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Response of rumen methane production and microbial community to different abatement strategies in yaks

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Abstract

Background Developing region-specific dietary strategies is crucial for mitigating methane (CH_4) emissions from yaks. However, there is a lack of tailored emission reduction strategies for yak production in the Qinghai-Tibet Plateau region. This study utilizes an in vitro rumen fermentation technique (Based on the ANKOMRF gas production measurement system) to investigate the effects of different dietary interventions on CH_4 production from regional yaks. The selected strategies—Sodium Nitrate solution, regional *Medicago sativa L.*, and regional *Helianthus tuberosus L.*—were chosen for their potential to reduce CH_4 production through various mechanisms: Sodium Nitrate as a methanogenesis inhibitor, *Medicago sativa L.* for its high nutritional value and its ability to modulate microbial fermentation, and *Helianthus tuberosus L.* due to its inulin content, which promotes beneficial microbial activity. These dietary interventions aim not only to reduce CH_4 production but also to support rumen health and productivity. In addition, gas chromatography and microbial sequencing techniques were employed to identify the optimal emission reduction strategy for regional yaks and to elucidate the key factors influencing the efficacy of these strategies.

Results The results indicate that supplementing the confined feeding ration (FR group) with Sodium Nitrate (12 mmol/L, FRN group), *Medicago sativa* L. (25%, FRM group), and *Helianthus tuberosus* L. (3%, FRH group) all have the effect of reducing CH_4 production from yak rumen. Among these interventions, the FRM group exhibits the most significant reduction, with a decrease in rumen CH_4 production by 42.76% compared to the FR group. The dry matter digestibility, total volatile fatty acids (TVFA), propionate, and butyrate levels in all groups were higher than those in the FR group. However, only the FRM group reached a significant level ($P < 0.01$). The pH values were significantly lower than those in the FR group ($P < 0.01$) across all groups. Each group exhibited distinct clustering patterns in bacterial and archaeal communities compared to the FR group ($P < 0.05$). The α diversity of bacterial communities was significantly lower than that of the FR group ($P < 0.01$), while the α diversity of archaeal communities was significantly higher than that of the FR group ($P < 0.01$). Taxa such as Lachnospiraceae, *Clostridium*, *Treponema*, Methanomicrobiaceae, *Methanosphaera*, and *Methanoplanus* were enriched in the FR group.

Conclusions CH_4 production from yak rumen were significantly negatively correlated with substrate crude protein (CP) levels, fermentation fluid TVFA levels, α diversity of archaeal communities, and the relative abundance of *Selenomonas* and *Megasphaera* in bacterial communities ($P < 0.01$). Conversely, CH_4 production were significantly positively correlated with the relative abundance of *Methanoplanus* in archaeal communities ($P < 0.01$). From the perspective

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of CH_4 gas production, the ranking of emission reduction effectiveness for different mitigation strategies is as follows: FRM group > FRH group > FRN group.

Keywords Qinghai-Tibet Plateau, Ruminants, In vitro fermentation, Dietary intervention, Emission reduction mechanisms

Background

Methane (CH_4) emissions from ruminants are a major contributor to global greenhouse gas emissions, accounting for approximately 30% of anthropogenic CH_4, with ruminal fermentation being the primary source [1, 2]. This CH_4 emissions not only accelerates climate change but also represents a loss of energy for ruminants, typically consuming 2% to 12% of their total energy intake, which severely impacts the efficiency of livestock production systems [3]. In the Tibetan Plateau of China, *Bos grunniens* (yaks) play a central role in the local pastoral economy, and their CH_4 emissions significantly contribute to the region's overall greenhouse gas output [4]. Therefore, reducing CH_4 emissions from yaks is crucial for achieving low-carbon livestock production in the Tibetan Plateau, promoting regional sustainable development, and supporting China's "dual carbon" goals.

Research on reducing CH_4 emissions from ruminants has primarily focused on dietary interventions, feed additives, and the regulation of rumen microbiota [5]. Feed additives, due to their economic feasibility and effectiveness, have been widely studied for their CH_4-reducing potential. Several studies have demonstrated that Nitrate, when used as a feed additive, can substitute carbon dioxide as an electron acceptor, inhibiting CH_4-producing bacteria and significantly reducing CH_4 emissions [6–10]. Both in vitro fermentation studies [6–8] and in vivo studies [9, 10] have confirmed that Nitrate supplementation in ruminant diets can effectively reduce CH_4 production. However, there is limited research on the application of Nitrate in yak systems in the Tibetan Plateau. Thus, exploring the suitability and effectiveness of Nitrate, especially in high-altitude environments, is particularly important.

In addition to Nitrate, *Medicago sativa* L. has gained attention for its potential to reduce CH_4 emissions from ruminants. This high-nutrient forage is not only rich in fiber but also supports the growth of beneficial microorganisms in the rumen, improving the fermentation of fibrous materials and reducing CH_4 production [11, 12]. Furthermore, the plant tannins can suppress the growth of CH_4-producing bacteria while promoting the growth of other beneficial microbes, enhancing its CH_4-reduction effects [13]. Given *Medicago sativa* L.'s strong adaptability to the Tibetan Plateau, investigating its incorporation into yak diets could improve both the ecological and economic sustainability of livestock farming in the region.

Moreover, *Helianthus tuberosus* L. has shown promise in reducing CH_4 emissions from ruminants, offering unique benefits. Rich in inulin, a fermentable carbohydrate, *Helianthus tuberosus* L. stimulates the fermentation of beneficial rumen bacteria and inhibits the activity of CH_4-producing microbes, thereby significantly reducing CH_4 emissions [14, 15]. Recent in vitro fermentation studies have shown that the addition of inulin can significantly reduce rumen CH_4 emissions in sheep and improve rumen fermentation processes [15]. However, some studies have indicated that adding inulin to the diet of calves can promote weight gain without affecting their CO_2 and CH_4 emissions [16]. Therefore, while inulin addition offers several benefits to ruminant animals, further research is needed to explore its effectiveness in reducing rumen CH_4 emissions. Moreover, *Helianthus tuberosus* L. exhibits good adaptability to the Qinghai-Tibet Plateau environment, is easy to cultivate, and has a high inulin content. Therefore, investigating the effect of *Helianthus tuberosus* L. addition on yak CH_4 emissions holds significant practical significance.

Although in vitro fermentation technology is widely used to study rumen fermentation and CH_4 production due to its controllability and simplicity [8, 9, 17], it has certain limitations. In vitro fermentation cannot fully replicate the dynamic and complex nature of the rumen ecosystem, nor can it accurately reflect the long-term effects of dietary interventions on rumen microbiota [18, 19]. Nevertheless, in vitro fermentation remains an essential preliminary tool for screening CH_4-reduction strategies, providing valuable data and theoretical support for subsequent in vivo studies.

Therefore, this study aims to (1) evaluate the CH_4-reducing effects of three feed additives—Nitrate, *Medicago sativa* L., and *Helianthus tuberosus* L.—using in vitro fermentation technology in yak rumen. (2) The study will investigate their impact on the rumen microbiota, elucidate the mechanisms of CH_4 reduction, and (3) assess their potential for mitigating CH_4 emissions in yaks on the Tibetan Plateau. The results will provide actionable strategies for CH_4 reduction and contribute to the development of sustainable, low-carbon livestock production systems in the region.

