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Effects of the Addition of Rumen Fluid and Molasses on the In Vitro Rumen Digestion Characteristics of *Caragana korshinskii* Kom. Silage

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Abstract

This study aimed to investigate the effects of molasses and rumen fluid addition on in vitro rumen fermentation, nutrient digestibility, and microbial community structure in *Caragana korshinskii* Kom. silage. The artificial rumen method was adopted to determine the in vitro rumen digestion indicators of four different kinds of silage: silage without additives (CK), silage with 7% rumen fluid (R), silage with 4% molasses (M), and silage with both 7% rumen fluid and 4% molasses (RM). Samples from each treatment were taken at days 15 and 60 to investigate the changes during ensiling. As for the 60-day silage, the rumen gas production and gas production from the insoluble but fermentable fraction were significantly lower in the R and RM groups than in the CK group ($p < 0.05$). Moreover, the methane production in the R group was the lowest. As for both the 15-day silage and 60-day silage, the M group exhibited a significantly higher gas production rate relative to the CK and R groups ($p < 0.05$). The digestibility of neutral detergent fiber and acid detergent fiber was significantly reduced in the M and RM treatments compared with the CK and R treatments. Conversely, the M and RM treatments had significantly lower propionate concentration but a significantly higher acetate-to-propionate ratio than the CK and R treatments ($p < 0.05$). R treatment exhibited greater relative *Prevotella* abundance than M and RM treatments ($p < 0.05$). In the 60-day silage, the M and RM treatments had higher *Methanobrevibacter* abundance but lower *Methanosphaera* abundance than CK treatment ($p < 0.05$). Silage with molasses reduced the dry matter, NDFD and ADFD, and reduced the total volatile fatty acids and the propionate content, but increased the A/P and CH_4 production. In contrast, silage with rumen fluid had a relatively small impact on rumen TVFA, NDFD and ADFD, but increased the dry matter digestibility and reduced the GP and CH_4 content.

Keywords: *Caragana korshinskii* Kom. silage; molasses; rumen fluid; in vitro digestibility

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1. Introduction

Caragana korshinskii Kom. (*C. korshinskii*), a small-leaved leguminous shrub rich in nutrients and various bioactive compounds, is widely distributed throughout arid and semiarid regions of northern China [1]. Its deep root system prevents wind erosion and promotes sand fixation for soil and water conservation [2], driving its extensive cultivation in pasturelands of northern China. The crude protein content of *C. korshinskii* during its growth stage is as high as that of fresh alfalfa [3,4], but the bitterness of tannins and the sharp thorns on the stem reduce its palatability and feed intake [5]. Silage is an effective pretreatment and preservation method for roughage, which can soften the thorns of *C. korshinskii*, produce aromatic odors, and improve palatability [5]. Molasses can effectively solve the buffering effect of the high protein content of *C. korshinskii* on pH and enhance the energy value of silage [6,7] by supplying soluble carbohydrates to lactic acid bacteria while suppressing fermentation by undesirable microbes [8]. In recent years, rumen fluid, with a more complex microbial community, including bacteria, methanogenic archaea and fungi, exhibits a higher ability to degrade lignocellulosic biomass than other normal anaerobic microorganisms [9]. However, relatively few studies have investigated the rumen degradation pattern and the changes in nutritional value by adding rumen fluid or a combination of rumen fluid and molasses to *C. korshinskii* silage. Therefore, this study aimed to investigate the effects of molasses and rumen fluid addition on in vitro rumen fermentation parameters, nutrient digestibility, and microbial community composition of *C. korshinskii* silage, with the goal of improving its digestibility and nutritional value. We further hypothesized that the complex microbial community in rumen fluid might exert a "pre-digestion" effect during ensiling and promote nutrient degradation, whereas molasses might provide abundant soluble carbohydrates to weaken the buffering effect of *C. korshinskii* protein on pH and facilitate a decrease in silage pH.

2. Materials and Methods

2.1. Ensiling and Sample Collection

C. korshinskii was harvested in September 2024 from a plantation in cooperation with Inner Mongolia Minzu University (longitude 122°11′22″–122°20′48″ E, latitude 43°33′59″–43°38′54″ N). Plant material harvested from 10 cm above the ground was wilted in the field for approximately 2 h. Subsequently, it was chopped into 1–2 cm lengths using a forage chopper and thoroughly mixed to ensure sample homogeneity. Four silage treatments were prepared: silage without additives (CK), silage with 7% rumen fluid (R), silage with 4% molasses (M), and silage with both 7% rumen fluid and 4% molasses (RM). The rumen fluid and molasses were diluted with a small amount of sterilized distilled water and then mixed uniformly with the chopped forage; the CK group received the same amount of sterilized distilled water to ensure comparable moisture content across all treatments. Fresh material (300 g) was sealed in specialized plastic bags with three replicates per treatment, and silage was stored in the dark at room-temperature (25 °C ± 2 °C) and sampled on days 15 (CK15, R15, M15, and RM15) and 60 (CK60, R60, M60, and RM60). Rumen fluid was obtained from three adult female Simmental crossbred cattle (fed a 4:6 concentrate-to-roughage diet) via an esophageal tube prior to morning feeding. A total of 2 L of rumen fluid was collected (discarding the initial 300 mL to avoid saliva contamination), pooled, filtered through four layers of cheesecloth, and immediately transferred to an anaerobic incubator at 39 °C for use as an inoculant. Molasses (42% sugar, Brix $\geq$ 60%) were obtained from Weifang Fengguan Biotechnology Co., Ltd., Weifang, China.

2.2. In Vitro Rumen Fermentation Profile and Gas Production

The in vitro rumen fermentation and gas production were evaluated using the artificial rumen method. The artificial rumen mixture I was prepared according to Menke and Steingass (1988) [10], consisting of a 1:2 volume ratio of rumen fluid (from the same donor animals described in Section 2.1) to buffer solution. The mixture was maintained at 39 °C under continuous CO_2 flushing to ensure anaerobic conditions.

For each replicate (n = 3), approximately 220 mg of the aforementioned silage sample (oven-dried at 70 ± 2 °C) was weighed into an individual 100 mL graduated glass syringe (Häberle, Germany), mixed with 30 mL of artificial rumen mixture I, and incubated in a shaking water bath (Suzhou Peiying Equipment Co., Ltd., Suzhou, China) at 39 °C and 120 rpm in order for fermentation to proceed. Gas production was recorded at 0, 0.5, 1, 2, 4, 6, 8, 12, 16, 24, 36, and 48 h of incubation. Blank incubations without substrate were included in parallel and treated in the same manner as the samples. Gas production values were corrected for gas originating from the inoculum by subtracting the corresponding blank values.

Rumen fermentation gas was collected individually into 200 mL aluminum foil bags (Shanghai Huibin Instrument Co., Ltd., Shanghai, China), and the proportions of methane (CH_4) and carbon dioxide (CO_2) were quantified using gas chromatography (TP-2060T; Beijing Analytical Instrument Co., Ltd., Beijing, China). Gas production kinetics were evaluated according to the method of Ørskov and McDonald (1979) [11]:

$$GP = A + B \times \left(1 - e^{-c(t-lag)}\right), \quad (1)$$

where GP is the gas production (mL/200 mg DM); A is the immediately soluble fraction; B is the insoluble but fermentable fraction; c is the gas production rate (h^{-1}); lag is the lag time (h), and t is the incubation time (h).

