.* Characterization of rumen bacterial diversity and fermentation parameters in concentrate fed cattle with and without forage. *J Appl Microbiol* 2012;**112**: 1152-62.
Petri RM, Pourazad P, Khiaosa-Ard R *et al.* Temporal dynamics of in-situ fiber-adherent bacterial community under ruminal acidotic conditions determined by 16S rRNA gene profiling. *PLoS One* 2017;**12**: e0182271.
Piao H, Lachman M, Malfatti S *et al.* Temporal dynamics of fibrolytic and methanogenic rumen microorganisms during in situ incubation of switchgrass determined by 16S rRNA gene profiling. *Front Microbiol* 2014;**5**: 307.
Rognes T, Flouri T, Nichols B *et al.* VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 2016;**4**: e2584.
Sadet S, Martin C, Meunier B *et al.* PCR-DGGE analysis reveals a distinct diversity in the bacterial population attached to the rumen epithelium. *Animal* 2007;**1**: 939-44.
Shinkai T, Ueki T, Kobayashi Y. Detection and identification of rumen bacteria constituting a fibrolytic consortium dominated by Fibrobacter succinogenes. *Anim Sci J* 2010;**81**: 72-9.
Suen G, Weimer PJ, Stevenson DM *et al.* The complete genome sequence of Fibrobacter succinogenes S85 reveals a cellulolytic and metabolic specialist. *PLoS One* 2011;**6**: e18814.
Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci* 1991;**74**: 3583-97.

Wang Q, Garrity GM, Tiedje JM *et al.* Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007;**73**: 5261-7.

Zened A, Combes S, Cauquil L *et al.* Microbial ecology of the rumen evaluated by 454 GS FLX pyrosequencing is affected by starch and oil supplementation of diets. *FEMS Microbiol Ecol* 2013;**83**: 504-14.