Results

Composition and nutritional characteristics of fermentation substrates

Analysis of the composition and nutritional content of substrates before in vitro fermentation revealed differences in the composition and nutritional levels of substrates among different mitigation strategies. As shown in Table 1, compared to the FR group, substrates in the FRM and FRH groups exhibited higher levels of crude protein (CP) and lower levels of neutral detergent fiber (NDF) and acid detergent fiber (ADF), while substrates in the FRN group had similar nutritional levels to those in the FR group. Additionally, compared to the FRH group, substrates in the FRM group also showed higher CP levels and lower NDF and ADF levels.

Dry matter disappearance rate of fermentation substrates and rumen fluid fermentation parameters

Analysis of substrates and fermentation fluid after 48 h of in vitro fermentation revealed differences in the dry matter disappearance rate (DMdr) of substrates and pH, as well as volatile fatty acids (VFA) concentrations in the fermentation fluid among different mitigation strategies. As shown in Table 2, compared to the FR group, the FRM group exhibited higher DMdr, TVFA, propionate, and butyrate levels, as well as lower pH and acetate-to-propionate ratio. The FRN groups showed lower pH values and acetate and similar levels of other parameters compared to the FR group. The FRH groups showed lower pH values and similar levels of other parameters compared to the FR group.

Cumulative gas production and CH_4 production during fermentation

Analysis of cumulative gas production during in vitro fermentation revealed that fermentation continued after adding substrates with different mitigation strategies compared to the K group (blank control), indicating that the buffer solution and rumen inoculum effectively simulated the rumen fermentation process in yaks (Fig. 1A, B). The gas production rate was higher in the first 12 h of fermentation compared to the later stages, and the cumulative gas production showed the trend FR group > FRN group > FRH group > FRM group (Fig. 1A, B). Analysis

Table 1 Composition and nutrient composition of in vitro fermentation substrates among the experimental groups (dry matter basis)

Items	Groups				SEM	*P*-value
	FRN	FRM	FRH	FR		
Nutrients						
DM (%)	95.98	96.10	96.45	96.22	0.15	0.652
CP (%)	9.80c	14.41a	11.52b	10.04c	0.39	< 0.001
NDF (%)	50.09a	39.95c	46.16b	50.19a	0.90	< 0.001
ADF (%)	28.09a	21.16c	25.71b	28.07a	0.63	< 0.001
Ash (%)	6.58	6.87	6.60	6.65	0.07	0.396

FRN group represents the Nitrate (12 mmol/L) added group, FRM group represents the *Medicago sativa* L. (25%) added group, FRH group represents the *Helianthus tuberosus* L. (3%) added group, and FR represents the control group (housed basal diet). DM represents dry matter, CP represents crude protein, NDF represents neutral detergent fiber, ADF represents acid detergent fiber, and Ash represents crude ash. SEM represents the standard error of the mean. Data in the same row without letters or with the same letters indicate that the differences are not significant, and different letters indicate significant differences ($P < 0.05$), similarly for the following

Table 2 Digestibility and fermentation parameters of in vitro fermentation among the experimental groups

Items	Groups				SEM	*P*-value
	FRN	FRM	FRH	FR		
DMdr (%)	60.50b	69.31a	65.07ab	59.34b	1.27	0.011
pH	6.74b	6.67b	6.76b	6.87a	0.21	0.003
TVFA (mmol L^{-1})	61.82b	64.47a	61.35b	58.32b	0.55	< 0.001
Acetate (%)	68.75bc	66.35c	70.20ab	72.22a	0.65	0.005
Propionate (%)	20.26b	22.68a	20.64ab	20.07b	0.37	0.037
Butyrate (%)	10.12b	11.28a	10.02b	9.11b	0.24	0.008
Acetate-to-Propionate ratio	3.44a	2.93b	3.42a	3.61a	0.09	0.005

FRN group represents the Nitrate (12 mmol/L) added group, FRM group represents the *Medicago sativa* L. (25%) added group, FRH group represents the *Helianthus tuberosus* L. (3%) added group, and FR represents the control group (housed basal diet). DMdr represents substrate dry matter digestibility, TVFA represents Total Volatile Fatty Acids

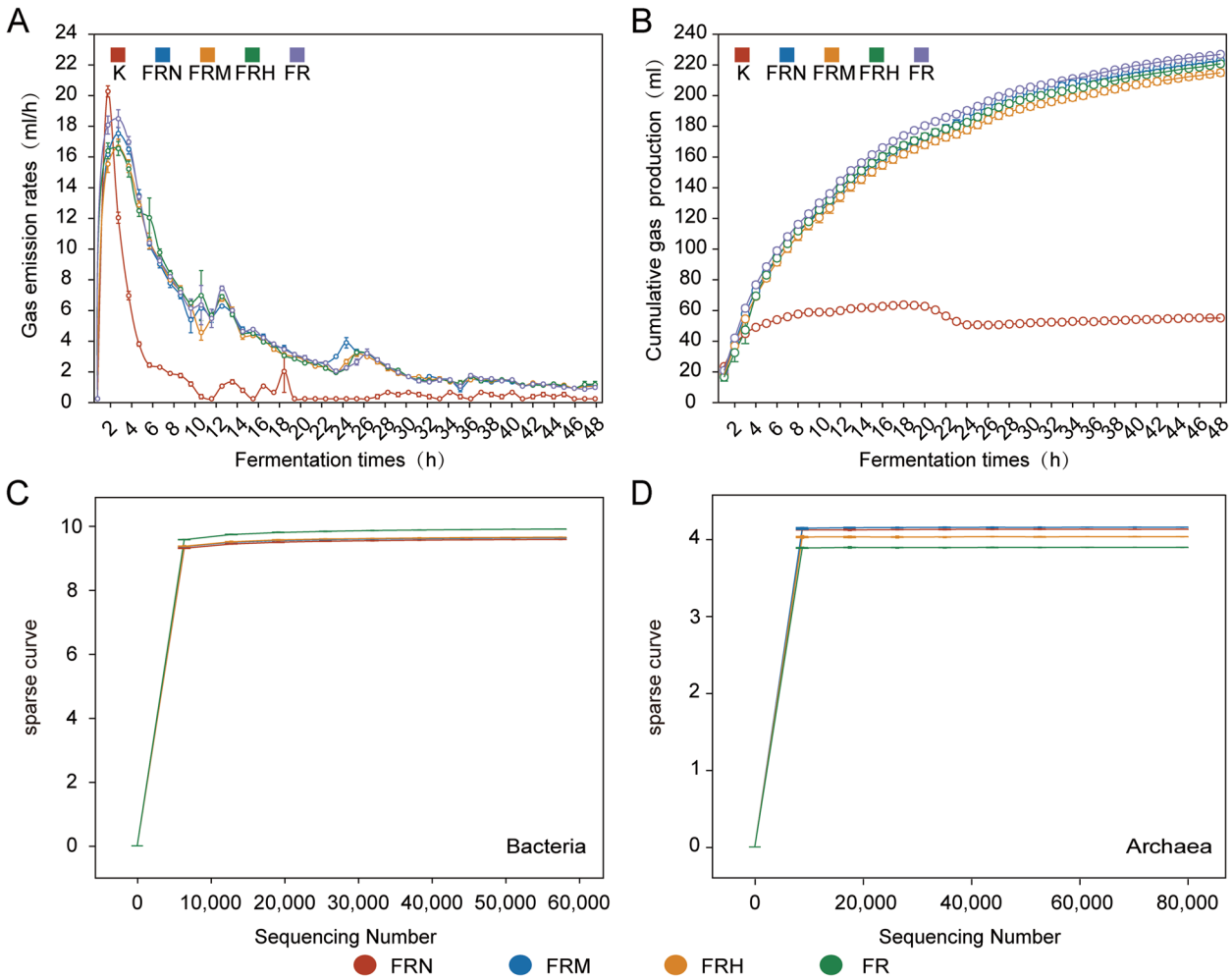

Fig. 1 Rate of change of gas production (**A**) and cumulative gas production curve (**B**) of *in vitro* fermentation, Sparse curve analysis of rumen fluid bacteria (**C**) and archaea (**D**) sequencing. FRN group represents the Nitrate (12 mmol/L) added group, FRM group represents the *Medicago sativa* L. (25%) added group, FRH group represents the *Helianthus tuberosus* L. (3%) added group, and FR represents the control group (housed basal diet)

