Animal Microbiome

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Temporal microbial colonization on different forages is driven by the rumen environmental conditions

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Abstract

The rumen is one of the four compartments of the ruminant stomach and houses a diverse array of anaerobic microbes that play a crucial role in feed digestion and volatile fatty acid (VFA) production. The aim of this study was to explore how two different in vivo rumen environmental conditions, AHR (created from sheep-fed alfalfa hay) and CSR (created from sheep-fed corn stover), affect fiber digestion and rumen bacterial colonization in relation to two types of forage, alfalfa hay (AH) and corn stover (CS). Both AH and CS forages were subjected to *in-sacco* incubation in AHR and CSR conditions for a period of 48 h. The results revealed that CSR exhibited a less variant pH, lower total VFA concentration, and higher acetate-to-propionate ratio than AHR. CSR significantly enhanced the degradation of neutral detergent fiber and acid detergent fiber in both incubated forages (AH and CS). Although CSR did not improve the degradation of dry matter (DM) or crude protein (CP) on AH, it improved the degradation of DM and CP on CS. Both CS and AH incubated under CSR were found to have a greater abundance of fibrolytic bacteria (e.g., *Fibrobacter* and *Butyrivibrio* 2) compared to the same forage incubated under AHR, especially during the initial stages of incubation. However, CS and AH incubated under AHR were colonized by bacteria specialized in breaking down soluble carbohydrates (e.g., *Prevotella* and *Succinivibrio*). Compared with AHR, CSR enhanced the degradation rates of both incubated forages (CS and AH). These findings underscore the role of the rumen microenvironment in affecting the composition of adherent microbial communities and enhancing the breakdown of forages. Therefore, optimizing the rumen microenvironment to promote the attachment of fibrolytic bacteria during the early fermentation stages while minimizing hydrogen accumulation to stabilize the pH could lead to improved forage fermentation and animal performance.

Keywords Rumen microbiota, Microbial succession, Rumen environment, Forage degradation

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Introduction

Forages comprise at least 50% of the ingredients used in ruminant diets and are the primary source of lignocellulose to be broken down in the rumen. However, their higher fiber content makes it less efficient for the host animal to extract nutrients and energy than concentrate-based diets. This poses a challenge in improving animal productivity because there is a need to increase the proportion of forages in ruminant diets to reduce the costs of feeding and the risk of metabolic and nutritional disorders [1]. Forage degradation efficiency is largely

influenced by the composition and functional capacity of the forage-adhering microbiota, which constitutes 75% of the total microbial population and enzymatic activities in the rumen [2, 3]. The process of microbial colonization during forage degradation is significantly affected by the incubation time, as evidenced by previous studies [4, 5]. Other factors that play a role in the incubation time include the type of forage [6, 7] and the physicochemical properties of the plant cell wall structure [6, 8]. A recent metagenomic survey revealed that microbial functions related to lignocellulose degradation were underrepresented in the early stages of incubation (at 1 and 4 h) [9], suggesting that limited microbial colonization in the initial phases of forage degradation hampers the breakdown of the plant cell wall.

The recalcitrant forages that ruminants rely on are the most decisive force driving diversity in the rumen environment and provide the plant cell wall components required for the growth of fibrolytic microbes. While some ruminant species have inherited a natural ability to digest forage diets more efficiently than others (e.g., bison and yak are more efficient at digesting cellulose and hemicellulose than cattle [10, 11]), the type of diet plays a significant role in shaping the microbial composition of the rumen environmental conditions. For example, forage-based diets encourage the growth of a diverse population of lignocellulolytic specialists that usually stabilize the structure of the rumen environmental conditions. In contrast, concentrate-based diets typically cause frequent changes in the microbiota composition due to constant shifts in the ruminal pH.

Building on this understanding, exploring the impact of rumen environmental conditions on microbial colonization processes during forage degradation is crucial to unlocking new insights into microbial dynamics and their functional roles. We hypothesized that AH (alfalfa hay) and CS (corn stover), two forages with different nutritional compositions, create two distinct rumen environmental conditions with specific ecological niches, forage digestion dynamics, and physico-chemical properties (e.g., pH). Therefore, the main objective of our study is to use these nutritionally divergent forages (AH vs. CS) to create unique rumen environmental conditions and provide a clearer picture of the microbial colonization process during forage degradation, particularly in the initial stages of incubation where limitations for an efficient breakdown of lignocellulose still exist.

Materials and methods

All animal procedures were approved by the Animal Care and Use Committee of Zhejiang University (Hangzhou, China) and were performed in accordance with the University's guidelines for animal research.

Animal experiment and sample collection

The overall experimental design is shown in Fig. 1. Three healthy ruminally cannulated Hu Sheep (body weight = 35.2 ± 0.8 kg) were used in this study. AH and CS were the primary forage sources used in the formulation of the diets (forage-to-concentration ratio of 7:3) (Additional file 3: Table S1). The animal trial was divided into two stages, each comprising a 30-day period for diet adaptation (Fig. 1A). In each stage, the three sheep were housed individually and fed AH- or CS-based diets ad libitum for 30 consecutive days twice a day (at 07:00 and 16:00 h) to create the desirable rumen environmental conditions: (1) AHR and (2) CSR. During the trial, all the animals had free access to water. The feed intake, ingredients, and refusals were recorded and collected during the last 7 days of each stage for the determination of dry matter intake (DMI) and nutrient intake.

After 30 days of diet adaptation in each stage, nylon bags containing AH or CS forage pellets were introduced into the rumen of the sheep for *in sacco* incubation, following the methodology described by Wang et al. (2014). Five grams of 3 mm air-dried AH or CS forage particles were weighed and placed into each 50 μm (pore size) nylon bag (10 × 20 cm, Arthur H. Thomas Co., Philadelphia, PA, USA). Then, two bags containing AH and two bags containing CS were fixed on a 40 cm long plastic tube (Φ = 0.3 cm) (Fig. 1B). A total of 7 plastic tubes fixed with 28 nylon bags (4 nylon bags/plastic tube) were placed into the rumen of each cannulated Hu sheep before the morning feeding. Each tube was retrieved from the rumen at 0.5, 2, 4, 8, 16, 24, and 48 h after incubation (Fig. 1C). Two of the four fixed bags with AH and CS were immediately washed with 2 × 50 ml of PBS (pH 7.0) to remove rumen fluid and loosely attached

(See figure on next page.)

Fig. 1 Animal experimental design. **A** Three ruminally cannulated Hu sheep were used in this experiment. The experiment was divided into two stages. In each stage, during the first 30 days, the animals were fed either alfalfa hay (AH)- or corn stover (CS)-based diets respectively in order to create a specific rumen environmental condition (AH rumen condition—AHR or CS rumen condition—CSR). After 30 days of adaptation, bags filled with AH or CS forage pellets were placed into the rumen of each cannulated animal before the morning feeding for a 2-day *in-sacco* rumen incubation. **B** Four nylon bags containing either AH or CS were fixed to both sides of a plastic tube. A total of 7 plastic tubes were placed into the rumen. **C** After the *in sacco* incubation, the nylon bags were retrieved sequentially from the rumen at 0.5, 2, 4, 8, 16, 24, and 48 h