2.3. Determination of Rumen Fermentation Parameters and Microbial Crude Protein

At 48 h of incubation, all samples for pH, MCP, NH_3-N, and VFA analyses were collected. The pH, MCP, and NH_3-N analyses were performed immediately after collection. The pH value was measured using a portable digital pH meter (Testo 205, Titisee-Neustadt, Germany). A 5 mL aliquot of the fermentation mixture was used to determine the microbial crude protein (MCP) concentration according to the method of Sedmak et al. (1977) [12], while another 5 mL was transferred into a cryovial and stored at −80 °C for subsequent microbial community analysis. The fermentation mixture was then centrifuged (8000 rpm, 4 °C, 15 min), and the supernatant was analyzed for ammonia nitrogen (NH_3-N) according to the method of Verdouw et al. (1978) [13]. For VFA analysis, metaphosphoric acid was added to the samples, and the samples were stored frozen. VFA analysis followed the method of Erwin et al. (1977) [14].

2.4. In Vitro Ruminal Digestibility Determination

Artificial rumen mixture II (rumen fluid: buffer solution = 1:1, v/v) was prepared according to the first step of the method of Tilley and Terry (1963) [15]. Only the ruminal incubation stage was performed for 48 h, and the acid–pepsin second stage was not included. The ruminal digestibilities (ND:DMD, NDFD and ADFD) were calculated using the following formula:

$$\mathrm{ND} = \frac{m - (m_2 - m_1 \times c)}{m} \times 100\%, \quad (2)$$

where ND is the in vitro ruminal digestibility (%); m is the nutrient weight of the sample (g); m_1 is the DM weight of the empty bag (g); m_2 is the DM weight of the bag plus residue after

incubation (g); and *c* is the bag correction factor of bag (the DM weight after incubation/the DM weight before incubation).

Dry matter (DM) was determined by oven-drying at 70 ± 2 °C. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured using a semi-automated fiber analyzer (ANKOM A200I; ANKOM Technology, Macedon, NY, USA) according to the procedures described by Van Soest (1991) [16].

2.5. Rumen Microbial Community Analysis

2.5.1. DNA Extraction and PCR Amplification

Frozen samples of rumen fluid were defrosted on ice under sterile conditions, and total genomic DNA was extracted via the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instructions. The V3–V4 regions of the bacterial 16S rRNA gene were amplified via the primers 338F and 806R, while the archaeal V4–V5 regions were amplified using primers 524F10extF and Arch958RmodR. Amplicons were subsequently purified and quantified via a Qubit 4.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Library preparation was carried out using the NEXTFLEX Rapid DNA-Seq Kit, followed by sequencing runs on an Illumina NextSeq 2000 platform (Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China).

2.5.2. Amplicon Sequence Processing and Analysis

Raw sequencing reads were first subjected to strict quality control and filtering using fastp. Sequences containing adapters, excessive unknown bases, or low quality were removed, and chimeric sequences were also discarded, ultimately yielding 1,293,091 high-quality effective sequences from all samples, with an average of 53,879 (range: 42,245–67,138) sequences per sample. To evaluate the reliability of the sequencing data, rarefaction curves were generated and Good's Coverage indices were calculated (made greater than 99% to ensure that the sequencing depth was sufficient to cover the microbial diversity in the sample), indicating that the sequencing depth was sufficient to capture the microbial diversity within the samples. Taxonomic assignment of ASVs was performed using the Naive Bayes consensus taxonomy classifier implemented in QIIME 2 and the SILVA 16S rRNA database (v138). The sequence counts for each taxon were then standardized to relative abundances to eliminate sequencing depth bias.

2.5.3. Microbial Diversity Analysis

The compositional structure of the microbial community was assessed through the online interface of Majorbio Cloud, https://cloud.majorbio.com (accessed on 14 November 2024). For diversity analysis, α-diversity indices, including Chao1 and ACE (richness indices) and Shannon and Simpson (diversity indices), were calculated, and inter-group differences were evaluated using the Kruskal–Wallis test. Meanwhile, principal coordinate analysis (PCoA) was conducted based on the Bray–Curtis distance matrix, and PERMANOVA was used to report the statistical significance of community differences among treatments. Furthermore, to identify biomarkers with significant differences between groups, LEfSe analysis was performed, with LDA score threshold of >3.5 for bacterial taxa and >2 for archaeal taxa set to screen for differentially abundant taxa ($p < 0.05$).

2.6. Statistical Analysis

A two-factor experimental arrangement (four levels by two levels) was adopted. Raw data were initially handled using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA), followed by statistical evaluation conducted with SPSS version 19.0 (SPSS

Inc., Chicago, IL, USA). Analysis of variance and multiple comparisons were performed employing the general linear model (GLM) combined with Tukey's post hoc test:

$$Y_{ij} = \mu + T_i + D_j + (T \times D)_{ij} + e_{ij}, \quad (3)$$

where Y_{ij}, the observation; μ, the overall mean; T_i, the treatment effect (i = 1, 2, 3, 4); D_j, the effect of ensiling duration (j = 1, 2); $(T \times D)_{ij}$, the interaction between treatment and ensiling duration; and e_{ij}, the random error. Statistical significance was declared when the p Value fell below 0.05.

2.7. Ethics Approval

The animal experiment protocol complies with the relevant laws, regulations and standards concerning animal welfare ethics. This project has been supervised and approved by the Laboratory Animal Ethics Committee of Inner Mongolia Minzu University. (IACUC Issue No. 2024060116001).

3. Results

3.1. Main Nutrient Composition of *C. korshinskii*

Table 1 shows that the M15 and M60 groups had significantly lower DM content than the CK group ($p < 0.05$). Furthermore, at both 15-day and 60-day silages, all treatment groups showed reduced NDF and ADF contents compared with the CK groups ($p < 0.05$). A significant treatment × time interaction was observed for DM and ADF ($p < 0.05$), but not for NDF ($p > 0.05$).

Table 1. Nutrient composition of *C. korshinskii* silage across treatments and ensiling periods.

Items	Times	Groups				SEM	*p* Value		
		CK	R	M	RM		T	D	T × D
DM g/kg	15 d	429.92 ^{a}	433.29 ^{a}	410.69 ^{b}	423.96 ^{ab}	3.453	<0.001	0.043	0.032
	60 d	432.99 ^{a}	427.07 ^{a}	407.22 ^{b}	418.87 ^{ab}				
NDF g/kg DM	15 d	511.72 ^{a}	471.48 ^{Ab}	435.91 ^{Ac}	429.62 ^{c}	6.861	<0.001	<0.001	0.093
	60 d	501.67 ^{a}	455.47 ^{Bb}	422.34 ^{Bc}	425.75 ^{c}				
ADF g/kg DM	15 d	463.17 ^{a}	445.81 ^{Ab}	409.10 ^{Ac}	402.55 ^{c}	5.965	<0.001	<0.001	<0.001
	60 d	466.28 ^{a}	416.04 ^{Bb}	391.61 ^{Bc}	395.06 ^{c}				

CK, silage without additives; R, silage with rumen fluid; M, silage with molasses; and RM, silage with both rumen fluid and molasses. DM, dry matter; NDF, neutral detergent fiber; ADF, acid detergent fiber. Different lowercase letters in the same row indicate significant differences among treatments at the same ensiling time ($p < 0.05$); different uppercase letters in the same column indicate significant differences between ensiling times ($p < 0.05$); identical or no letters indicate no significant difference ($p > 0.05$).

3.2. Gas Production Dynamics During In Vitro Rumen Fermentation

As shown in Figure 1, GP in all groups plateaued at 48 h, indicating the completion of fermentation. Table 2 shows that 15-day silages generally exhibited higher GP than 60-day silages. Specifically, R60 and RM60 had lower GP than CK60 ($p < 0.05$). Notably, R60 displayed significantly lower GP from the immediately soluble fraction (A fraction) compared with CK60, M60 and RM60, and exhibited the longest lag phase, which was significantly longer than that of RM60 ($p < 0.05$). GP from the insoluble but fermentable fraction (B) was significantly lower in RM15 than in CK15, and was also lower in all 60-day silages compared to CK60 ($p < 0.05$). Conversely, M15, M60, and RM60 showed a higher gas production rate (c) but significantly greater in vitro ruminal CH_4 proportion than the

CK groups ($p < 0.05$). A significant treatment × time interaction was observed for GP, A, B, c and CH_4 proportion ($p < 0.05$), but not for lag ($p > 0.05$).