of the net CH_4 production per unit at 12, 24, and 48 h of fermentation showed that all three mitigation strategies had certain mitigation effects (Table 3). As shown in Table 3, at 12, 24, and 48 h of fermentation, net CH_4 production per unit was significantly higher in the FR group than in the FRN, FRH, and FRM groups ($P<0.01$), with FRN and FRH groups significantly higher than the FRM group ($P<0.01$), and no significant difference between the FRN and FRH groups. Analysis of the unit CH_4 production after 48 h as a measure of the mitigation effects of different strategies revealed that the unit CH_4 production showed the trend FR group > FRN group > FRH group > FRM group. Compared to the FR group, the FRN group reduced by 22.66%, the FRM group reduced by 42.76%, and the FRH group reduced by 27.46%. Thus, it can be concluded that the FRM group had the best mitigation effect, followed by the FRH group, with the mitigation effect of the FRH group being similar to that of the FRN group.

Impact of different mitigation strategies on rumen microbial community characteristics

Quantity and quality of sequencing data: After quality control and filtering of sequencing data of bacteria and archaea from fermentation liquid samples after 48 h of fermentation with different mitigation strategies, a total of 3,100,772 raw reads and 1,779,709 high-quality sequences of bacteria were obtained from 24 samples, with an average of 74,154 high-quality sequences per sample. A total of 3,312,261 raw reads and 2,203,333 high-quality sequences of archaea were obtained, with an average of 83,472 high-quality sequences per sample. Sparse analysis showed that the sequencing depth of samples from each experimental group was sufficient

Table 3 Cumulative gas and CH_4 emissions of in vitro fermentation substrates among the experimental groups

Items	Groups				SEM	*P*-value
	FRN	FRM	FRH	FR		
12 h						
Cumulative gas production (ml)	137.02	133.95	139.38	144.16	1.50	0.092
Blank calibration gas production (ml)	59.59	59.59	59.59	59.59	-	-
Net gas production per unit (ml/g DMdr)	127.99b	107.29c	122.62b	142.52a	3.30	< 0.001
CH_4 concentration (%)	5.94bc	5.53c	6.35b	8.06a	0.22	< 0.001
Unit production of CH_4 (ml/g DMdr)	7.62b	5.96c	7.80b	11.50a	0.46	< 0.001
24 h						
Cumulative gas production (ml)	185.35ab	177.35c	182.30bc	189.98a	1.41	0.005
Blank calibration gas production (ml)	50.37	50.37	50.37	50.37	-	-
Net gas production per unit (ml/g DMdr)	223.10b	183.22d	202.73c	235.27a	4.45	< 0.001
CH_4 concentration (%)	8.91a	8.16b	9.25a	9.53a	0.15	0.003
Unit production of CH_4 (ml/g DMdr)	19.84b	14.97c	18.76b	22.39a	0.60	< 0.001
48 h						
Cumulative gas production (ml)	222.50ab	214.59c	220.41b	226.58a	1.27	0.002
Blank calibration gas production (ml)	54.82	54.82	54.82	54.82	-	-
Net gas production per unit (ml/g DMdr)	277.14b	230.53d	254.47c	289.47a	4.90	< 0.001
CH_4 concentration (%)	8.30b	7.39c	8.47b	10.27a	0.25	< 0.001
Unit production of CH_4 (ml/g DMdr)	22.98b	17.01c	21.56b	29.72a	1.00	< 0.001

FRN group represents the Nitrate (12 mmol/L) added group, FRM group represents the *Medicago sativa* L. (25%) added group, FRH group represents the *Helianthus tuberosus* L. (3%) added group, and FR represents the control group (housed basal diet). DMdr represents dry matter digestibility

to reflect the ASV information contained in the samples, capturing the majority of microbial community information from in vitro fermented rumen fluid samples (Fig. 1C, D).

Diversity of bacterial and archaeal communities: Statistical analysis of the ASV table after rarefaction (depth set at 95% of the lowest sample sequence count) revealed that different mitigation strategies affected the microbial community characteristics in yak rumen fermentation fluid. Alpha diversity (Chao 1 and Shannon index) was used to characterize the α-diversity of bacterial and archaeal communities among different mitigation strategies (Fig. 2). In bacterial communities, compared to the FR group, the α-diversity of the FRN, FRM, and FRH groups was significantly reduced ($P < 0.01$); there was no significant difference in α-diversity among the FRN, FRM, and FRH groups (Fig. 2A, B). In archaeal communities, compared to the FR group, the α-diversity of the FRN, FRM, and FRH groups was significantly increased ($P < 0.01$); there was no significant difference in α-diversity among the FRN, FRM, and FRH groups (Fig. 2D, E). Unconstrained principal coordinates analysis (PCoA) based on Bray–Curtis distance was used to characterize the β-diversity of bacterial and archaeal communities among different mitigation strategies. In bacterial communities, clear clustering was observed within each experimental group; PCo1 and PCo2 explained 32.1% and 15.5% of the total variation, respectively. PERMANOVA analysis further showed significant differences in community composition among all experimental groups (FR vs. FRN, FRM, FRH) ($P < 0.05$), particularly between the FR group and the other experimental groups (Fig. 2C). In archaeal communities, clear clustering was observed within each experimental group; PCo1 and PCo2 explained 79.0% and 5.3% of the total variation, respectively. PERMANOVA analysis further showed significant differences in community composition between the FR group and the other groups ($P < 0.05$), with no significant difference in community composition between the FRN and FRM groups (Fig. 2F).

Composition of bacterial and archaeal communities: In bacterial communities, Venn diagrams revealed that the FRN, FRM, FRH, and FR groups had 3780, 3402, 3727, and 6118 specific ASVs, respectively, with 2350 common ASVs (Fig. 3A). In archaeal communities, Venn diagrams showed that the FRN, FRM, FRH, and FR groups had 80, 56, 62, and 60 specific ASVs, respectively, with 157 common ASVs (Fig. 3B). At the family level in bacterial communities, Ruminococcaceae (13.05—14.63%), Lachnospiraceae (7.91—11.22%), BS11 (6.54—9.87%), Sphaerochaetaceae (2.97—8.89%), Prevotellaceae (3.80—4.90%), and Veillonellaceae (3.54—4.36%) were the predominant bacterial families (Fig. 3C). At the genus level, *Sphaerochaeta* (2.97—8.89%), *Prevotella* (3.78—5.85%),