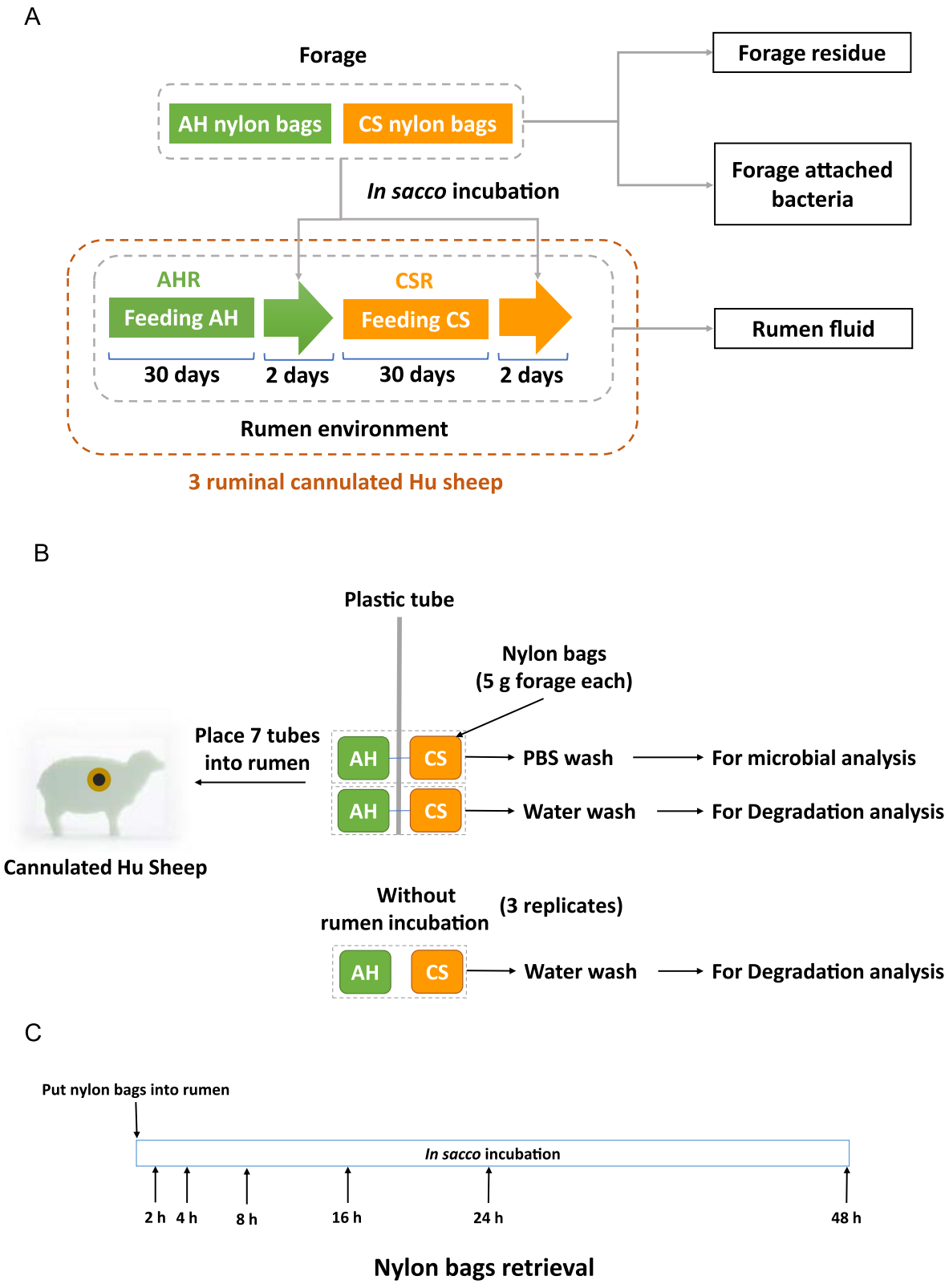

Fig. 1 (See legend on previous page.)

microbes. Then, these samples were transferred to sterile 25 ml tubes, immediately frozen in liquid nitrogen and stored at -80 °C until DNA extraction for microbial analysis. The remaining two nylon bags containing AH and CS residues were rinsed in cold water until the water ran clear and then were dried at 65 °C for nutritional degradation analysis. Moreover, two additional bags (control) containing AH and CS in three replicates were subjected to the procedures described above but without rumen incubation (0 h) (Fig. 1B). Rumen fluid was collected from ventral sac at each time point after the nylon bags were retrieved and placed on ice, after which the rumen pH was immediately measured with a portable pH meter (Starter 300; Ohaus Instruments Co. Ltd., Shanghai, China).

Forage degradation analysis

The feed and refusal samples were dried at 65 °C in a forced-air oven for 48 h and then ground in a Cyclotec mill (Tecator 1093; Tecator AB, Höganäs, Sweden) through a 1 mm screen. The feed, refusal and incubated forage residues were analyzed for dry matter (DM), crude protein (CP) and acid detergent fiber (ADF) according to the procedures of the Association of Official Analytical Chemists (AOAC) (#934.01, #927.02, #988.05 and #973.18, respectively) [12]. Neutral detergent fiber (NDF) was determined without a heat-stable amylase and with residual ash included [13].

The *in sacco* degradation constants were estimated using the exponential model described by E Orskov, et al. [14]: $p = a + b\left(1 - e^{-ct}\right)$[14], where p represents the degradation rate of the forage; a represents the rapidly degrading proportion of the forage; b represents the slow degrading proportion of the forage;c represents the degradation rate of the slow degrading component of the forage; and t represents the forage retention time in the rumen. The model was fitted by nonlinear least squares using the Gauss–Newton algorithm. The effective rumen degradability (ERD $= a + bc/(c + kp)$) was calculated by a passage rate (kp) of 5%/h from the equations suggested by the NRC (2001) for wet forage [15].

Ruminal volatile fatty acids analysis

The supernatants were collected by centrifuging the rumen fluid samples at 13,000×g for 15 min. One microlitter supernatant was then added to 200 µl of 25% phosphoric acid and transferred to a gas chromatograph (GC-2010; Shimadzu, Kyoto, Japan) equipped with a capillary column (HP-INNOWAX, 1909 N-133) and a flame ionization detector with N_2 as the carrier gas. The initial column temperature was 90 °C, and the temperature was then increased gradually to 170 °C at a rate of 10 °C/min and held for 2 min at a split ratio of 20:1. The total flow of N_2 in the column was 63.8 m/min, and the temperature of the injector/detector was 200 °C. A mixture of VFAs containing 10 mmol/L acetic, propionic, isobutyric, butyric, valeric and isovaleric acids was used as the standard sample.

DNA extraction

Total DNA was extracted from the forage contents present in the nylon bags following the cetyltrimethyl ammonium bromide (CTAB)-based method [16], with minor modifications. To ensure that the extracted DNA is representative of the entire microbiota associated with the forage, the five-point sampling method was employed. Approximately 0.5 g of incubated forage residue collected from the in situ nylon bags was mixed with 1 ml of CTAB DNA extraction buffer, and the samples were mixed with 0.5- and 0.1-mm zirconium beads and vortexed on a Beat Beater (FastPrep-24, M. P. Biomedicals, Santa Ana, CA, United States). The homogenates were transferred to a 72 °C water bath for 20 min and vortexed once every 5 min. RNA contamination was removed by adding 5 µl of RNase to the supernatant, followed by incubation at 37 °C for 15 min. The DNA was purified with an equal volume of phenol–chloroform-isopentanol (25:24:1), precipitated using 1 ml of isopropanol, washed several times with 75% ethanol, and finally dried and dissolved in ddH_2O. The extracted DNA was quantified using a Qubit dsDNA HS Assay Kit (Invitrogen, Eugene, OR, United States) on a Qubit 2.0 fluorimeter (Invitrogen, Carlsbad, CA, United States).

16S rRNA gene sequencing and bioinformatic analysis

The hypervariable V3-V4 regions of the 16S rRNA gene were amplified using the primer set F341 (forward primer 5′-ACTCCTACGGGRSGCAGCAG-3′) and R806 (reverse primer 5′-GGACTACVVGGGTAT CTAATC-3′) [17]. PCR products were verified by electrophoresis on a 2.0% agarose gel for 40 min at 80 V in TAE buffer (Tris base, acetic acid and EDTA) and then retrieved and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, United States). The amplicon library was constructed and sequenced on an Illumina HiSeq PE 250 platform (Illumina, San Diego, CA, United States) to generate 250-bp paired-end reads. The raw reads were demultiplexed by the q2-demux function in QIIME2 [18], and Cutadapt [19] was used to trim 23-bp adaptors on the 3' ends of the reads in paired-end mode. Quality control, sequence variant inference, paired-end merging and chimera filtering were performed using the *dada2* package [20] in R [21]. Reads with a quality score lower than 2 and a maximum number of expected errors larger than 2 were removed. The error rates of each sample were estimated via

self-consisting nonsupervised machine learning for true sequence variance estimation, as proposed by *dada2*. Merging of sequences was performed by aligning the denoised paired-end reads with at least 15 overlapping bases. The OTU-like amplicon sequence variant (ASV) table was constructed based on sequence variance estimations. Chimeric sequences in the ASV table were identified and discarded. The nonchimeric sequences were then processed in QIIME2 and classified taxonomically using the q2-feature classifier with a customized training set of reference sequences built from the SILVA 132 database [22]. The ASVs assigned as chloroplasts and mitochondria were filtered out and regarded as contaminants, with bacteria and archaea being analyzed separately.

Statistical analysis

The 16S rRNA amplicon sequence variant tables were analyzed using the *phyloseq* package in R [23]. The alpha diversity indices, including the Shannon, Simpson, Chao1 and observed species indices, in each sample were calculated using the *estimate_richness* function. The variations in alpha diversity were analyzed using a linear model, and multiple comparisons among time points were tested by the Waller-Duncan k-ratio test adjusted to the false discovery rate (FDR) using R [21] as implemented in the *agricolae* package [24]. Beta diversity was calculated by Bray–Curtis dissimilarity, and microbial communities with greater similarity were clustered via principal coordinate analysis using *phyloseq* [23] and visualized with *ggplot2* [25].