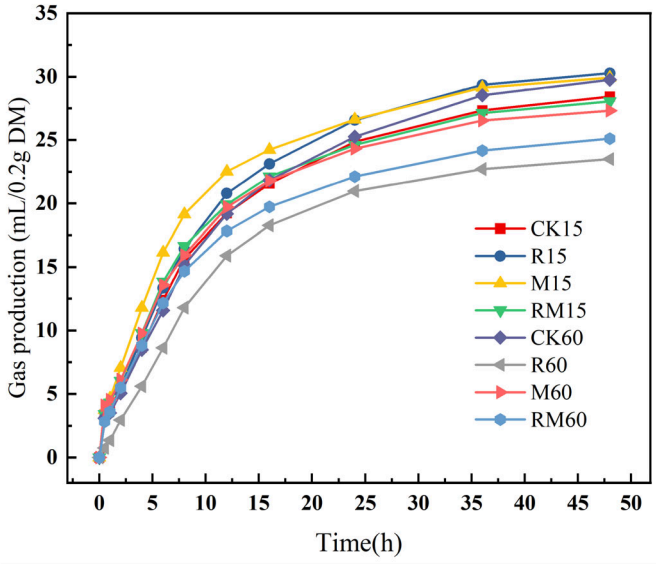

Figure 1. Cumulative gas production over 48 h during in vitro fermentation of *C. korshinskii* silage under different treatments.

Table 2. Kinetic parameters of gas production during 48 h of in vitro fermentation of *C. korshinskii* silage.

Items	Times	Groups				SEM	*p* Value		
		CK	R	M	RM		T	D	T × D
GP (mL)	15 d	28.47	30.28 ^{A}	29.39 ^{A}	28.04 ^{A}	0.505	0.002	<0.001	<0.001
	60 d	29.76 ^{a}	23.50 ^{Bc}	27.20 ^{Bab}	24.79 ^{Bbc}				
A (mL)	15 d	3.70	3.33 ^{A}	3.95	3.63	0.217	0.016	<0.001	0.013
	60 d	3.46 ^{a}	1.14 ^{Bb}	4.39 ^{a}	2.94 ^{a}				
B (mL)	15 d	26.23 ^{ab}	28.73 ^{Aa}	26.59 ^{Aab}	25.34 ^{b}	0.444	<0.001	<0.001	<0.001
	60 d	27.95 ^{a}	23.59 ^{Bb}	23.81 ^{Bb}	22.89 ^{b}				
c (h^{-1})	15 d	0.09 ^{Ab}	0.09 ^{b}	0.12 ^{Aa}	0.10 ^{b}	0.002	0.007	<0.001	0.005
	60 d	0.08 ^{Bb}	0.10 ^{a}	0.10 ^{Ba}	0.10 ^{a}				
Lag (h)	15 d	1.05	0.95	0.77	0.74	0.063	0.129	0.026	0.417
	60 d	1.17 ^{a}	1.34 ^{a}	1.03 ^{ab}	0.64 ^{b}				
CH_4 (%)	15 d	10.99 ^{bc}	10.47 ^{Bc}	12.98 ^{a}	11.86 ^{Bb}	0.25	<0.001	0.029	0.002
	60 d	10.76 ^{c}	11.35 ^{Abc}	12.38 ^{b}	13.93 ^{Aa}				
CO_2 (%)	15 d	85.78	85.16	84.84	85.93	0.301	0.419	0.253	0.16
	60 d	86.50 ^{a}	87.52 ^{a}	85.88 ^{ab}	84.48 ^{b}				
CH_4 (mL)	15 d	3.13 ^{b}	3.17 ^{Ab}	3.82 ^{a}	3.32 ^{ab}	0.074	0.001	0.059	0.043
	60 d	3.21 ^{ab}	2.67 ^{Bb}	3.37 ^{a}	3.45 ^{a}				
CO_2 (mL)	15 d	24.43	25.78 ^{A}	24.94	24.10 ^{A}	0.432	0.004	<0.001	0.001
	60 d	25.75 ^{a}	20.56 ^{Bc}	23.37 ^{ab}	20.94 ^{Bbc}				

GP, cumulative gas production at 48 h; *A*, gas production from the immediately soluble fraction; *B*, gas production from the insoluble but fermentable fraction; *c*, the gas production rate (h^{-1}); and *lag*, lag time (h). Different lowercase letters in the same row indicate significant differences among treatments at the same ensiling time ($p < 0.05$); different uppercase letters in the same column indicate significant differences between ensiling times ($p < 0.05$); identical or no letters indicate no significant difference ($p > 0.05$).

3.3. In Vitro Ruminal Digestibility of *C. korshinskii* Silage

Table 3 shows that DMD was increased in R15, M15, and R60 ($p < 0.05$), whereas NDFD and ADFD were decreased in all M and RM treatments ($p < 0.05$). However, R15 showed a higher ADFD than the other groups ($p < 0.05$). A significant treatment × time interaction was observed for DMD, NDFD and ADFD ($p < 0.05$).

Table 3. Effects of 48 h of in vitro fermentation on the ruminal digestibility of *C. korshinskii* silage.

Items	Times	Groups				SEM	*p* Value		
		CK	R	M	RM		T	D	T × D
DMD (%)	15 d	52.94 ^{Bb}	58.62 ^{Ba}	57.59 ^{a}	54.22 ^{b}	0.515	<0.001	<0.001	<0.001
	60 d	58.42 ^{Aab}	60.38 ^{Aa}	56.02 ^{bc}	54.98 ^{c}				
NDFD (%)	15 d	25.00 ^{Ba}	27.27 ^{a}	19.04 ^{Ab}	11.29 ^{c}	1.478	<0.001	0.082	<0.001
	60 d	28.61 ^{Aa}	25.80 ^{a}	13.41 ^{Bb}	10.70 ^{b}				
ADFD (%)	15 d	20.35 ^{Bb}	25.80 ^{a}	16.12 ^{Ac}	7.81 ^{Ad}	1.551	<0.001	0.068	<0.001
	60 d	25.85 ^{Aa}	20.97 ^{a}	8.29 ^{Bb}	8.28 ^{Ab}				

DMD, dry matter digestibility; NDFD, neutral detergent fiber digestibility; ADFD, acid detergent fiber digestibility. Different lowercase letters in the same row indicate significant differences among treatments at the same ensiling time ($p < 0.05$); different uppercase letters in the same column indicate significant differences between ensiling times ($p < 0.05$); identical or no letters indicate no significant difference ($p > 0.05$).

3.4. In Vitro Rumen Fermentation Parameters of *C. korshinskii* Silage

As summarized in Table 4, ruminal pH was significantly higher in R15 and M15 ($p < 0.05$). NH_3-N and MCP concentrations increased significantly in R15, M15 and RM15 ($p < 0.05$), with R60 exhibiting even greater NH_3-N levels than R15 ($p < 0.05$). In the 15-day silage, total volatile fatty acids (TVFAs) and individual VFAs (except butyrate) decreased in the M group ($p < 0.05$), whereas in the 60-day silage, only propionate and branched-chain VFAs were reduced. The RM group showed a similar reduction pattern in branched-chain VFAs. Notably, for the 15-day silage, acetate and propionate did not show significant decreases, while the TVFA of 60-day silage decreased significantly ($p < 0.05$). Furthermore, the acetate-to-propionate (A/P) ratio followed the order: R15 and R60 < CK15 and CK60 < M15 and M60 ($p < 0.05$). A significant treatment × time interaction was observed for NH_3-N, MCP, TVFA, Acetate, Propionate, Butyrate, Isobutyrate, Valerate and Isovalerate ($p < 0.05$), but not for pH and A/P ($p > 0.05$).