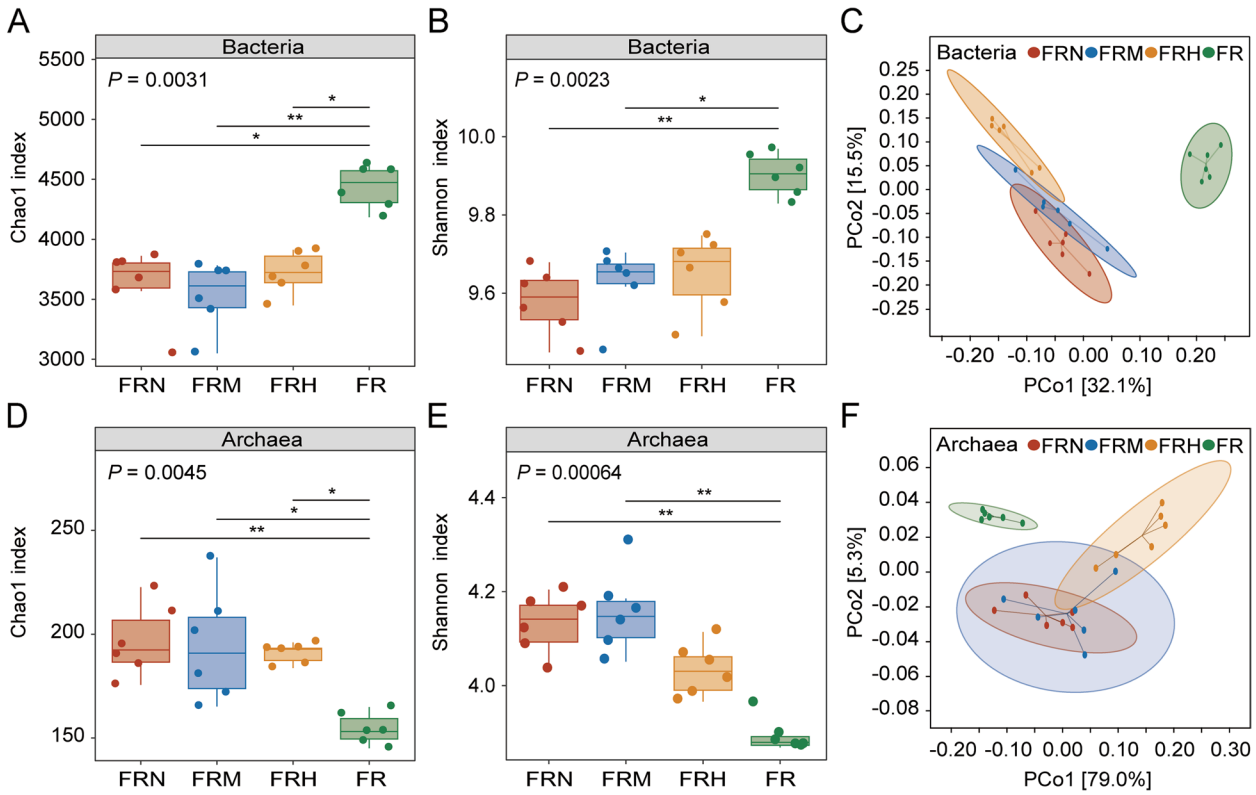

Fig. 2 The α diversity of rumen fluid bacteria (**A**, **B**) and archaea (**D**, **E**), The β diversity of rumen fluid bacteria (**D**) and archaea (**F**). FRN group represents the Nitrate (12 mmol/L) added group, FRM group represents the *Medicago sativa* L. (25%) added group, FRH group represents the *Helianthus tuberosus* L. (3%) added group, and FR represents the control group (housed basal diet)

Succiniclasticum (2.78—3.28%), *Ruminococcus* (1.88—2.52%), *Clostridium* (1.85—2.97%), and *Treponema* (1.03—5.3%) were the predominant bacterial genera (Fig. 3D). In archaeal communities, at the family level, Methanobacteriaceae (99.41—99.51%) and Methanomicrobiaceae (0.05—0.20%) were the predominant archaeal families (Fig. 3E). At the genus level, *Methanobrevibacter* (97.69—98.34%), *Methanosphaera* (1.12—1.80%), and *Methanoplanus* (0.05—0.24%) were the predominant archaeal genera (Fig. 3F).

Differences in composition of bacterial and archaeal communities: At the family level in bacterial communities, compared to the FR group, the relative abundance of Lachnospiraceae was significantly decreased in the FRN and FRH groups ($P < 0.01$), while Veillonellaceae was significantly increased ($P < 0.01$) in the FRN and FRH groups, and the relative abundance of Sphaerochaetaceae was significantly increased ($P < 0.01$) in the FRH group. The relative abundance of Prevotellaceae was significantly decreased ($P < 0.01$) in the FRM group (Fig. 4A). At the genus level, compared to the FR group, the relative abundance of *Clostridium* and *Treponema* was significantly decreased ($P < 0.01$) in the FRN, FRM, and FRH groups, while the relative abundance of *Prevotella* was significantly decreased ($P < 0.01$) in the FRM group, and the relative abundance of *Sphaerochaeta* was significantly increased ($P < 0.01$) in the FRH group (Fig. 4B). In archaeal communities, at the family level, compared to the FR group, the relative abundance of Methanomicrobiaceae was significantly decreased ($P < 0.01$) in the FRN, FRM, and FRH groups (Fig. 4C). At the genus level, compared to the FR group, the relative abundance of *Methanosphaera* was significantly decreased ($P < 0.01$) in the FRN, FRM, and FRH groups, and the relative abundance of *Methanoplanus* was significantly decreased ($P < 0.01$) in the FRN and FRM groups (Fig. 4D).

Differential genera in bacterial and archaeal communities: Using the Random Forests algorithm, differential genera between experimental groups were filtered at the genus level for both bacterial and archaeal communities. In bacterial communities, the FR group was enriched with *Fibrobacter* and *Clostridium*. The FRN group was enriched with *Ruminobacter*, *Prevotella*, and