The *lme4* package [26] was used to perform linear mixed effects analysis of the forage degradation, rumen pH and VFA measurements, and the model was parameterized as follows:

$$Y_{ijkl} = \mu + R_j + F_j + T_l + S_i + \varepsilon_{ijkl},$$

where Y_{ijkl} represents the variable of interest and μ represents the mean values obtained from the three sheep. The rumen environmental condition (R_j), forages in nylon bags (F_j) and time (T_l) were treated as fixed effects; animals (S_i) were regarded as a random effect, and ε_{ijkl} was the residual error. Visual inspection of the residual plots did not reveal any obvious deviation from homoscedasticity or normality, and P values were obtained by the maximum likelihood ratio test. Differences between time points were compared by Tukey's honest significant differences test using the *agricolae* package [24]. Statistical significance was defined as $P \leq 0.05$, with highly significant values at $P \leq 0.01$; trends were defined as $0.05 < P \leq 0.10$.

The similarity of microbial compositions among samples was tested by *adonis2* implemented in the *vegan* package in R [27]. Furthermore, analysis of the composition of the microbiota (ANCOM) [28] was performed to test the compositional differences in the forage-adhering microbiota according to the two rumen environments. Mixed linear models were constructed in ANCOM and adjusted for an FDR less than or equal to 0.05 using the Benjamin-Hochberg algorithm. The relative abundances of the 15 most significant bacteria were analyzed in conjunction with the changes in the rumen fermentation variables and the average degradation rate of forage components in each time interval using canonical correspondence analysis (CCA). Monte Carlo permutation tests were used to estimate the influence of the rumen fermentation variables on the microbial composition. The relative abundance matrix of the significant genera was normalized by z scores and visualized in a heatmap using the *pheatmap* package [29]. Hierarchical clustering was then performed to investigate variations in each genus (based on Euclidean distances) associated with the shifts in the microbial composition.

Results

Dynamics of rumen parameters and forage degradation rates

The average DMI recorded for sheep fed AH- or CS-based diets during the experiment was 1.68 ± 0.04 and 1.21 ± 0.02 kg/day, respectively. The ruminal pH decreased immediately after feeding and reached the lowest values after four (pH = 5.67; AHR) and eight hours (pH = 5.71; CSR), respectively. The pH levels returned to their 0 h levels after 24 h (pH = 6.32 for AHR and 6.28 for CSR) (Table 1). Linear mixed model analysis indicated that time, but not forage type, had a significant effect on ruminal pH ($P < 0.01$), and the forage × time interaction was significant ($P < 0.05$). Except for valerate, the concentrations of total VFAs and the molar proportions of acetate, propionate, butyrate, isobutyrate, and isovalerate were significantly affected by forage type, sampling time and their interactions ($P < 0.01$) (Table 2). Compared to CSR, AHR had a greater concentration of total volatile fatty acids (VFAs) and a greater molar proportion of propionate, butyrate, and other short-branched fatty acids (including valeric and isovaleric acids). On the other hand, CSR exhibited a greater molar proportion of acetate than AHR. Detailed information on the variations in each VFA component is presented in Table 2.

This study revealed that the degradation rates of both forages can be categorized into three phases: (1) the first 0.5 h, (2) between 2 to 4 h, and (3) after 4 h (Fig. 2). During the first 0.5 h, the disappearance rate of DM was notably faster in both forage types, with 34.1% and 34.0% of the DM disappearing in AH and 19.3% and 25.0% of the DM disappearing in CS, respectively. The crude protein of the forages was degraded by up to 50% and 30%

Table 1 Ruminal pH variations in the different rumen environmental conditions after feeding

Time		pH[1]	
		AHR	CSR
0 h		6.39[a]	6.27[a]
0.5 h		6.18[ab]	6.28[a]
2 h		5.91[bc]	6.03[ab]
4 h		5.67[c]	5.73[b]
8 h		5.99[bc]	5.71[b]
16 h		5.70[c]	5.75[b]
24 h		6.32[a]	6.28[a]
SEM		0.05	0.05
P value[2]	Time	< 0.01	
	Treatment	0.24	
	Time × Treatment	0.04	

[a –c]Means within the same column with different superscripts denote significant differences ($P < 0.05$)

[1] AHR: alfalfa hay rumen environmental condition; CSR: corn stover rumen environmental condition

[2] Linear mixed model analysis was used to test the effects of incubation time and the rumen environment on the ruminal pH

for AHR and CSR, correspondingly, while the degradation of NDF and ADF was much slower in both rumen environments. The second phase was observed between 2 to 4 h, when the degradation rates of all forages were halted in both rumen environments, except for CS incubated in AHR, which remained halted until 8 h. After 4 h of incubation, biomass degradation was linear but at a lower rate than that in the first 0.5 h. However, the disappearance rate of NDF and ADF was faster after 4 h of incubation than in the first 0.5 h.

The degradation of DM and CP in AH was not significantly influenced by the AHR or CSR. While ADF and NDF degradation of AH was faster in CSR than in AHR until 24 h, it was surpassed at 48 h when AH was incubated in AHR ($P < 0.05$). When CS was incubated in AHR, AHR significantly decreased not only DM degradation but also the CP, NDF, and ADF degradation rates of CS ($P < 0.05$). These results were in line with the effective rumen digestibility (ERD) parameter calculated from the rumen degradation model (Additional file 3: Table S2), which showed that CSR significantly increased the degradability of fractions *a* (fast-degrading component) and *c* (slow-degrading component) of the forages. Ultimately, they increased ERD assuming a passage rate of 5%/h rumen flow rate ($P < 0.05$).

Temporal dynamics of microbial diversity

A total of 68,124 ± 101 reads/sample were generated after concatenation and quality control, which were then clustered into 9,383 distinct bacterial variants using the *dada2* algorithm. The observed and Chao1 indices differed significantly among forages incubated in the different rumen environmental conditions (Fig. 3, $P < 0.05$). The diversity and richness of the microbial communities were greater for forages incubated in CSR than for those incubated in AHR at 0.5 and 2 h (AH-AHR *vs.* AH-CSR, CS-AHR *vs.* CS-CSR, both $P < 0.05$). The lowest values of α-diversity indices were observed at 4 and 8 h after incubation for AHR and CSR, respectively. An increase in Observed species, Chao1 and Shannon diversity, was also observed in 4, 8 to 48 h. Notably, when forages were incubated in a rumen environmental condition different from that of their forage type (e.g., CS-AHR *vs.* AH-AHR, AH-CSR *vs.* CS-CSR), the diversity of bacteria associated with it significantly increased ($P < 0.05$).

Principal coordinate analysis based on Bray–Curtis similarity distances showed that bacterial taxa were influenced by the forage type, the rumen environmental conditions, and individual variations in the cannulated sheep, with the rumen environmental condition exhibiting the greatest impact on the bacterial phylotypes (Fig. 4A). Adonis testing further confirmed this observation, which showed significant differences between treatments with respect to the bacterial taxa ($R = 0.4688$, $P < 0.01$). However, samples collected from the same rumen environmental condition were very similar to each other, with no significant difference detected (AH-AHR *vs.* CS-AHR: $R = 0.037$, $P = 0.123$; AH-CSR *vs.* CS-CSR: $R = 0.031$, $P = 0.146$). The difference in the microbiota structure was less pronounced when the forages were incubated under CSR than under AHR. Additionally, the effect of time on the compositional variation of the microbes was concealed by the individual variation in the sheep (Fig. 4B).

Effect of the rumen environmental conditions on the bacterial microbiota colonizing forages in nylon bags

Of all the sequencing amplicon variants (ASVs) detected, 13 bacterial phyla, 24 classes, 45 orders, 85 families, and 187 genera were successfully classified. *Prevotella* 1, unclassified Christensenelacea R-7 group, Ruminococcaceae NK4A214 group, *Ruminococcus* 1, *Treponema* 2, *Ruminoccocus* 2, *Pseudobutyrivibrio* 2, *Butyrivibrio* 2, *Saccharofermentansn,* Rikenellaceae RC9 gut group, and *Roseburia* were the most abundant genera in the forage types examined in this study (Additional file 1: Fig. S1B). Analysis of the microbiota composition using ANCOM showed that forage types and rumen environments significantly affected two phyla, three classes, six orders, 14 families, and 36 genera (Table 3).