Table 4. Fermentation parameters of *C. korshinskii* silage after 48 h of in vitro rumen fermentation.

Items	Times	Groups				SEM	*p* Value		
		CK	R	M	RM		T	D	T × D
pH	15 d	6.88 ^{b}	6.94 ^{a}	6.97 ^{a}	6.93 ^{ab}	0.007	0.007	0.934	0.057
	60 d	6.91	6.92	6.93	6.96				
NH_3-N (mg/dL)	15 d	18.11 ^{Bc}	19.32 ^{Bb}	20.11 ^{ab}	20.84 ^{a}	0.221	0.013	<0.001	0.002
	60 d	20.00 ^{A}	20.85 ^{A}	20.9	20.58				
MCP (mg/dL)	15 d	10.75 ^{Bb}	14.26 ^{a}	13.95 ^{a}	14.51 ^{a}	0.513	0.003	0.044	0.008
	60 d	13.91 ^{A}	14.03	14.64	14				
TVFA (mmol/L)	15 d	49.78 ^{Ba}	50.76 ^{a}	44.59 ^{Bb}	52.02 ^{Aa}	0.591	<0.001	<0.001	<0.001
	60 d	53.97 ^{Aa}	52.65 ^{ab}	51.76 ^{Aab}	50.57 ^{Bb}				

Table 4. *Cont.*

Items	Times	Groups				SEM	*p* Value		
		CK	R	M	RM		T	D	T × D
Acetate (mmol/L)	15 d	31.79 ^{Bb}	32.05 ^{b}	28.90 ^{Bc}	33.98 ^{Aa}	0.343	0.002	<0.001	<0.001
	60 d	33.70 ^{A}	32.61	33.32 ^{A}	32.82 ^{B}				
Propionate (mmol/L)	15 d	11.29 ^{Bab}	11.76 ^{a}	9.43 ^{Bc}	11.12 ^{Ab}	0.209	<0.001	<0.001	<0.001
	60 d	12.74 ^{Aa}	12.37 ^{a}	11.22 ^{Ab}	10.70 ^{Bb}				
Butyrate (mmol/L)	15 d	3.44 ^{Bb}	3.52 ^{Bb}	3.40 ^{Bb}	3.77 ^{a}	0.046	0.106	<0.001	0.009
	60 d	3.79 ^{A}	3.82 ^{A}	3.99 ^{A}	3.81				
Isobutyrate (mmol/L)	15 d	1.01 ^{Bb}	1.04 ^{Ba}	0.94 ^{Bd}	0.98 ^{Bc}	0.013	<0.001	<0.001	0.037
	60 d	1.12 ^{Aa}	1.13 ^{Aa}	1.02 ^{Ab}	1.01 ^{Ab}				
Valerate (mmol/L)	15 d	0.86 ^{Bab}	0.91 ^{Ba}	0.67 ^{Bc}	0.82 ^{b}	0.025	<0.001	<0.001	0.016
	60 d	1.02 ^{Aa}	1.05 ^{Aa}	0.80 ^{Ab}	0.83 ^{b}				
Isovalerate (mmol/L)	15 d	1.39 ^{Bb}	1.48 ^{Ba}	1.26 ^{Bc}	1.35 ^{b}	0.026	<0.001	<0.001	0.026
	60 d	1.59 ^{Aa}	1.66 ^{Aa}	1.41 ^{Ab}	1.40 ^{b}				
A/P	15 d	2.81 ^{Ab}	2.73 ^{Ab}	3.06 ^{a}	3.05 ^{a}	0.039	<0.001	<0.001	0.199
	60 d	2.65 ^{Bb}	2.63 ^{Bb}	2.97 ^{a}	3.07 ^{a}				

A/P, acetate-to-propionate ratio. Different lowercase letters in the same row indicate significant differences among treatments at the same ensiling time ($p < 0.05$); different uppercase letters in the same column indicate significant differences between ensiling times ($p < 0.05$); identical or no letters indicate no significant difference ($p > 0.05$).

3.5. Changes in In Vitro Rumen Fermentation Microbial Diversity of C. korshinskii Silage

3.5.1. Alpha Diversity Analysis

As shown in Tables 5 and 6, the sequencing coverage exceeded 99% in all the groups. Regarding the archaeal communities, the Simpson index varied significantly among the groups; specifically, the CK60 and R60 groups exhibited significantly lower values compared to the M60 and RM60 groups ($p < 0.05$).

Table 5. Effects of rumen fluid and molasses on the alpha diversity of bacterial communities during in vitro fermentation of *C. korshinskii* silage.

Items	Times	Group				SEM	*p* Value		
		CK	R	M	RM		T	D	T × D
ACE	15 d	1043.22	1296.96	1489.74	1526.68	45.412	0.122	0.807	0.225
	60 d	1310.93	1263.30	1408.68	1291.30				
Chao1	15 d	1023.19	1260.69	1456.67	1490.29	43.830	0.103	0.839	0.213
	60 d	1283.51	1231.23	1391.33	1259.42				
Shannon	15 d	4.89	5.17	6.01	5.72	0.110	0.046	0.696	0.331
	60 d	5.38	5.14	5.77	5.19				
Simpson	15 d	0.05	0.04	0.01	0.03	0.005	0.133	0.427	0.500
	60 d	0.04	0.05	0.02	0.05				
Coverage	15 d	1.00	0.99	0.99	0.99	—	—	—	—
	60 d	0.99	0.99	1.00	1.00				

Table 6. Effects of rumen fluid and molasses on the alpha diversity of archaeal communities during in vitro fermentation of *C. korshinskii* silage.

Items	Times	Group				SEM	*p* Value		
		CK	R	M	RM		T	D	T × D
ACE	15 d	29.00	30.33	28.67	32.00	0.810	0.983	0.527	0.597
	60 d	31.33	32.00	32.33	29.00				
Chao1	15 d	29.00	30.33	28.67	32.00	0.810	0.983	0.527	0.597
	60 d	31.33	32.00	32.33	29.00				
Shannon	15 d	2.12	2.27	2.20	2.18	0.021	0.417	0.431	0.296
	60 d	2.23	2.19	2.13	2.09				
Simpson	15 d	0.17	0.14	0.16	0.17	0.005	0.036	0.190	0.140
	60 d	0.15 ^{b}	0.16 ^{b}	0.18 ^{a}	0.19 ^{a}				
Coverage	15 d	1.00	1.00	1.00	1.00	—	—	—	—
	60 d	1.00	1.00	1.00	1.00				

Different lowercase letters in the same row indicate significant differences among treatments at the same ensiling time ($p < 0.05$); identical or no letters indicate no significant difference ($p > 0.05$).

3.5.2. Beta Diversity Analysis

As revealed by principal coordinate analysis (PCoA) (Figure 2), microbial communities separated distinctly, with PC1 and PC2 explaining 19.18% and 16.48% of the total variation for bacteria (Figure 2A), respectively. The samples from the CK and R groups clustered distinctly from those subjected to M and RM groups. For the archaeal communities (Figure 2B), PC1 and PC2 explained 50.80% and 20.89% of the total variation, respectively. Specifically, samples from the CK15, R15, and R60 groups clustered separately from those in the M15, M60, RM15, and RM60 groups. Community composition differed significantly among treatments for both bacteria ($p = 0.004$) and archaea ($p = 0.001$).