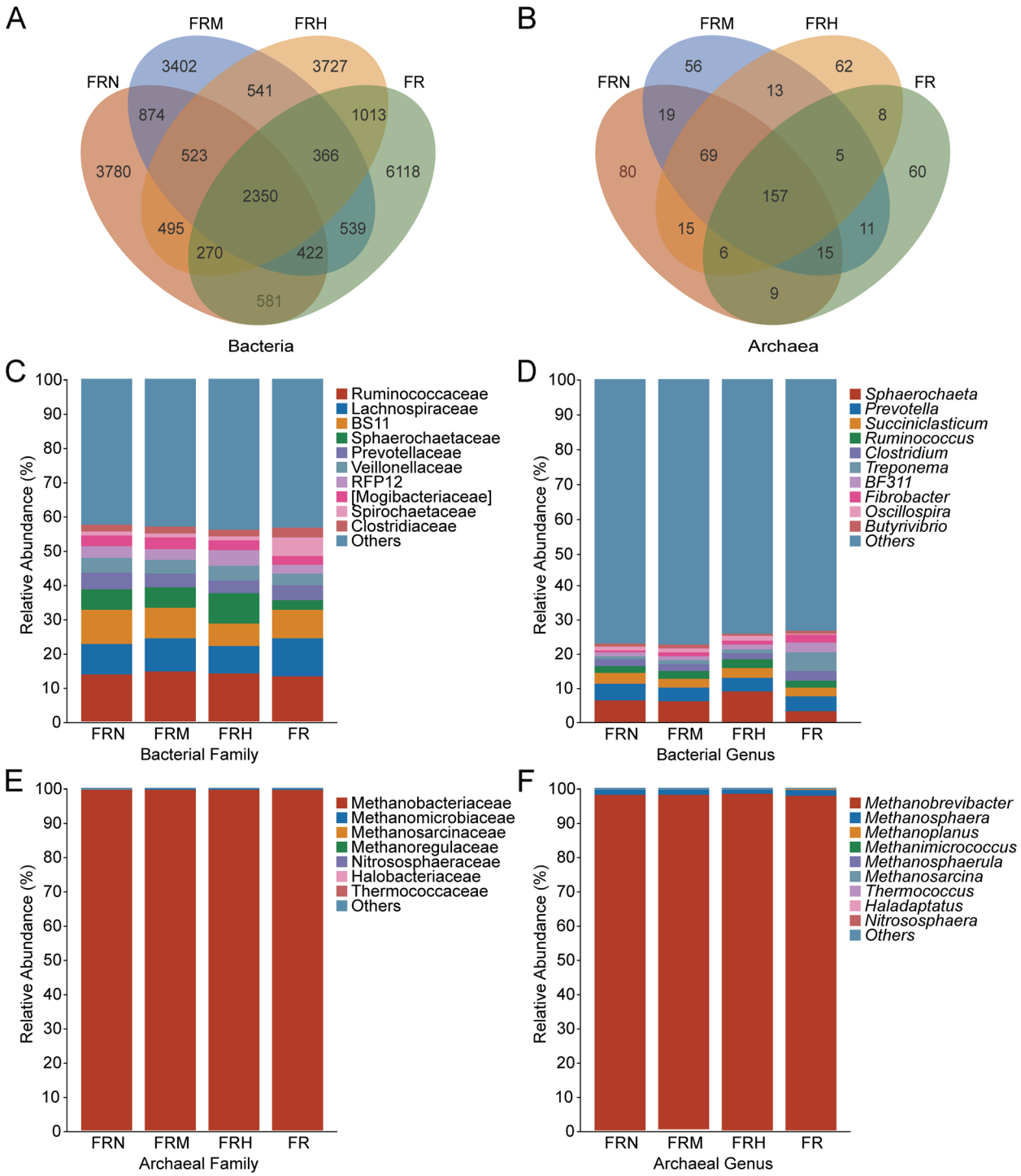

Fig. 3 Venn diagram of ASV levels of rumen fluid bacteria (**A**) and archaea (**B**), Community composition of rumen fluid bacteria (**C**, **D**) and archaea (**E**, **F**). FRN group represents the Nitrate (12 mmol/L) added group, FRM group represents the *Medicago sativa* L. (25%) added group, FRH group represents the *Helianthus tuberosus* L. (3%) added group, and FR represents the control group (housed basal diet)

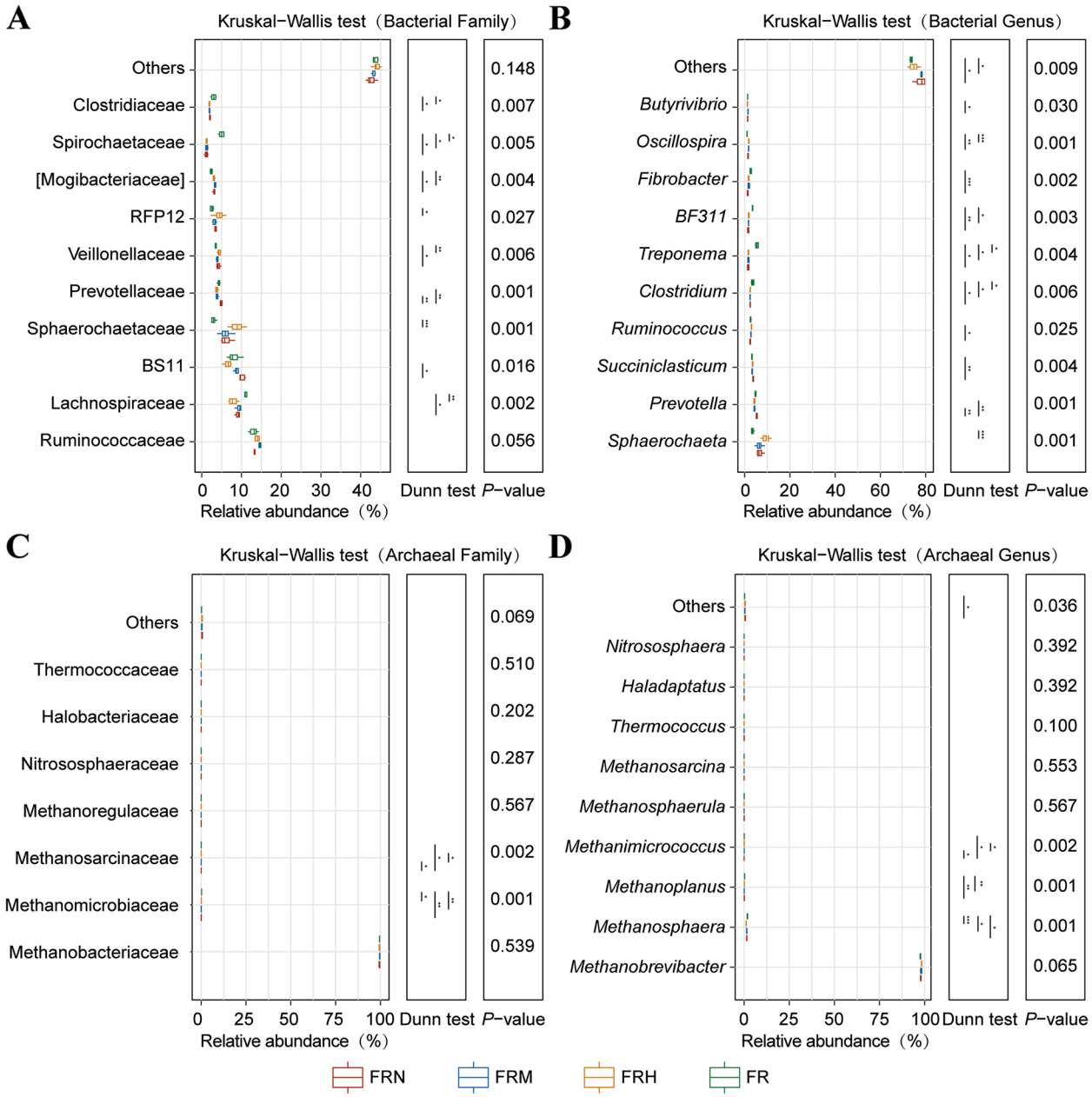

Fig. 4 Differential analysis of community composition of rumen fluid bacteria (**A**, **B**) and archaea (**C**, **D**). FRN group represents the Nitrate (12 mmol/L) added group, FRM group represents the *Medicago sativa* L. (25%) added group, FRH group represents the *Helianthus tuberosus* L. (3%) added group, and FR represents the control group (housed basal diet)

Succiniclasticum; while the FRM and FRH groups were enriched with *Bacillus*, *Selenomonas*, and others (Fig. 5A). In archaeal communities, the FR group was most significantly enriched with *Methanoplanus* (Fig. 5B).