Table 2 Ruminal volatile fatty acids variations in the different rumen environmental conditions after feeding

Time	AHR[1]							CSR[1]							SEM	*P* value[2]		
	0	0.5	2	4	8	16	24	0	0.5	2	4	8	16	24		R	T	R×T
Total VFA, mM/L	116^{abc}	127^{a}	107^{bc}	126^{ab}	100^{c}	117^{abc}	116^{abc}	90^{b}	105^{a}	85^{bc}	83^{bc}	80^{bc}	76^{c}	90^{b}	3.07	< 0.01	< 0.01	< 0.01
Molar proportion, mM/100 mM																		
Acetate	66.6^{ab}	67.1^{a}	66.4^{ab}	65.8^{b}	63.8^{c}	63.4^{c}	66.6^{ab}	70.5^{a}	70.4^{a}	68.3^{b}	67.3^{b}	68.0^{b}	67.7^{b}	70.5^{a}	0.24	< 0.01	< 0.01	< 0.01
Propionate	17.7^{c}	18.7^{bc}	18.5^{bc}	19.7^{ab}	18.7^{bc}	21.1^{a}	17.7^{c}	15.9^{d}	15.5^{d}	20.1^{ab}	21.1^{a}	18.4^{c}	19.0^{bc}	15.9^{d}	0.29	< 0.01	< 0.01	< 0.01
Butyrate	11.4^{b}	10.9^{b}	12.1^{ab}	11.5^{b}	13.6^{a}	12.5^{ab}	11.4^{b}	10.9^{b}	11.8^{a}	8.9^{d}	9.2^{d}	10.3^{c}	10.4^{bc}	10.9^{b}	0.24	< 0.01	< 0.01	< 0.01
Isobutyrate	1.3^{a}	0.8^{b}	0.8^{b}	0.8^{b}	1.2^{a}	0.8^{b}	1.3^{a}	0.9^{bc}	0.7^{d}	0.8^{bcd}	0.8^{cd}	1.1^{a}	1.0^{ab}	0.9^{bc}	0.04	< 0.01	< 0.01	< 0.01
Valerate	1.5^{a}	1.4^{a}	1.3^{a}	1.5^{a}	1.4^{a}	1.4^{a}	1.5^{a}	0.7^{b}	0.7^{b}	0.8^{b}	0.8^{b}	0.9^{a}	0.8^{ab}	0.7^{b}	0.04	< 0.01	0.15	< 0.01
Isovalerate	1.5^{a}	1.0^{bc}	1.0^{c}	0.8^{c}	1.4^{ab}	0.7^{c}	1.5^{a}	1.2^{ab}	0.9^{c}	1.2^{b}	0.9^{c}	1.2^{a}	1.1^{bc}	1.2^{ab}	0.05	< 0.01	< 0.01	< 0.01
Acetate/Propionate	3.76^{a}	3.59^{ab}	3.60^{ab}	3.34^{b}	3.44^{b}	3.01^{c}	3.76^{a}	4.44^{a}	4.55^{a}	3.41^{bc}	3.19^{c}	3.69^{b}	3.57^{b}	4.44^{a}	0.07	< 0.01	< 0.01	< 0.01

[a] –[d]Means within the same column with different superscripts are significantly different ($P < 0.05$)

[1] AHR: alfalfa hay rumen environmental condition; CSR: corn stover rumen environmental condition

[2] R = treatment effect, T = time effect, F × T = interactive effect of treatment and time

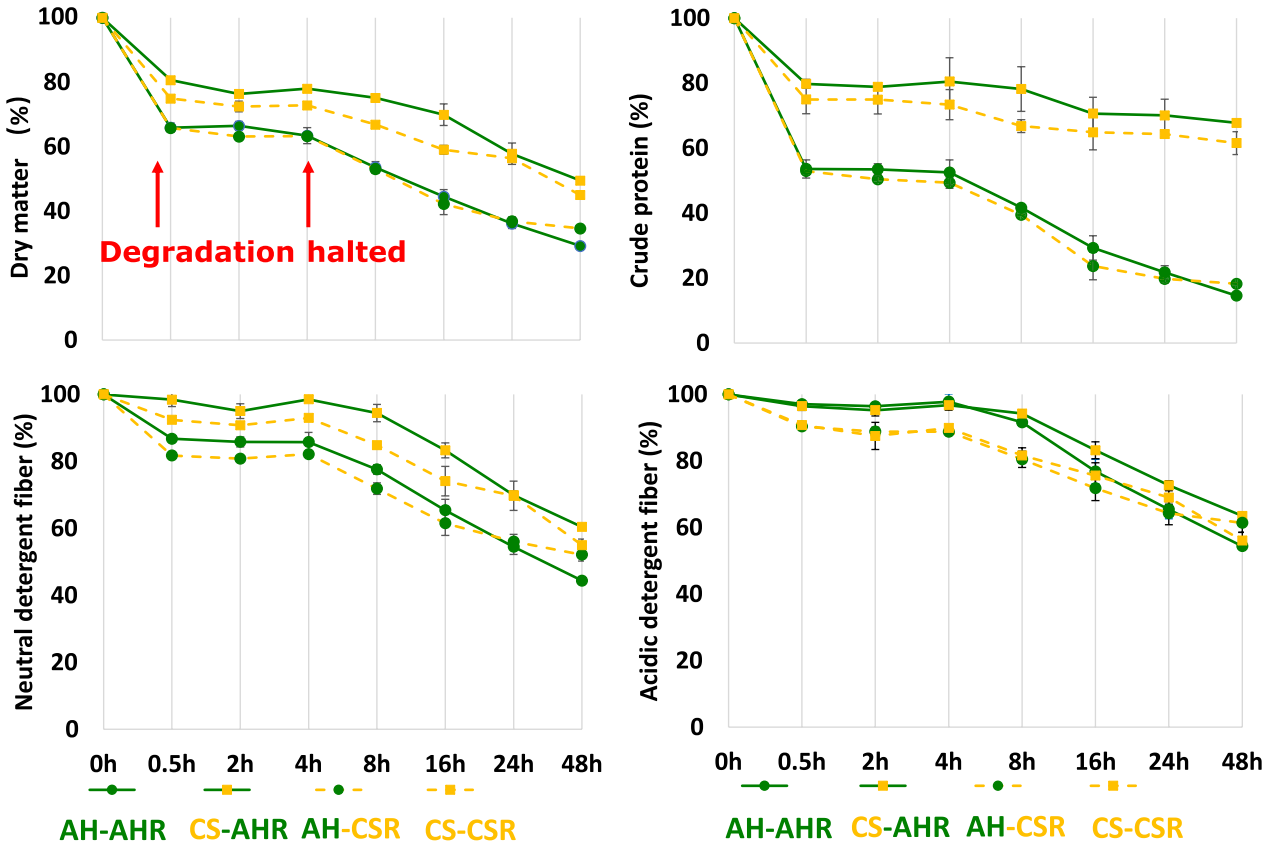

Fig. 2 Temporal degradation of forages incubated in the different rumen environmental conditions. Line graphs showing temporal changes in the proportions of residual dry matter (**A**), crude protein (**B**), neutral detergent fiber (**C**), and acid detergent fiber (**D**) after rumen incubation. The solid green lines represent the AHR (a rumen environment created from animals fed an alfalfa hay-based diet), and the dashed yellow lines represent the CSR (a rumen environmental condition created from animals fed a corn stover-based diet). Yellow squares and green dots in each line represent CS (corn stover pellets in nylon bags) and AH (alfalfa hay pellets in nylon bags) incubated in the respective rumen environmental condition

Our findings underscore the unique association between the rumen environmental conditions and the relative abundances of certain bacterial genera. For instance, when comparing the microbiota colonizing both AH and CS with respect to AHR and CSR (AH-AHR *vs.* CS-CSR), we observed that *Butyrivibrio* 2 and *Fibrobacter* were more abundant on CS, while *Roseburia*, *Succiniclasticum, Prevotella* 1, *Atopobium*, *and Mucorella* were more abundant on AH (Additional file 3: Table S3). This shift in the rumen environmental conditions led to a change in the composition of 12 genera colonizing AH (AH-AHR *vs.* AH-CSR) and 24 genera colonizing CS (CS-AHR *vs.* CS-CSR) (Additional file 2: Fig. S2). Among these genera, 11 were sensitive to changes in the rumen environmental conditions for both forages, including *Roseburia*, *Butyrivibrio* 2, *Succiniclasticum*, *Fibrobacter*, *Moryella*, *Atopobiu*, Rikenellaceae RC9 gut group, Lachnospiraceae NK3A20 group, Prevotellaceae UCG-004, Ruminococcaceae UCG-014, and Lachnospiraceae XPB1014 group (Additional file 3: Table S4). This shift from AHR to CSR increased the relative abundances of *Fibrobacter*, *Butyrivibrio* 2 and Lachnospiraceae XPB1014 group but decreased the relative abundances of *Roseburia*, *Succiniclasticum*, *Moryella*, *Atopobium*, Lachnospiraceae NK3A20 group, Prevotellaceae UCG-004 and Ruminococcaceae UCG-014. The relative abundances of one single genus on AH and 13 genera on CS were uniquely associated with the rumen conditions in which AH and CS were incubated (Additional file 2: Fig. S2).