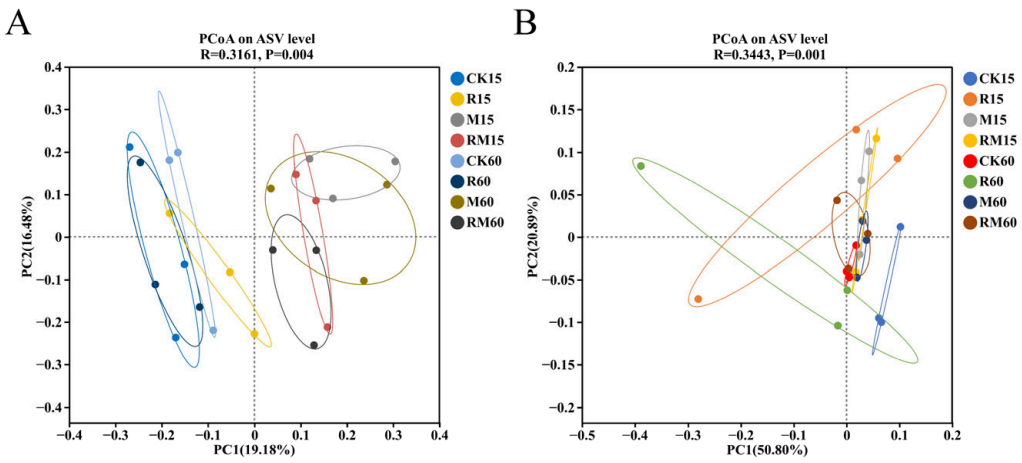

Figure 2. Principal coordinate analysis (PCoA) of bacterial and archaeal communities during in vitro fermentation of *C. korshinskii* silage with rumen fluid and molasses addition. (**A**) PCoA of bacterial communities during in vitro fermentation of *C. korshinskii* silage with rumen fluid and molasses addition; (**B**) PCoA of archaeal communities during in vitro fermentation of *C. korshinskii* silage with rumen fluid and molasses addition.

3.5.3. Taxonomic Composition Analysis

Taxonomic profiling indicated that Firmicutes (35.98%), Proteobacteria (27.08%), Bacteroidota (29.54%), Patescibacteria (2.56%), Actinobacteria (0.86%), Desulfobacterota (1.15%), and Verrucomicrobiota (0.52%) constituted the dominant bacterial phyla (Figure 3A). At the

genus level, the community was dominated by *Comamonas* (17.33%), U29-B03 (10.79%), *Prevotella* (10.89%), and *Succiniclasticum* (11.53%) (Figure 3B). Among archaeal communities, Euryarchaeota (99.87%) was identified as the dominant phylum (relative abundance > 1%; Figure 4A). At the genus level, *Methanobrevibacter* (88.92%) and *Methanosphaera* (10.96%) were the most prevalent, with relative abundances exceeding 1% (Figure 4B).

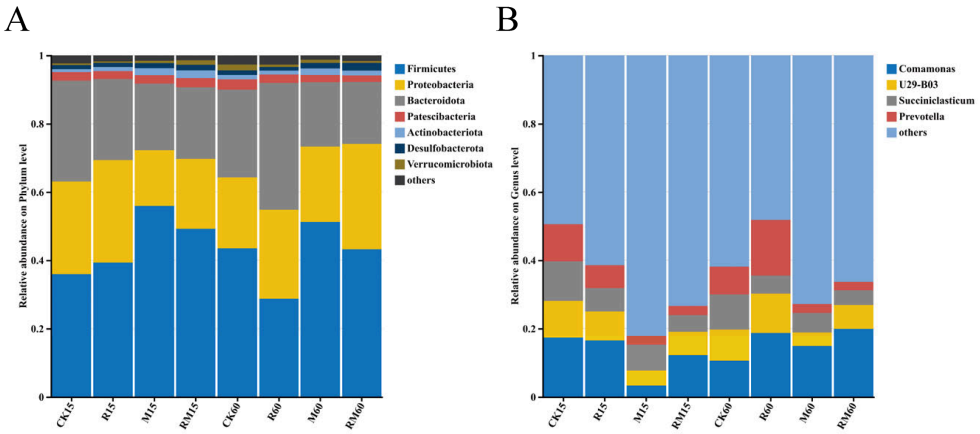

Figure 3. Composition of bacterial communities during in vitro fermentation of *C. korshinskii* silage with rumen fluid and molasses addition at the phylum and genus levels. (**A**) Bacterial community composition at the phylum level during in vitro fermentation of *C. korshinskii* silage with rumen fluid and molasses addition; (**B**) bacterial community composition at the genus level during in vitro fermentation of *C. korshinskii* silage with rumen fluid and molasses addition.

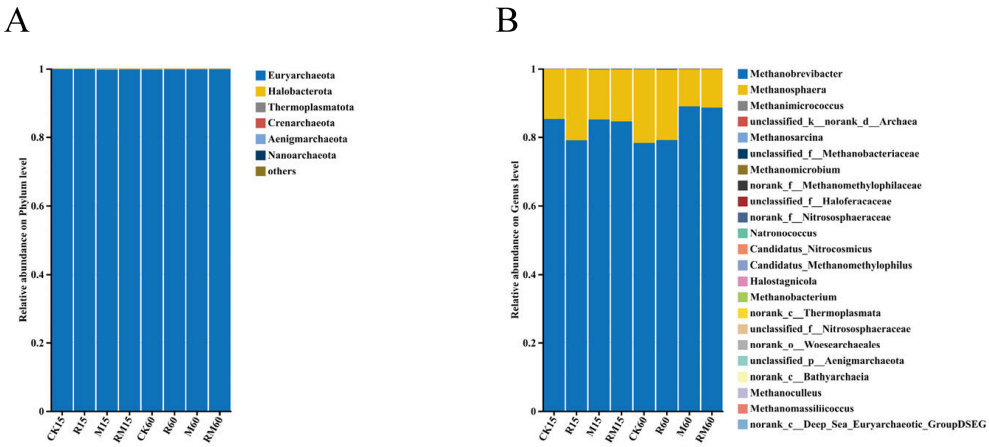

Figure 4. Composition of archaeal communities during in vitro fermentation of *C. korshinskii* silage, with rumen fluid and molasses addition at the phylum and genus levels. (**A**) Archaeal community composition at the phylum level; (**B**) archaeal community composition at the genus level.

As depicted in Figure 5, the molasses-treated groups were characterized by an enrichment of fiber-degrading bacteria (including NK4A214_group, Rikenellaceae_RC9_gut_group, Christensenellaceae_R-7_group, and Lachnospiraceae) and fermentative bacteria (including *Acetitomaculum*, norank_f_F082, and Lachnospiraceae_NK3A20_group). In contrast, *Prevotella* was more abundant in the rumen fluid-treated groups. The relative abundance of *Methanobrevibacter* in the M60 (89.04 ± 1.37%) and RM60 (88.64 ± 2.79%) groups was significantly higher than that in the CK60 (78.28 ± 1.25%) and R60 (80.32 ± 3.32%) groups, representing approximately 1.14-fold and 1.13-fold increases compared to the CK60 group, respectively ($p < 0.05$).