Correlation analysis between gas production indicators and influencing factors

Through multivariate stepwise regression analysis of nutritional indicators of fermentation substrates, fermentation parameters of rumen fluid, differential microbial genera, and gas production indicators, key driving factors related to the unit CH_4 production of in vitro fermentation were screened, including the CP level of substrates, TVFA level of rumen fluid, α-diversity of archaeal communities, relative abundance of *Fibrobacter* and *Selenomonas* in bacterial communities, and relative abundance of *Methanoplanus* in archaeal communities. Further correlation analysis between these factors and gas production

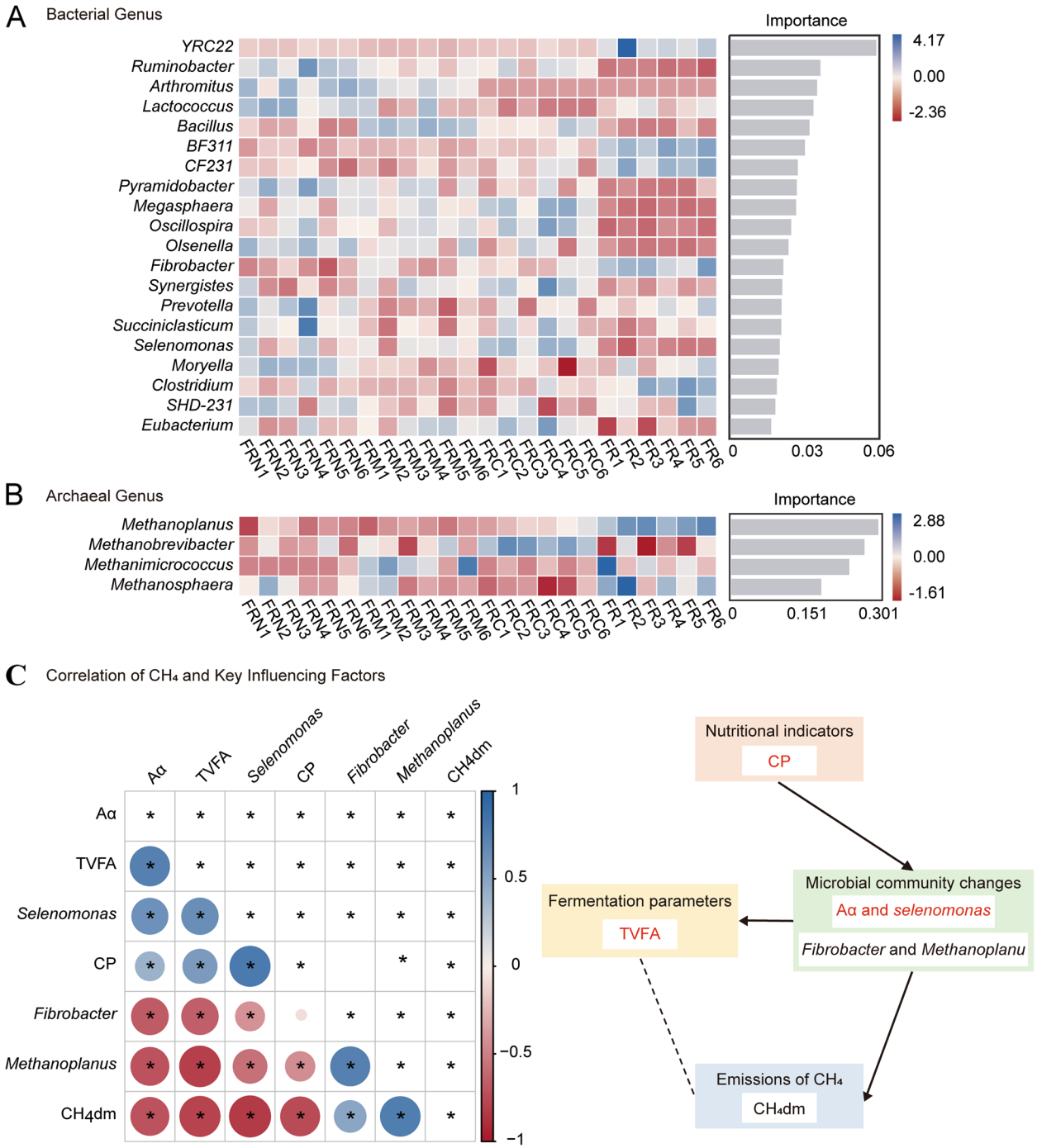

Fig. 5 Differential genus analysis of rumen fluid bacteria (**A**) and archaea (**B**), Correlation analysis of in vitro fermentation gas production index and influencing factors (**C**). In (**C**), CH_4dm represents unit CH_4 production, red font represents a negative correlation, black font represents a positive correlation, and the solid line represents a direct effect and the dashed line represents an indirect effect

indicators revealed significant negative correlations ($P<0.05$) between the unit CH_4 production of in vitro fermentation and the CP level of substrates, TVFA level of rumen fluid, α-diversity of archaeal communities, and relative abundance of *Selenomonas* in bacterial communities (Fig. 5C); while significant positive correlations ($P<0.05$) were observed between the unit CH_4 production and the relative abundance of *Fibrobacter* in bacterial communities and *Methanoplanus* in archaeal communities (Fig. 5C).

Discussion

Analysis of the CH_4 mitigation effects and adaptability of different mitigation strategies

Based on an in vitro fermentation system that simulates ruminal fermentation in cold-season housed yaks, with the basic diet for yaks (FR) serving as the control, this study found that the strategies FRN, FRM, and FRH all exhibited effective CH_4 mitigation effects. Previous in vitro studies have mainly focused on the addition of Nitrate, which has been shown to reduce CH_4 production in ruminants by 23.3%–70.0%, with the specific effect depending on the dosage and experimental conditions [6–8, 20]. In this study, the CH_4 reduction effect of Nitrate was slightly lower (22.66%), which may be attributed to differences in Nitrate concentration, substrate types, or rumen inoculum sources. Moreover, the addition of Nitrate in this study had minimal impact on ruminal fermentation parameters and dry matter digestibility, indicating that Nitrate can effectively reduce CH_4 production without compromising fermentation efficiency. This result aligns with most previous studies [6–8, 20]. In fact, recent in vivo studies have also reported that short-term Nitrate supplementation in dairy cow diets reduced ruminal CH_4 production by approximately 20%, with no significant effects on dry matter digestibility, TVFA, or acetic acid levels in the rumen [9, 21]. A study on grazing beef cattle also concluded that feeding a Nitrate-containing diet did not significantly affect dry matter intake, while CH_4 production per kilogram of dry matter consumed decreased by 18.5% [10]. These results suggest that Nitrate supplementation can effectively mitigate CH_4 production in ruminants under practical conditions. However, the potential risk of Nitrate toxicity remains a concern, and further dose-gradient studies are needed before applying this strategy to yaks.

Leguminous forages have demonstrated notable advantages in reducing ruminal CH_4 production and enhancing fermentation characteristics in ruminants. For instance, Zhong et al. [22] employed the in vitro gas production technique to examine the effects of grass and legume forages on CH_4 production and fermentation in goat rumen. They found that legume forages produced less CH_4 during fermentation compared to grasses, while also yielding higher concentrations of volatile fatty acids (VFAs). Similarly, Peng et al. [23] observed that feeding a 25% legume inclusion in the diet of dairy calves resulted in a 13.15% reduction in total gas production, along with improved dry matter digestibility and elevated VFA concentrations. Suybeng et al. [24] reported that supplementing beef cattle diets with 31% legume plants led to a 10% reduction in CH_4 production. These findings align with the results of the current study, where the inclusion of *Medicago sativa* L. significantly reduced CH_4 production by 42.76%, while simultaneously improving DMdr and enhancing concentrations of VFA, propionate, and butyrate. Additionally, Roca-Fernández et al. [17] conducted continuous culture in vitro fermentation trials on four legume forages, including *Medicago sativa* L. to assess nutrient digestibility, VFA concentrations, and ruminal CH_4 production. Their study found that legumes with higher tannin content significantly reduced CH_4 production but at the expense of some fermentation product yields. In contrast, *Medicago sativa* L. with relatively low tannin levels, managed to strike a favorable balance between reducing CH_4 production and maintaining good fermentation performance. In the present study, the addition of *Medicago sativa* L. not only reduced CH_4 production in in vitro fermentation but also ensured higher VFA concentrations in the fermentation liquid, indirectly supporting the choice of additive and its inclusion level. An in vivo study by Jonker et al. [12] demonstrated that feeding *Medicago sativa* L. pellets to sheep reduced enteric CH_4 production, with a positive correlation between ruminal VFA concentrations and CH_4 production, which is consistent with the findings of this study. Therefore, based on the in vitro results, the inclusion of *Medicago sativa* L. with low tannin content appears to be an effective strategy for reducing ruminal CH_4 production in yaks. However, further validation through in vivo studies is needed to confirm these results.