The microbiota colonizing AH and CS differed within the same rumen environmental condition, with the influence of forage types on microbiota abundance being a key factor. Genera detected between AH and CS were more abundant in AHR than in CSR (AH-AHR *vs.* CS-AHR: 13 differential genera; AH-CSR *vs.* CS-CSR: 5 differential genera, Additional file 2: Fig. S2), which is in line with the results presented in Fig. 4, where the microbiota colonizing the different forage types clustered according to the rumen environmental conditions. *Prevotella* 1

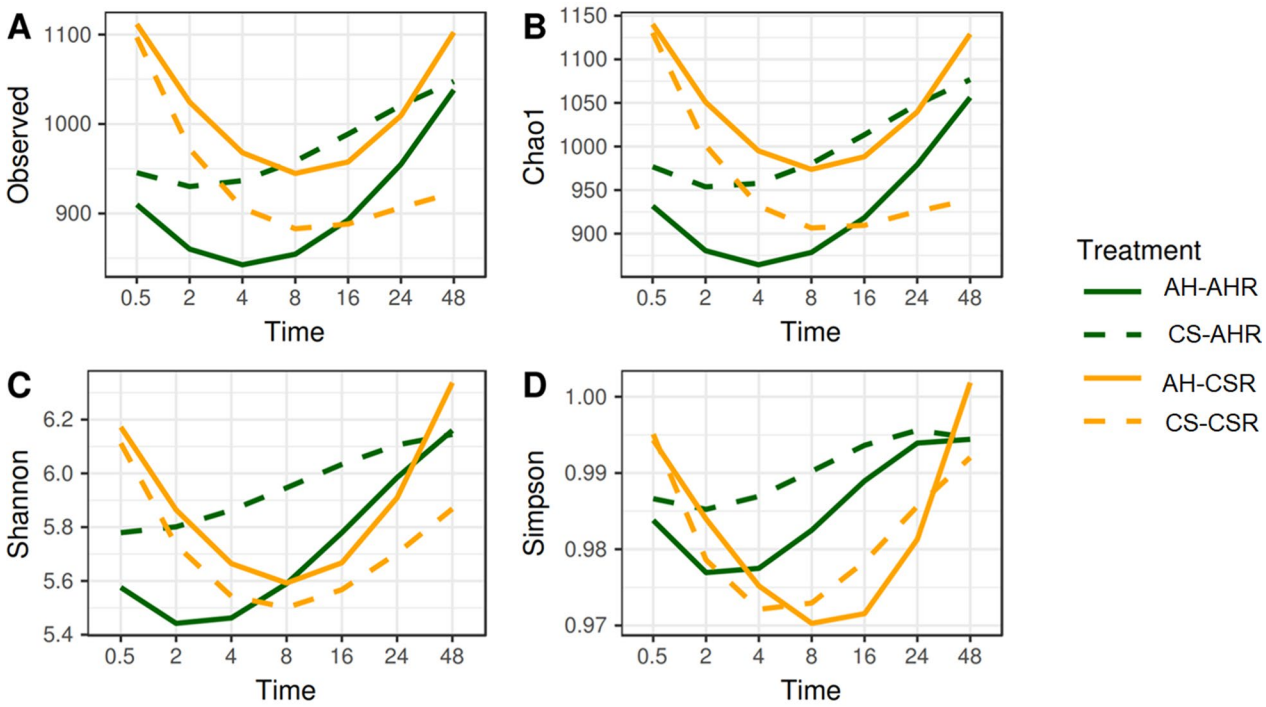

Fig. 3 Variation in the alpha diversity of the microbiota attached to the forage surface. The lines show the changes in microbial alpha diversity during forage incubation in the different rumen environmental conditions. The green and yellow solid lines represent the microbiota attached to AH (alfalfa hay pellets in nylon bags) that was incubated in either AHR (a rumen environmental condition created from animals fed an alfalfa hay-based diet) or CSR (a rumen environmental condition created from animals fed a corn stover-based diet). The green and yellow dashed lines represent bacteria attached to CS incubated under AHR and CSR, respectively

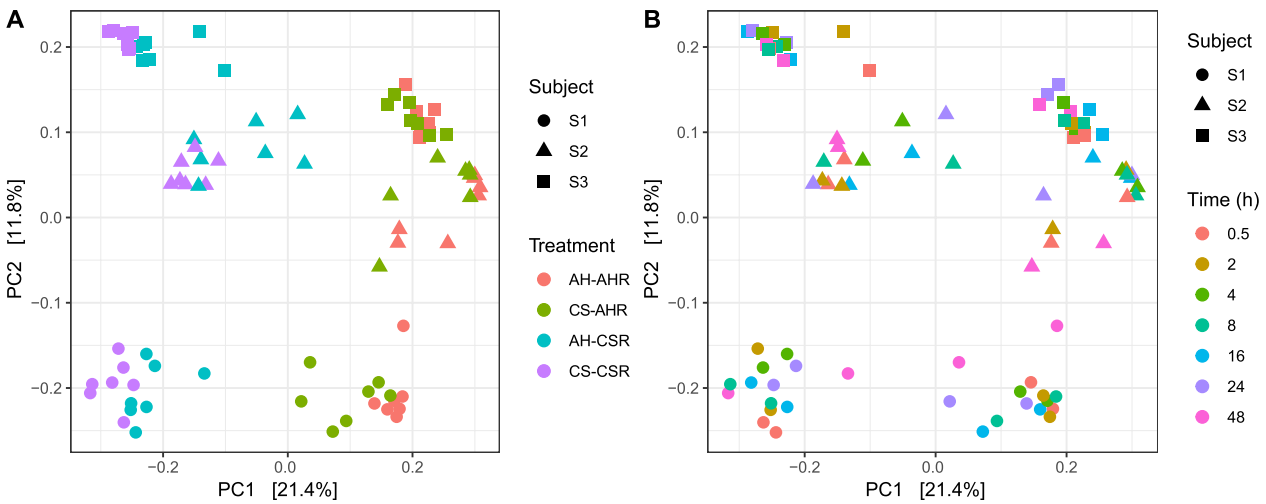

Fig. 4 Principal coordinate analysis of the forage adherent microbial community. Points with different shapes represent samples from different subjects. Samples from forages incubated in different rumen environmental conditions are represented by different colors in section A, while samples collected at different time points after incubation are represented by different colors in section B. Samples were clustered according to the rumen environmental conditions ($R = 0.4688$, $P < 0.01$). Within the four different conditions, samples collected from the same rumen environment were similar to each other, with no significant differences detected (AH-AHR *vs.* CS-AHR: $R = 0.037$, $P = 0.123$; AH-CSR *vs.* CS-AHR: $R = 0.031$, $P = 0.146$)

Table 3 Bacterial taxa affected by forage type and rumen environmental conditions