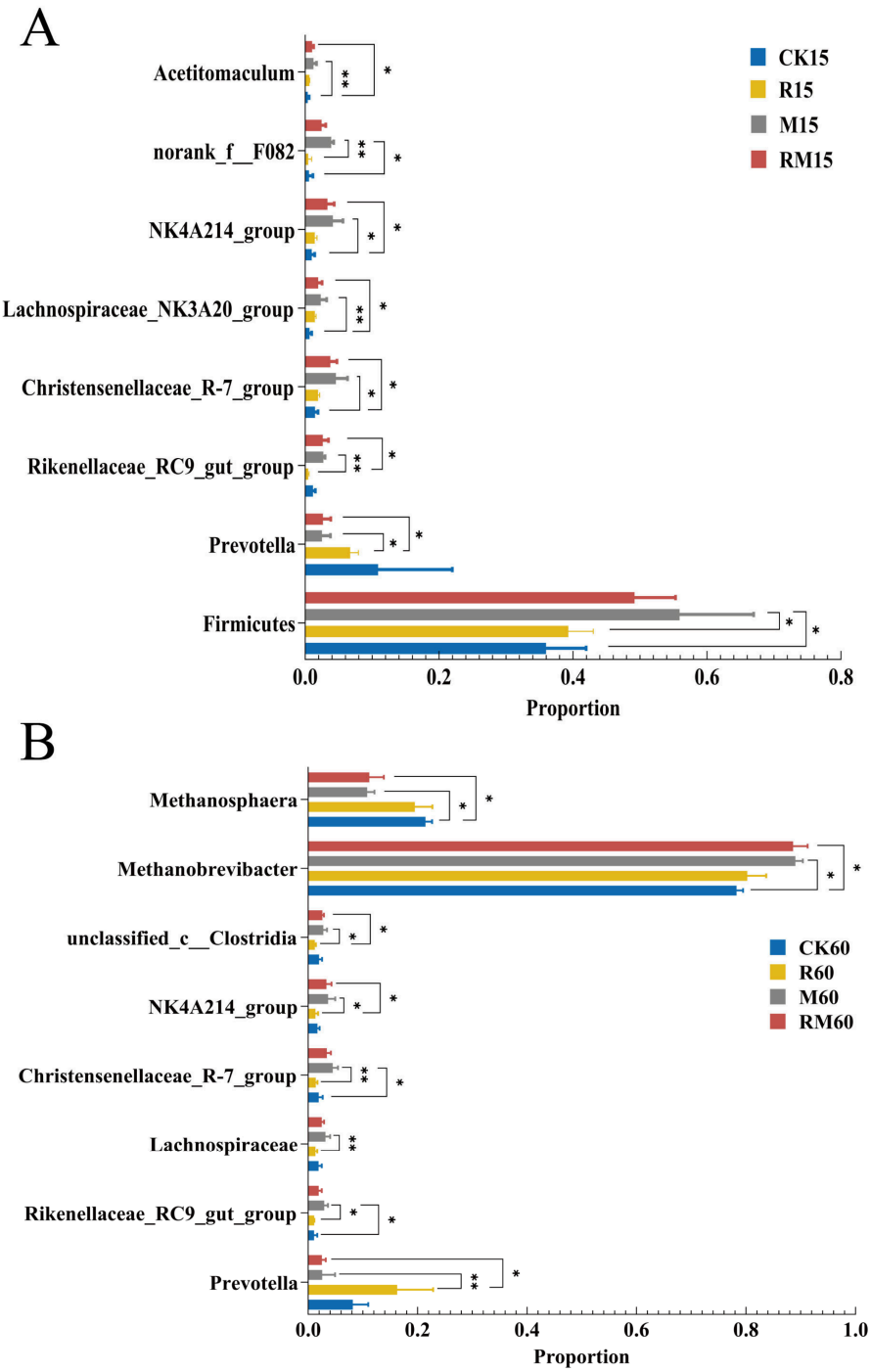

Figure 5. Multiple comparisons of rumen microbial communities during in vitro fermentation of *C. korshinskii* silage with rumen fluid and molasses addition. (**A**) Multiple comparisons of rumen microbial communities after 15 days of in vitro fermentation; (**B**) multiple comparisons of rumen microbial communities after 60 days of in vitro fermentation. * indicates $0.01 < p \leq 0.05$; ** indicates $0.001 < p \leq 0.01$.

3.5.4. Indicator Species Analysis

Linear discriminant analysis effect size (LEfSe) identified 30 bacterial indicator taxa ($p < 0.05$, LDA > 3.5) across groups (Figure 6). Ten taxa were significantly enriched in M15, including Firmicutes-related taxa, whereas the R60 group promoted the enrichment of *Prevotella* and *Fibrobacter*. Archaeal LEfSe analysis ($p < 0.05$, LDA > 2) revealed the enrichment of *Methanosphaera* in R15 and *Methanobrevibacter* in M60 (Figure 7).

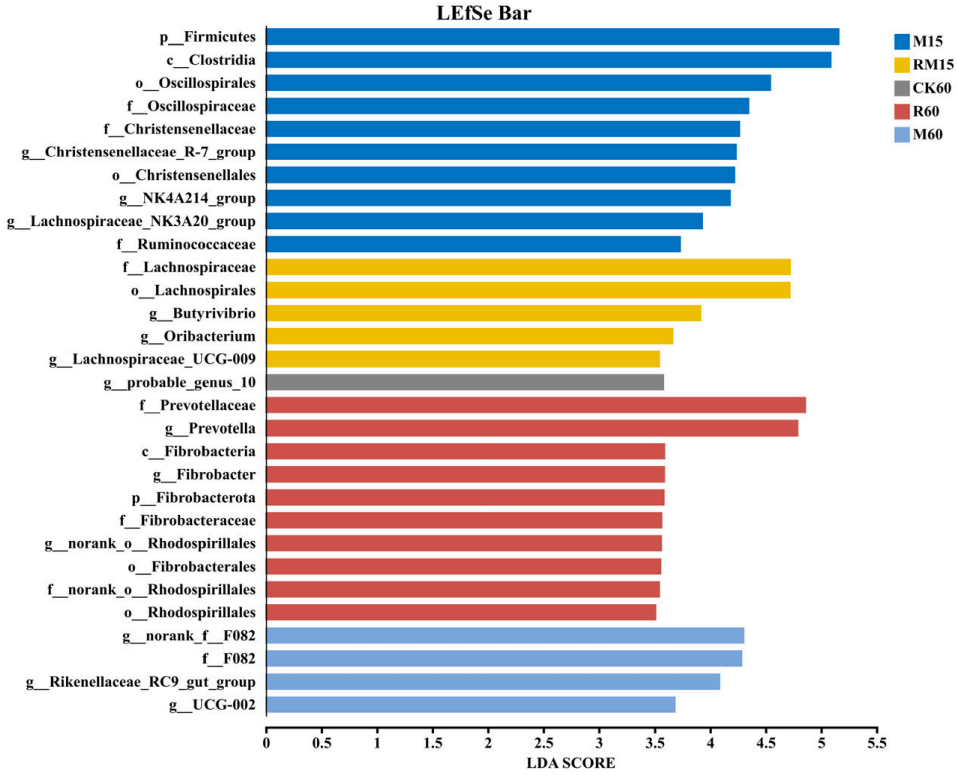

Figure 6. LEfSe analysis of bacterial communities during in vitro fermentation of *C. korshinskii* silage with rumen fluid and molasses addition.

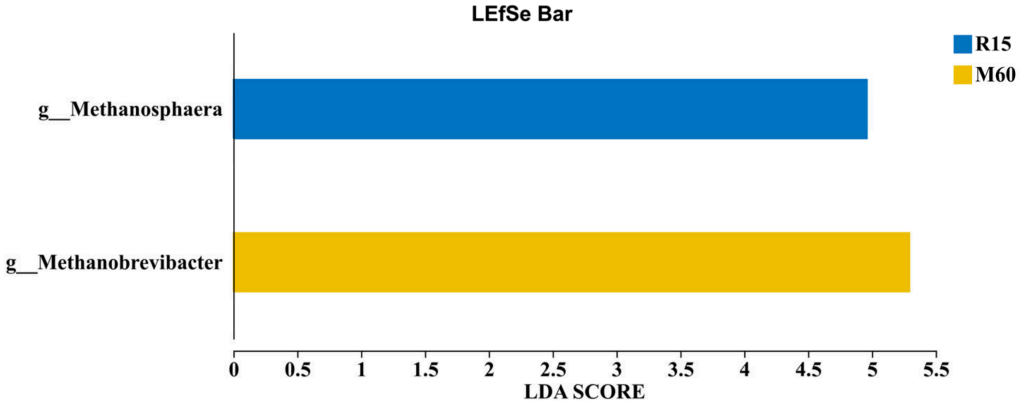

Figure 7. LEfSe analysis of archaeal communities during in vitro fermentation of *C. korshinskii* silage with rumen fluid and molasses addition.