The effectiveness of inulin supplementation in ruminant health, particularly in terms of growth performance and modulation of rumen environment, has garnered considerable attention [25, 26]. However, limited information is available regarding the effects of inulin on ruminal CH_4 production in ruminant animals, and there are discrepancies in the results [15, 16, 27, 28]. Previous studies utilizing in vitro rumen simulation techniques have found that supplementation with inulin-containing additives (e.g., *Helianthus tuberosus* L.) has a positive effect on reducing ruminal CH_4 production in ruminant animals, with its mitigating effect diminishing under conditions of higher ruminal pH [27]. Similarly, a study on beef cattle found that inulin supplementation reduced ruminal CH_4 production [28]. More recently, a study on dairy cows demonstrated that inulin, as a dietary additive, decreased CH_4 production produced by ruminal microbial fermentation of feed and positively influenced the ruminal fermentation process [15]. These findings are consistent with the results of the present study. However, an in vivo study involving calves fed diets supplemented with *Helianthus tuberosus* L. powder (at 1.2% of dietary dry matter basis) found that *Helianthus tuberosus* L. powder addition did not alter ruminal CH_4 production [16]. These discrepancies may be due to variations in the amount of inulin supplementation and dietary

composition, but it can still be believed that *Helianthus tuberosus* L. powder supplementation has a positive effect on reducing ruminal CH_4 emissions in ruminant animals, at least from the perspective of this study. Furthermore, further experimentation with additional gradients of *Helianthus tuberosus* L. supplementation is essential for accurately assessing its mitigation effects and providing more precise reference for in vivo investigations.

Potential mechanisms analysis of different mitigation strategies in reducing CH_4 emission

An important factor influencing changes in ruminal fermentation gas emissions is the rumen microbial community [29], which ferments plant material into metabolic end products such as short-chain fatty acids (SCFAs) and CH_4 under anaerobic conditions [30]. It has been reported that differences in ruminal CH_4 emissions in ruminant animals are also observed in their rumen microbial communities [31–33]. Therefore, studying changes in rumen microbial communities can help explain the differences in gas emissions caused by mitigation strategies. In this study, Mitigation strategies exhibited lower α-diversity of bacterial communities compared to the control group, reflected in the reduced diversity of bacterial amplicon sequence variants (ASVs) and phylogeny observed in rumen fermentation fluid samples. Gruninger et al. [34] also found that the addition of 3-nitrooxypropanol (3-NOP) and canola oil significantly reduced enteric CH_4 emissions in beef cattle and decreased bacterial community α-diversity. This phenomenon can be explained from the perspective that the efficiency of ruminant energy harvest depends on specific rumen microbial community compositions [35, 36]. One fact is that the production of ruminal CH_4 represents an energy loss from feed [37], while bacterial communities in the rumen typically act as energy producers [30]. In other words, efficient rumen microbes are not as complex, but more specialized to support the energy needs of ruminants. In this study, mitigation strategies exhibited higher digestibility and VFA levels compared to the control group, confirming this explanation. The rumen archaeal community is the microbial group most directly related to CH_4 emissions, as they typically utilize products from bacterial communities (such as CO_2 and H_2) to produce CH_4 [38]. Studies have shown that the α-diversity of archaeal communities in the rumen of ruminants with low CH_4 emissions is higher [39], indicating that a diversified methanogenic archaeal community weakens the specialized CH_4-producing function, thereby reducing CH_4 production. This is consistent with the results of this study. This may be because fewer specialized methanogenic taxa are involved in CH_4 production pathways in ruminants with high CH_4 emissions [29].

The differences in CH_4 production can be attributed to the distinct microbial compositions in the rumen fermentation fluid between the control group and the mitigation strategies, as the pathways of CH_4 generation are closely associated with the enrichment of rumen microbial communities [40]. The results of this study indicate that, compared to the FR group with higher CH_4 emissions, the relative abundances of Lachnospiraceae, *Clostridium*, and *Treponema* were generally lower in various mitigation strategy groups with lower CH_4 emissions. Previous studies on cellulolytic bacteria have shown that Lachnospiraceae primarily participate in cellulose degradation, possessing various endo-glucanases and glycoside hydrolases [41]. In a study exploring goats, it was found that *Clostridium* is a cellulolytic bacterium with anaerobic probiotic characteristics capable of producing short-chain fatty acids, including propionic and butyric acids [42]. Another study on goats found that *Treponema*, similarly, is an anaerobic bacterial genus specialized in degrading recalcitrant fibers [43], typically providing VFA required by ruminants while releasing hydrogen. They are also closely associated with ruminant rumen CH_4. It has been reported that when studying rumen microbial communities under different CH_4 production phenotypes in dairy cows and sheep, Lachnospiraceae were enriched in rumen types with high CH_4 emissions [44]. When evaluating the effect of perilla seed extract on ruminal CH_4 mitigation, a positive correlation was found between the relative abundance of Lachnospiraceae and CH_4 production [45]. Similarly, significant enrichment of *Clostridium* was observed in the rumen of cows with high CH_4 emissions [46]. When Nitrate was added to the diets of calves and water buffaloes, it was found that ruminal CH_4 emissions decreased with lower relative abundance of *Treponema* [21, 47]. These studies have demonstrated that the relative abundances of Lachnospiraceae, *Clostridium*, and *Treponema* are generally positively correlated with ruminal CH_4 emissions. Furthermore, compared to the FR group with higher CH_4 emissions, the relative abundances of Methanomicrobiaceae, *Methanosphaera*, and *Methanoplanus* were generally lower in various mitigation strategy groups with lower CH_4 emissions. These microbial taxa are directly involved in rumen CH_4 production. For instance, Khiaosa-ard et al. [48] found that a decrease in the relative abundance of Methanomicrobiaceae resulted in reduced ruminal CH_4 production. Jiang et al. [49] found that an increase in Methanomicrobiaceae during anaerobic fermentation led to an increase in CH_4 production during the anaerobic digestion process. Li et al. [39] found a significant increase in the relative abundance of *Methanoplanus* in the rumen fermentation fluid of groups with high CH_4 emissions. This suggests that, from the

perspective of rumen microbial community composition, the reasons for CH_4 emission reduction by mitigation strategies can be reasonably explained.