Phylogeny[2]	AHR[1]		CSR[1]		*W* statistic	SEM
	AH	CS	AH	CS		
Phylum						
Firmicutes	53.23	56.29	53.91	48.51	12	0.98
Fibrobacteres	1.57	1.74	3.18	4.51	11	0.29
Class						
Clostridia	49.27	52.43	45.63	42.81	21	0.92
Fibrobacteria	1.57	1.74	3.18	4.51	19	0.29
Alphaproteobacteria	0.14	0.20	0.74	0.61	18	0.08
Order						
Clostridiales	49.27	52.42	45.61	42.81	37	0.92
Fibrobacterales	1.57	1.74	3.18	4.51	35	0.29
Coriobacteriales	1.23	1.53	0.97	0.76	33	0.07
Selenomonadales	2.79	2.85	2.23	1.84	33	0.11
Desulfovibrionales	0.32	0.40	0.21	0.14	32	0.02
Synergistales	0.13	0.15	0.09	0.05	31	0.01
Family						
Lachnospiraceae	25.27	24.11	20.19	21.52	66	0.62
Ruminococcaceae	16.94	19.97	16.76	13.90	66	0.48
Fibrobacteraceae	1.57	1.74	3.18	4.51	65	0.29
Rikenellaceae	4.45	5.12	6.00	3.37	65	0.26
Prevotellaceae	25.48	21.20	21.33	30.17	65	1.06
Acidaminococcaceae	1.87	1.89	1.11	0.61	63	0.09
Coriobacteriaceae	1.23	1.53	0.97	0.76	62	0.07
Bacteroidales BS11 gut group	2.52	2.73	2.23	1.47	61	0.11
Bacteroidales S24-7 group	2.65	3.43	2.14	1.47	61	0.16
Family XIII	1.14	1.46	1.11	0.70	60	0.07
Defluviitaleaceae	0.28	0.28	0.17	0.13	60	0.02
Desulfovibrionaceae	0.32	0.40	0.21	0.14	59	0.02
Synergistaceae	0.13	0.15	0.09	0.05	58	0.01
Bacteroidales UCG-001	0.02	0.03	0.18	0.29	58	0.02
Genus						
Roseburia	8.21	6.20	1.90	1.46	147	0.48
Prevotella 1	19.04	15.72	17.09	25.47	147	0.94
Rikenellaceae RC9 gut group	4.18	4.78	5.62	3.05	146	0.24
Fibrobacter	1.57	1.74	3.18	4.51	145	0.29
Butyrivibrio 2	2.28	1.71	4.17	5.04	145	0.25
Ruminococcaceae UCG-014	1.58	1.96	0.93	0.61	143	0.09
Pseudobutyrivibrio	2.29	1.79	2.81	3.43	143	0.17
Succiniclasticum	1.87	1.89	1.11	0.61	141	0.09
Lachnospiraceae XPB1014 group	0.75	1.19	1.44	1.71	141	0.07
Lachnospiraceae NK3A20 group	1.66	1.72	1.19	1.21	140	0.04
Oribacterium	1.35	0.78	0.75	0.73	140	0.09
Prevotellaceae UCG-001	1.40	1.46	1.33	1.84	140	0.08
Prevotellaceae UCG-004	1.01	1.27	0.56	0.39	139	0.06
Ruminococcaceae NK4A214 group	4.37	5.16	4.10	3.67	136	0.18
Atopobium	0.39	0.43	0.16	0.08	133	0.02
[*Eubacterium*] *hallii* group	0.36	0.35	0.26	0.15	133	0.02
Moryella	0.40	0.48	0.19	0.15	132	0.02
Ruminococcaceae UCG-013	0.18	0.10	0.07	0.05	131	0.01

Table 3 (continued)

Phylogeny[2]	AHR[1]		CSR[1]		*W* statistic	SEM
	AH	CS	AH	CS		
[*Eubacterium*] *coprostanoligenes* group	0.75	0.94	0.66	0.51	131	0.04
Lachnospiraceae NK4A136 group	0.31	0.61	0.54	0.54	129	0.03
Family XIII AD3011 group	0.31	0.35	0.29	0.17	126	0.02
[*Eubacterium*] *xylanophilum* group	0.08	0.08	0.16	0.26	125	0.01
Desulfovibrio	0.32	0.40	0.21	0.14	124	0.02
Lachnoclostridium 1	0.13	0.15	0.04	0.02	124	0.01
Lachnospiraceae AC2044 group	1.39	1.64	0.94	1.04	124	0.11
Ruminococcaceae V9D2013 group	0.53	0.63	0.15	0.16	124	0.06
Papillibacter	0.13	0.18	0.37	0.34	124	0.03
Ruminobacter	0.54	0.77	0.50	0.44	123	0.04
Saccharofermentans	1.53	2.40	1.73	1.70	123	0.13
Succinimonas	0.27	0.30	0.16	0.10	122	0.02
Mogibacterium	0.20	0.25	0.14	0.10	122	0.01
Defluviitaleaceae UCG-011	0.28	0.28	0.17	0.13	122	0.02
Lachnospiraceae UCG-008	0.24	0.28	0.17	0.13	122	0.01
probable genus 10	0.15	0.28	0.26	0.27	119	0.02
[*Ruminococcus*] *gauvreauii* group	0.49	0.55	0.38	0.28	119	0.03
Prevotellaceae UCG-003	0.58	0.76	0.75	0.87	119	0.04

[1] AHR: alfalfa hay rumen environmental condition; CSR: corn stover rumen environmental condition; AH: alfalfa hay; CS: corn stover

[2] Microbes significantly affected by forage types and rumen environmental conditions tested by ANCOM

was the only genus significantly influenced by the forage types incubated in the same rumen environmental conditions; it was more abundant on AH than on CS under AHR but less abundant on AH than on CS under CSR (Table 3). However, *Succiniclasticum* was more abundant in AH than in CS when these forages were incubated in the CSR. *Roseburia*, *Oribacterium*, and *Saccharofermentans* were more abundant on AH, while *Fibrobacter*, *Treponema* 2, and *Butyrivibrio* 2 were more abundant on CS incubated in the AHR (Table 3).

The dynamic colonization of the bacterial and archaeal microbiota

Our analysis identified variations in bacterial genera over time, with 25 genera influenced by the incubation time (Additional file 3: Table S5). The relative abundances of *Prevotella* 1, Succinivibrionaceae UCG-002 and *Succinicibrio* peaked at 0.5 h and gradually declined thereafter. These strains were then succeeded by *Fibrobacter*, *Treponema* 2 and *Ruminococcus* 1. Importantly, we observed a similar pattern of variation in the bacterial taxa across time and in the rumen environmental conditions, with certain genera (e.g., *Fibrobacter*, *Butyrivibrio* 2, and *Pseudobutyrivibrio*) clustering together (Fig. 5A). The archaeal genera we detected were classified as *Methanobrevibacter* and *Methanosphaera*. The ratio of archaea to bacteria initially increased after 0.5 h of incubation, peaked at 2 h, and then gradually decreased over time in all the samples (Fig. 5B). Compared with the AHR, the CSR exhibited a greater relative abundance of *Methanobrevibacter* but a lower relative abundance of *Methanosphaera* (Additional file 3: Table S5).

Correlations among rumen fermentation, forage degradation and bacteria

The degradation of DM, CP, NDF, ADF, pH, total VFAs, acetate, propionate, butyrate, isovalerate, and valerate were used as environmental explanatory variables in the CCA. The results of the Monte Carlo permutation tests indicated that the variables (degradation of DM, CP, NDF, and ADF; pH; total VFAs; acetate, propionate, butyrate, isovalerate, and valerate) could be used to explain the variations in the microbial communities ($P < 0.01$). Total VFAs, pH, acetate, butyrate, and valerate had a significant impact on the changes in the microbiota ($P < 0.05$) (Fig. 6A). There was also a trend toward significance for isobutyrate ($P = 0.085$). The total VFAs, ADF, acetate, isobutyrate, butyrate, and valerate explained more than 90% of the variation in the first axis of the CCA. In comparison, NDF explained more than 90% of the variation in the direction of the second axis of the CCA (Fig. 6A).

We also performed Spearman rank correlations to reveal possible relationships between the environmental