3.5.5. Correlation Heatmap Analysis Between Rumen Microorganisms and Fermentation Parameters

As illustrated in Figures 8 and 9, fiber-degrading bacteria (e.g., *Ruminococcus* and NK4A214_group) and fermentative bacteria (e.g., Lachnospiraceae_NK3A20_group and *Acetitomaculum*) correlated positively with pH and negatively with propionate concentration. Conversely, *Prevotella* and related genera (including *Succinivibrio*, *Selenomonas*, *Succiniclasticum*, and *Veillonellaceae*_UCG-001) showed positive correlations with propionate. Additionally, the dominant archaeal genus *Methanosphaera* was positively correlated with propionate levels.

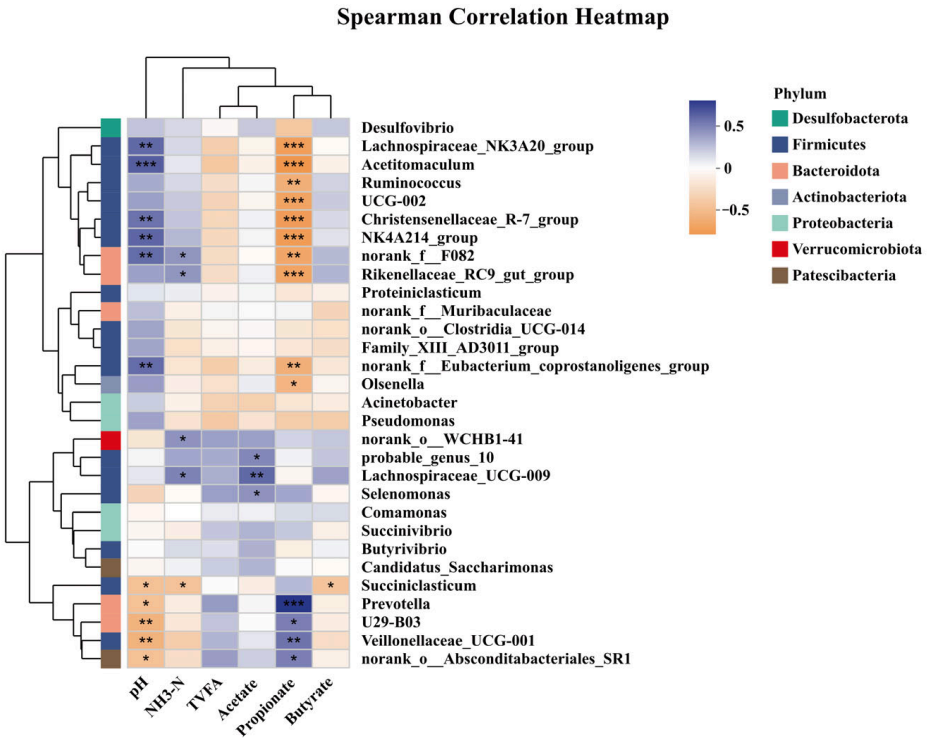

Figure 8. Correlation analysis between bacterial communities and rumen fermentation parameters during in vitro fermentation of *C. korshinskii* silage supplemented with rumen fluid and molasses. * indicates $0.01 < p \leq 0.05$; ** indicates $0.001 < p \leq 0.01$; *** indicates $p \leq 0.001$.

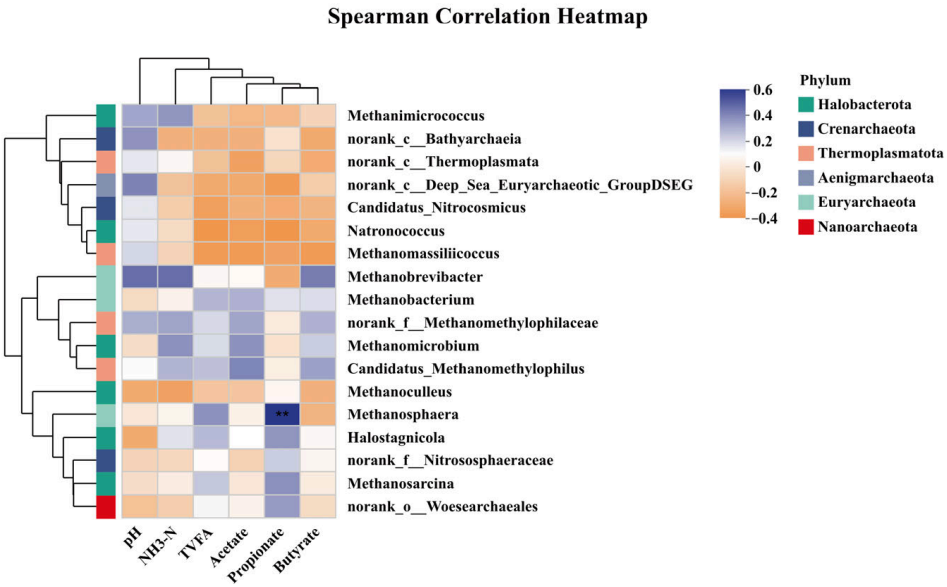

Figure 9. Correlation analysis between archaeal communities and rumen fermentation parameters during in vitro fermentation of *C. korshinskii* silage supplemented with rumen fluid and molasses. ** indicates $0.001 < p \leq 0.01$.

4. Discussion

4.1. In Vitro Ruminal Gas Production of C. korshinskii Silage with Rumen Fluid and Molasses

GP is a key indicator of rumen fermentation activity and is influenced primarily by rumen microbial metabolic activity and substrate carbohydrate content [17]. In the present study, R60 exhibited the lowest GP from A and the longest lag phase among all groups, suggesting that the pre-fermentation process depleted readily fermentable substrates, thereby

delaying the onset of gas production. The lower GP in R60 and RM60 was primarily attributable to reduced GP from B, which was linked to lower silage fiber content (NDF and ADF). This indicates that the rumen fluid inoculation exerted a strong fiber-decomposing effect during ensiling, reducing the fiber content available for subsequent rumen fermentation. As a result, fewer soluble carbohydrates were available for microbial digestion through the rumen fermentation stage. During rumen fermentation, both enzymatic activity and microbial dynamics are subject to temporal variation [18]. Microorganisms preferentially utilize soluble sugars through glycolysis and the pentose phosphate pathway [19], leading to rapid GP, accumulation of VFAs, and lowered pH. In this study, rumen fluid was used to "pre-ferment" *C. korshinskii*, thereby depleting the carbohydrates that would otherwise be available to microorganisms. This is similar to the situation of low-starch feeds (e.g., rice straw, cassava hay, and corn cobs); when readily fermentable carbohydrates are insufficient to support immediate gas production, microorganisms must first degrade fiber to access sugar substrates, resulting in a significant lag phase [20]. Consequently, the significantly prolonged lag phase observed in R60 indicates that the pre-fermentation process induced a scarcity of immediately available carbon sources, leading to the observed delay in gas production. Notably, although M15, M60 and RM60 showed a higher c than the CK groups, they exhibited a significantly greater ruminal CH_4 proportion. The observed increase in the rumen gas production rate of silage upon adding molasses is largely due to the abundance of soluble carbohydrates it provides. By offering a readily available energy source, molasses accelerate microbial metabolic processes. Previous studies proved that an increased carbohydrate availability stimulates microbial fermentation, consequently shifting the fermentation dynamics in the rumen [21]. However, despite the higher gas production rate in the M and RM, the GP from A did not increase accordingly, as the degradation of molasses generates large amounts of H_2 and formic acid, which serve as key substrates for *Methanobrevibacter* to synthesize CH_4 [22]. This also accounts for the increased proportion and yield of CH_4 observed in the M and RM groups.

4.2. In Vitro Rumen Digestibility of C. korshinskii Silage with Rumen Fluid and Molasses

Rumen digestibility reflects the capacity of rumen microorganisms to utilize a variety of nutrients in feed substrates. In the present study, R15 had higher DMD and ADFD, exhibiting the effect of rumen fluid inoculation in degrading short-term fermented silage fiber. This can be attributed to the rich microbial population, enzymatic activity, and their synergistic effects present in the rumen fluid [23]. As shown in Figure 5A, the results of the microbial community analysis indicate that the abundance of *Prevotella* was higher in the R15. This genus is primarily involved in the degradation of hemicellulose and other non-cellulosic polysaccharides, contributing to the complex lignocellulolytic degradation cascade in the rumen [24].

In contrast, silage with molasses added had significantly reduced NDFD and ADFD regardless of sampling time. This may be explained by the significantly reduced NDF and ADF in the silage. Abundant fermentable carbohydrates from molasses promote the activity of microorganisms (especially sugar-utilizing bacteria such as lactic acid bacteria) in the very early stage of silage. Concurrently, the structural carbohydrates (NDF and ADF) had also been extensively decomposed by the microorganisms to produce more easily fermentable sugars, which are abundant in M and RM groups. Rumen microorganisms may preferentially utilize these easily fermentable sugars rather than the rough structural carbohydrates left over from the previous silage process, consequently resulting in reduced fiber degradation efficiency [25]. This situation was more pronounced in the RM group, as the rumen microorganisms in the early stage intensified the decomposition of degradable

components, resulting in fewer remaining degradable substances, which finally led to the decrease in DMD.

4.3. In Vitro Rumen Fermentation Parameters of C. korshinskii Silage Treated with Rumen Fluid and Molasses

The rumen pH reflects the integrated effects of feed degradation, microbial activity, and the absorption and secretion of the rumen wall. Maintaining the pH at 6.0–7.0 is essential for optimal microbial activity [26]. In the present work, the pH value of each group remained within the normal range (6.88–6.97), indicating that adding rumen fluid and molasses to silage did not affect the stability of rumen microecology.

NH_3-N, an intermediate product of dietary protein metabolism, serves as an important nitrogen source for the protein synthesis and activity of rumen microorganisms [27–29]. Therefore, ruminal NH_3-N concentration is closely associated with the MCP level. The 15-day silage with rumen fluid and molasses added showed significantly increased NH_3-N and MCP during rumen degradation, approaching the levels of the 60-day silage in the CK group. This indicated that adding rumen fluid and molasses can enable *C. korshinskii* silage to reach a relatively high protein nutritional level in a relatively short period of time.

VFAs, produced from carbohydrate fermentation, provide 40–70% of the digestible energy required by ruminants [30]. In this study, the changes in rumen VFAs and TVFA in each group were also consistent with the results of NDFD and ADFD in silage. Furthermore, molasses alone or combined with rumen fluid significantly reduced the propionate yield of silage in the rumen and subsequently increased the A/P [31]. Since higher ruminal acetate concentrations and A/P are usually associated with methane release [32], the increased A/P in this study led to the higher CH_4 proportion and yield in the M and RM groups. On the other hand, 15-day silage with rumen fluid added increased the yield of rumen branched-chain fatty acids, whereas 60-day silage did not differ significantly from the CK group. The TVFA of 15-day silage was quite close to that of 60-day silage, which means that 15 days is sufficient to meet the silage requirements when rumen fluid is added. Considering that the MCP in group R was also significantly improved, rumen fluid may become a very efficient silage inoculum. However, more research is still needed to determine the effects of different dosages of rumen fluid.

4.4. The Microbial Diversity of Rumen Fermentation in C. korshinskii Silage

Firmicutes are key contributors to fiber degradation and VFA production in the rumen [33,34]. Although M15 had the highest relative abundance of Firmicutes, its NDFD, ADFD, and TVFA were quite low. The high relative abundance of Firmicutes may stem from WSC degradation. On the other hand, *Prevotella*, a dominant rumen genus involved in carbohydrate and protein degradation, can also promote propionate production via the succinate or acrylate pathway and reduce A/P [35]. Furthermore, *Prevotella* abundance is positively associated with VFA production [35]. In the R group, higher *Prevotella* abundance accounted for the increased DMD, whereas lower *Prevotella* abundance in groups M and RM accounted for their decreased digestibility and VFA reduction. In addition, the two predominant ruminal methanogenic archaeal genera in this study, *Methanobrevibacter* and *Methanosphaera*, can utilize H_2 as an electron donor to transfer CO_2 to CH_4 or convert methanol to CH_4 using H_2 [36]. The increased abundance of *Methanobrevibacter* explains the rise in the CH_4 proportion and yield in M60 and RM60 groups. Moreover, since the propionate synthesis in the rumen may reduce the CH_4 production by consuming hydrogen (H_2) [37], the reduced propionate in the M and RM groups was consistent with the results of the microbial community. In contrast, *Methanosphaera* was significantly enriched in R15 and was also positively correlated with the propionate concentration, which confirms the theory that *Methanosphaera* may indirectly promote propionate production through

alternative hydrogen utilization or microbial interactions, thereby reducing hydrogen availability for methanogenesis [38]. As for Group R, the reduction in GP due to the decrease in immediately soluble fraction led to a decrease in CO_2 yield.

5. Conclusions

In vitro rumen fermentation results showed that adding 7% rumen fluid within 15 days of ensiling improved the ruminal digestibility of dry matter and fiber components, and enhanced ruminal nitrogen utilization and microbial protein synthesis. However, addition with 4% molasses alone or combined with rumen fluid decreased the rumen digestibility of the fiber fraction and TVFA, despite increasing ammonia nitrogen and MCP in 15-day silage. In summary, the addition of 7% rumen fluid improved subsequent ruminal digestibility and nitrogen utilization, whereas 4% molasses addition, alone or combined with rumen fluid, reduced subsequent ruminal fiber digestibility in 15-day silage.

These diverse fermentation outcomes may be associated with changes in the rumen microbial community. The increased digestibility in the R group may be related to a higher relative abundance of *Prevotella*, while the higher CH_4 production in the M60 and RM60 groups may be associated with an enrichment of *Methanobrevibacter*.

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Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request. The rumen content sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject IDs PRJNA1280656 (bacteria) and PRJNA1280544 (archaea). Direct links: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1280656 (accessed on 22 June 2025) and https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1280544 (accessed on 22 June 2025).

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

C. korshinskii	*Caragana korshinskii* Kom.
GP	Gas production
A	The immediately soluble fraction
B	The insoluble but fermentable fraction
c	Gas production rate
DM	Dry matter
NDF	Neutral detergent fiber
ADF	Acid detergent fiber
DMD	Dry matter digestibility
NDFD	Neutral detergent fiber digestibility

ADFD	Acid detergent fiber digestibility
TVFA	Total volatile fatty acids
VFAs	Volatile fatty acids
A/P	Acetate to propionate ratio
MCP	Microbial crude protein
NH_3-N	Ammonia nitrogen
GLM	General linear model
PCoA	Principal coordinate analysis
LEfSe	Linear discriminant analysis effect size

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