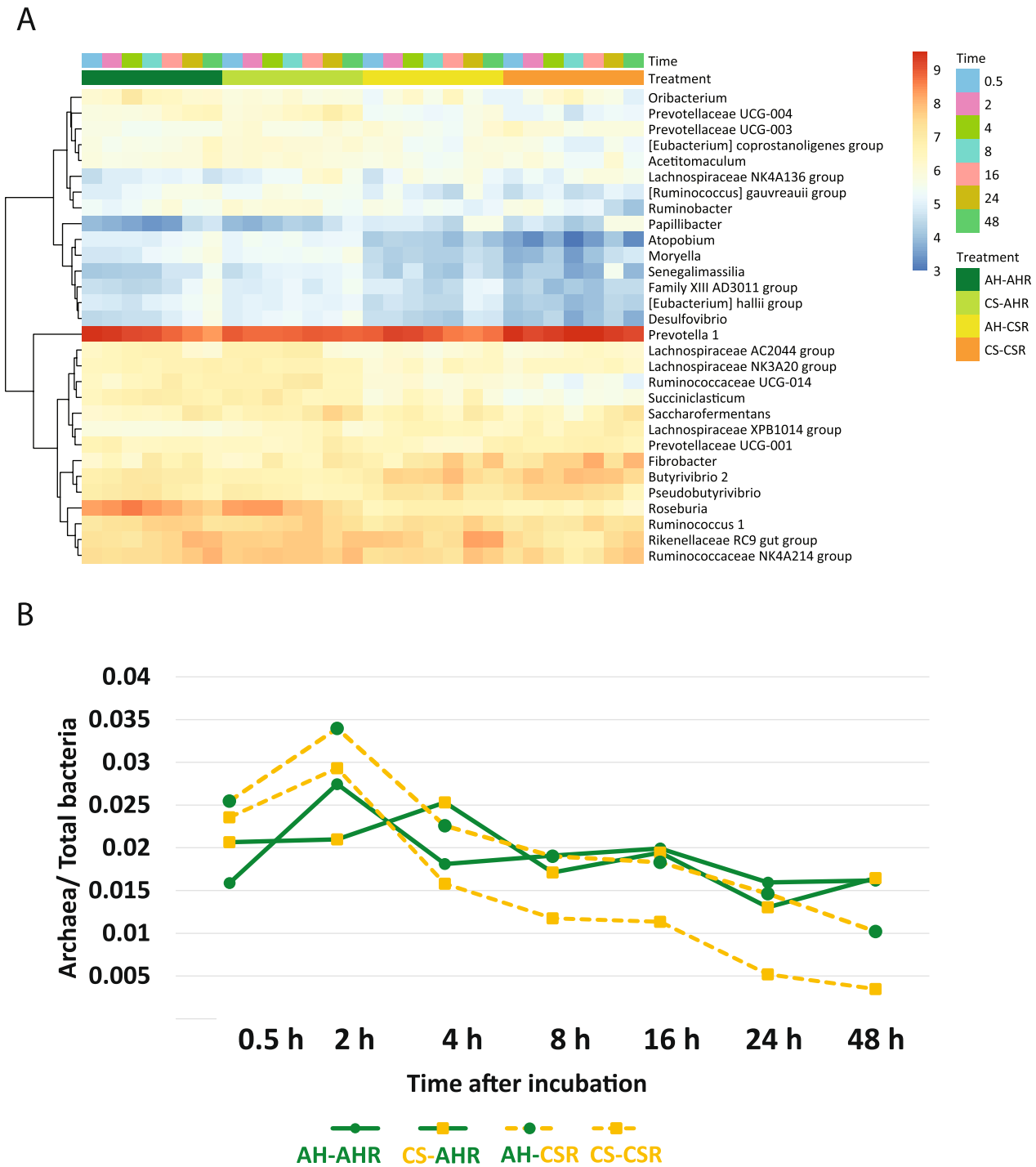

Fig. 5 Forage adherent microbial community variations in the different rumen environmental conditions. **A** Variations in genus abundance within the rumen environmental conditions. The columns of the heatmap represent the samples collected from individual animals on different days, and the rows correspond to the genera that varied significantly during forage incubation. The relative abundances were normalized by *z*-scores for easier visualization of variation patterns in each genus across different time points. Genera were clustered according to the unweighted pair group method with arithmetic mean (UPGMA) method and calculated based on Spearman's correlation of scaled abundances. **B** Variations in the bacteria-to-archaea ratio. The lines show the changes in the total bacteria-to-archaea ratio during forage incubation in different rumen environmental conditions. The solid green lines represent the AHR (a rumen environmental condition created from animals fed an alfalfa hay-based diet), and the dashed yellow lines represent the CSR (a rumen environmental condition created from animals fed a corn stover-based diet). Yellow squares and green dots in each line represent CS (corn stover pellets in nylon bags) and AH (alfalfa hay pellets in nylon bags) incubated in the respective rumen environmental condition

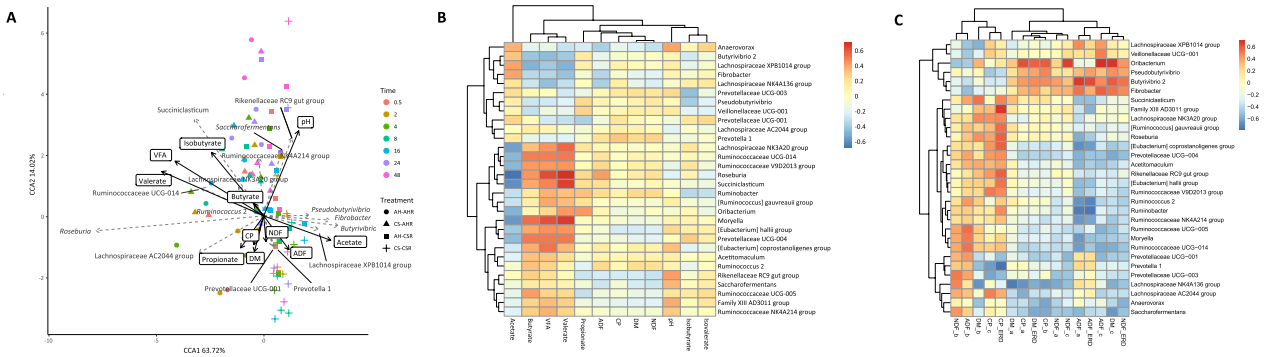

Fig. 6 Analysis of the microbial community in relation to rumen fermentation variables and forage degradation. **A** Canonical correlation analysis. The top 10 most differentially abundant genera are indicated by triangle arrows with dashed lines, and the rumen fermentation variables and degradation of forage components are indicated by triangle arrows with solid lines. The samples collected at different time points and from different rumen environmental conditions are presented in various colors and shapes. **B** Spearman correlations of the differentially abundant genera and fermentation parameters. The columns represent the rumen fermentation variables and forage degradation efficiency, and the rows correspond to the genera significantly affected by the rumen environmental conditions and forage types. Gradient colors indicate the correlation coefficients. Genera were clustered according to the Euclidean distances calculated based on the correlation coefficients. **C** Spearman correlation of differentially abundant genera and Orskov's fractions. Columns represent Orskov's fractions of forage components, and rows correspond to the differentially abundant genera. The gradient colors indicate the correlation coefficients. Genera were clustered according to the Euclidean distances calculated based on the correlation coefficients

variables and the changes in the microbiota (Fig. 6B). The results showed that valerate was positively correlated with *Roseburia*, *Succiniclasticum*, and *Moryella*, while *Roseburia* was negatively correlated with acetate ($R > 0.6$). The correlations between Orskov's degradation fractions and the microbial composition showed that bacteria can be divided into two major clusters. The first cluster (e.g., *Fibrobacter*, *Butyrivibrio* 2) was positively correlated with the rapidly degrading component of the forage (fraction *a*), the degradation rate of the slowly degrading component of the forage (fraction *c*), and ERD of DM, ADF, and NDF and negatively correlated with the slowly degrading portion of the forage (*b* fraction). The second cluster (e.g., *Succiniclasticum* and *Saccharomerments*) was positively correlated with the *c* fraction and ERD of the CP (Fig. 6C).

Discussion

The metabolic profile of the rumen environment is primarily affected by the concentration of fermentation end-products (e.g., VFAs) released during feed degradation. By comparing the chemical composition of AH and CS, we concluded that AH contains a much greater concentration of nonfibrous carbohydrates and more fermentable constituents than CS, which aligns with previous findings on forage composition and degradability [34]. During the first 0.5 h of fermentation, the rumen pH rapidly decreased in both feeding regimens, along with a rapid increase in VFA concentrations. Approximately 30% of the forage biomass was degraded within this initial period, highlighting the efficient adherence of microbes to feed particles and enhanced metabolite production, similar to previous reports using switchgrass and ryegrass substrates [5, 30]

The overall colonization process, which was subdivided into three stages in this study (0–0.5 h, 0.5–4 h, and 4–48 h), aligns with previous studies on incubation times in switchgrass [5], ryegrass [30], and rice stover [31] in the rumen of cows. During the initial colonization stages (0—0.5 h), the most dominant genera were *Prevotella*, *Succinivibrio*, and Succinivibrionaceae UCG-002, which are typically more abundant in high-concentrate diets than in forage-based diets [32]. These genera, which are highly efficient at metabolizing soluble carbohydrates and tolerating a low rumen pH, may be responsible for the rapid degradation of 30% of the forage biomass within the first 0.5 h of incubation. After the rapid degradation of readily fermentable carbohydrates, the overall metabolism shifted toward the degradation of the recalcitrant portion of the forage biomass. Consequently, the bacterial genera were gradually replaced by *Fibrobacter*, *Treponema* 2, Rikenellaceae RC9 gut group, Prevotellaceae NK3B31 group, *and Ruminococcus* 1, which are mostly involved in cellulose and hemicellulose degradation after 4 h of incubation [33]. This shift in metabolism highlights the functional difference between the primarily attached bacteria and the secondary fermenters, reinforcing findings from earlier work on bacterial succession during fiber degradation [6]. It also highlights the importance of considering the different stages of forage degradation to optimize feed fermentation by the rumen microbiota.

The α-diversity indices (observed, Chao1 and Shannon) decreased significantly in AH and CS. They reached the lowest values at 4 h and 8 h in the AHR and CSR treatments, respectively. However, Huws, et al. [4] observed a linear increase in bacterial diversity over time after incubation in a study of ryegrass microbial colonization in the rumen. The rapid decrease in biodiversity observed at 4 or 8 h in our study is likely a result of the decreased pH recorded in the AHR or CSR rumen environments. Since AH contains a greater abundance of readily fermentable polysaccharides and its fiber components are metabolized into VFAs more quickly [34], the rumen pH rapidly decreased within the first 4 h in AH compared to that in CS. The lower pH may have possibly suppressed the activities of the microbes that were sensitive to a lower pH and decreased the overall microbial community diversity, as is usually observed in ruminant-fed high-concentrate diets [35].

The attachment of fibrolytic bacteria on the forage surface, a process crucial for forage degradation, relies mainly on polyglycoprotein complexes that are extremely sensitive to pH variations [36]. Our study suggested that these protein complexes may have hindered the initial colonization of fibrolytic bacteria in the AHR (where a more pronounced pH reduction was observed) and ultimately decreased the degradation efficiency of the incubated forages. This finding raises questions about the role of pH in the rumen environmental conditions. For CSR, the rumen was enriched with a greater number of fibrolytic bacteria (and possibly fungi commonly found in the rumen of animals fed low-quality forage that has higher fiber content, lower digestibility and nutritional value) and was freely suspended in the rumen fluid. Fungal enrichment in low-quality forage is known to reduce the internal tension of plant cell walls through the growth of hyphae, which separates the lignified fibrous tissue and exposes the substrate to the adhesion of fibrolytic bacteria [37, 38].

Moreover, bacteria adhering to the forage surface were significantly affected by individual variations among sheep, which biased the impact of time on the bacterial changes. In this context, no pattern or regularity was observed for bacterial composition over the examined time points, which is consistent with previous reports showing strong host-specific effects on rumen microbial dynamics [5, 6, 30]. Despite the individual variations among animals, differences in the rates of forage degradation were small, which may be related to functional redundancy in the rumen microbiota [39].

The predominant rumen cellulolytic bacteria, such as *Fibrobacter,* lack flagella or cilia and are nonmotile, which limits the rates of bacterial attachment to substrates [3], suggesting that the initial attachment to feed particles relies mainly on the magnitude of the free-suspended cellulolytic population. The first 0.5 h of AH fermentation under CSR in the present study showed a *greater* abundance of Fibrobacter than under AHR. However, previous reports have demonstrated that differences in the abundance of *Fibrobacter* are significant at 16 h post-feeding of alfalfa hay and rice straw in cows [6, 30]. Metagenomic investigations of fresh perennial ryegrass revealed that adherent bacteria lack cellulolytic activity during early incubation (within the first 4 h) [9], demonstrating the importance of increasing the rate of cellulose degradation at the beginning of fermentation. The PCoA results showed that the rumen environment affected the bacterial composition more significantly than the incubated forage type, suggesting that manipulating the rumen environment could be a potential strategy for enhancing bacterial adhesion and accelerating the colonization of feed by fibrolytic bacteria.

The possible reasons why CSR is more favorable for forage degradation than AHR include different factors, such as a less fluctuating pH, greater bacterial diversity, and a faster colonization rate in the initial stages of incubation promoted by fibrolytic bacteria. However, limiting factors still exist in the initial stages of forage degradation that need to be overcome. Thus, the CS rumen environmental condition, which plays a decisive role in microbial colonization of forage, can be optimized. For instance, adjusting feed formulas by decreasing the proportion of less fermentable carbohydrates and providing feed additives, such as live fungal cultures at appropriate time points, to enhance forage degradation [40]. Combining high-quality and low-quality forages offers a promising strategy to prevent nutritional disorders while improving forage degradation efficiency in ruminants. Additionally, early dietary interventions for young animals may support rumen maturation, fostering optimal conditions to enhance its function [41, 42].

It is important to note that the primer sets used in this study are universal for bacteria and may introduce bias in archaeal sequence amplification. Nonetheless, the relative abundance data for archaea can still provide insights into general abundance trends. It is worth noting that the ratio of archaea to total bacteria increased and peaked two hours after incubation, a finding that aligns with the research of Piao, et al. [30], who also observed a peak in archaeal abundance in the rumen two hours after switchgrass incubation. This pattern likely reflects the rapid degradation of readily fermentable polysaccharides and the resulting accumulation of hydrogen (H_2) in the rumen. Elevated H_2 partial pressure is known to inhibit polysaccharide hydrolysis by rumen bacteria and fungi. The subsequent proliferation of methanogens, which

consume H_2 through hydrogenotrophic methanogenesis, helps to relieve polysaccharide hydrolysis inhibition. By lowering H_2 concentrations, methanogens contribute to restoring conditions favorable for fibrolytic microbial activity and continued fermentation, particularly under substrates rich in fermentable carbohydrates such as AHR [43, 44].

Methanobrevibacter exhibited a greater abundance in the samples incubated in the corn stover rumen environmental condition (AH-CSR and CS-CSR) than did its counterpart, *Methanosphaera*. *Methanobrevibacter* belongs to a group of archaea that recycle H_2 by combining it with formic acid or carbon dioxide to produce methane, and this finding has been reported in the rumen of cows that were fed high-fiber diets containing wheat stover as the primary forage [45]. *Methanospharea* use methanol as the primary carbon source, the end-product of pectin microbial fermentation in the rumen [46]. The high pectin content in AH possibly favored *Methanosphaeara* growth in relation to that of other methanogens. The differences in the archaeal composition observed in the examined rumen environmental conditions may ultimately influence the degradation efficiency of forage biomasses and, consequently, methane production.

While in situ incubation is widely used for estimating forage degradation rates in the rumen, forage particles in nylon bags do not undergo real mastication, rumination, or passage through the gastrointestinal tract [14]. Due to these limitations, indices such as total digestible nutrients and net energy, which directly reflect the energy supply to animals under the influence of the rumen environment, cannot be evaluated. Therefore, further investigations are needed to verify the superiority of CSR over AHR for forage degradation in terms of total tract digestibility. In addition to the measures of significant fermentation products, such as VFAs and pH, other microbial metabolites and plant chemicals (e.g., phenolic compounds) are also critical in determining the colonization process of forage-adhering microbes and thus need to be further investigated to uncover the interplay between rumen metabolites and microbial colonization [2, 9].

Conclusion

This study highlights the significant impact of rumen environmental conditions on microbial colonization and forage degradation dynamics. Compared to the alfalfa hay feeding, corn stover accelerated the degradation of NDF and ADF in *in-sacco*-incubated-forages within the first 16 h. This acceleration was driven by a greater abundance of free-suspended fibrolytic bacteria (such as *Fibrobacter*, *Butyrivibrio* 2, and *Pseudobutyrivibrio*), a more diverse bacterial composition, and a more stable rumen pH, which also influenced the forage-adherent bacterial population. Corn stover feeding further minimized differences in microbial adherence on incubated forages between alfalfa hay and corn stover. These findings suggest that optimizing the early-stage colonization of fibrolytic microbes could address key limitations in forage degradation efficiency. Moreover, enriching the rumen environment with low-quality forages may promote the growth of specialized microbes in deconstructing lignocellulosic components, and improve the overall efficiency of ruminant diets. It is worth noting that the 48-h in situ incubation period may not fully capture the plateau phase of fiber degradation, highlighting the need for longer incubation periods (>72 h) to better understand the degradation kinetics of recalcitrant forages. Additionally, as a future direction, the use of updated microbial taxonomy databases, such as Silva 138, could enhance the resolution of taxonomic classification, offering deeper insights into rumen microbial dynamics and their functional implications.

Declaration

Competing interests

The authors declare no competing interests.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-025-00407-x.

Additional file 1. Relative abundance of forage-adherent bacterial communities at the phylum and genus levels over time.

Additional file 2. Venn diagrams of differential bacterial genera across forage types and rumen environments.

Additional file 3. Supplementary tables detailing diet composition, forage degradation, and microbial community analyses.

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Author contributions

XX, JW, JL, and LG conceived and designed the study. XX performed the animal feeding and laboratory experiments, analyzed the sequencing data, interpreted the data, prepared the figures and tables, and wrote the draft manuscript. ALAN was involved in the data processing and analysis with XX. ALAN and XX wrote the final version of the manuscript. JW, JL, and LG also contributed to the writing of the manuscript. All the authors have read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files. The raw data from the 16S rRNA amplicon sequencing has been deposited in the Genome Sequence Archive in China National Center for Bioinformatics (CNCB) with the accession No. CRA017708.

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