Through differential microbial community and their correlation with gases, this study found that some minor microbial taxa were also associated with rumen CH_4 emissions. For example, *Selenomonas*, which was found to be enriched in the FRM and FRH groups, showed a negative correlation with CH_4 emissions. *Selenomonas* is a protein-degrading bacterium and a hydrogenophilic bacterium, mainly involved in the fumarate and Nitrate reduction pathways in the rumen, competing with methanogens for H_2. In conditions where it is enriched, ruminant cattle CH_4 emissions are typically lower [50]. The relative abundance of *Megasphaera* was also significantly negatively correlated with gas emissions in this study. Studies have shown that the gene systems enriched in the highly efficient rumen microbiome are dominated by *Megasphaera*, a bacterium that efficiently produces butyric and propionic acids from lactate [35]. Kamke et al. [51] found this type of bacterium in the rumen of sheep with low CH_4 production, confirming the results of this study. In summary, the mitigation effects exerted by rumen fermentation strategies in yaks are driven by differential microbial community diversity, composition, and differential microbial taxa between the mitigation strategy groups and the control group.

While this study offers valuable insights into the application of three mitigation strategies to reduce CH_4 emissions in yaks, it is important to note that the in vitro fermentation system employed, though capable of replicating fundamental ruminal fermentation processes, cannot fully capture the complex dynamics of the actual rumen environment, particularly the intricate interactions between the host and its microbial community. To address this limitation, future research should prioritize in vivo validation to evaluate the practical effectiveness of these strategies under real-world farming conditions. Additionally, this study focused primarily on the short-term effects of these mitigation approaches. The long-term impacts of Nitrate, *Medicago sativa* L., and *Helianthus tuberosus* L. supplementation on animal health, productivity, and microbial community adaptation remain uncertain and require further investigation.

Conclusion

The addition of Sodium Nitrate solution, *Medicago sativa* L., and *Helianthus tuberosus* L. to the shed-feed diet of regional yaks effectively mitigates CH_4 production from their rumens. The analysis suggests that the key mechanism underlying the effectiveness of these mitigation strategies involves the nutritional composition of fermentation substrates, particularly the crude protein (CP) content. These nutritional factors drive shifts in the microbial community diversity and changes in the relative abundance of specific bacterial genera within the fermentation fluid. In turn, these microbial changes influence fermentation parameters and gas production, with observed indirect correlations between fermentation parameters and CH_4 production. The findings from this study may offer valuable insights for future CH_4 reduction strategies in yaks. However, as the results are based on in vitro fermentation models, which cannot fully replicate the complex dynamics of the rumen environment, further multi-dimensional and multi-gradient investigations, both in vivo and in vitro, are necessary to validate the effectiveness of these mitigation strategies comprehensively.

Materials and Methods

Experimental design and preparations

Experimental design: 6 mature housed yaks from the Qinghai-Tibet Plateau region were selected for this experiment. These animals were privately owned by a local farm and were fed a diet consisting of oat hay and concentrate (60:40 ratio) for three months prior to the experiment to ensure stable rumen conditions. The yaks served as donors of rumen fluid for this study, providing the fluid used in the in vitro fermentation process. The slaughter of these yaks was carried out with the utmost regard for animal welfare. A high-voltage electrical stunning method was employed to induce instantaneous loss of consciousness, ensuring minimal suffering. This was followed by halal slaughter, in accordance with local cultural and religious practices. All procedures were conducted in strict compliance with national animal welfare regulations and ethical standards to ensure humane treatment throughout the process. providing the rumen fluid used for in vitro fermentation. The study comprised four treatment groups: FR group (n = 6), consisting of a mixture of oat hay and concentrate (60:40), which represents a common dietary composition for locallyed yaks. FRN group (n = 6), supplemented with Nitrate solution to achieve a concentration of 12 mmol/L in the mixture [6–8]. FRM group (n = 6), with 25% of *Medicago sativa* L. replacing the substrate of the FR group [12, 23]. FRH (n = 6) group, with 3% *Helianthus tuberosus* L. replacing the substrate of the FR group [15, 16]. K group (n = 3), serving as a blank control group without experimental samples, only including 90 ml of buffer/rumen fluid mixture (Fig. 6), Its purpose was to provide a baseline for comparing the fermentation results of the other treatment groups.

Substrate preparation: Oat hay, concentrate, Sodium Nitrate, *Medicago sativa* L., and *Helianthus tuberosus* L. were obtained from Haiyan County, Haibei Tibetan

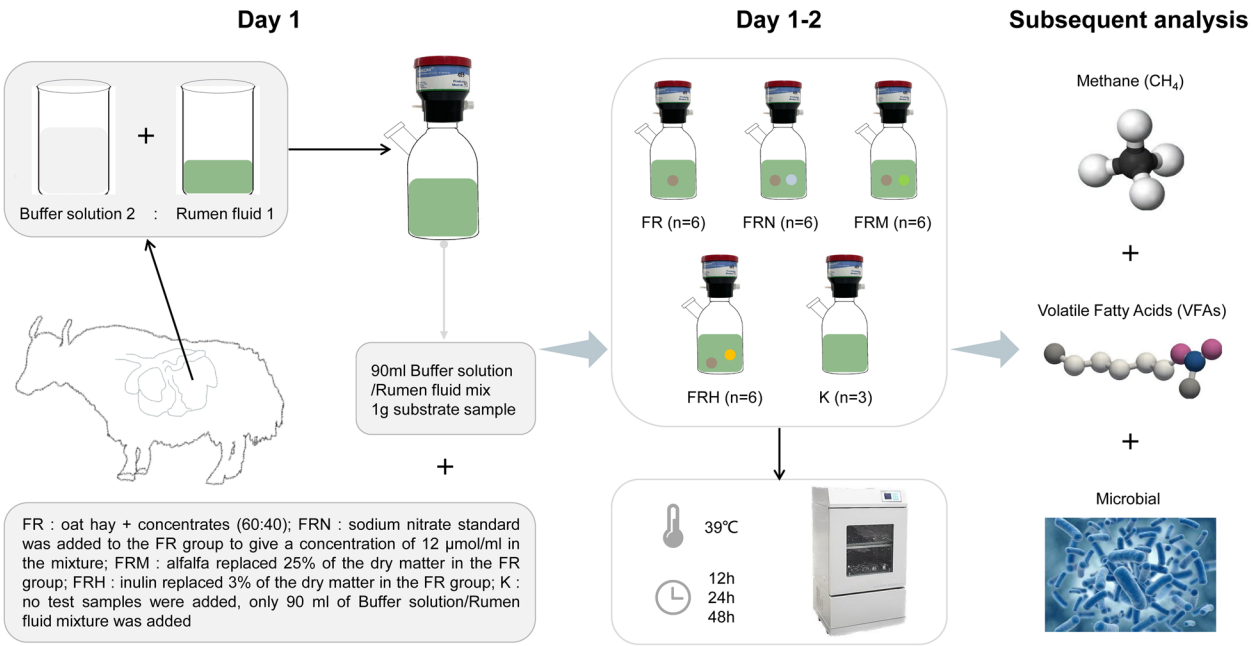

Fig.6 Schematic diagram of in vitro rumen fermentation experiments. FRN group represents the Nitrate (12 mmol/L) added group, FRM group represents the *Medicago sativa* L. (25%) added group, FRH group represents the *Helianthus tuberosus* L. (3%) added group, and FR represents the control group (housed basal diet)

Autonomous Prefecture, Qinghai Province. The Oat hay, *Medicago sativa* L. and *Helianthus tuberosus* L. used in this study were provided by the Qinghai Province Gui'nan County Jiacang Ecological Animal Husbandry Cooperative. These three plant species are commonly cultivated as forage crops in the Qinghai-Tibetan Plateau region. As they are widely known and locally cultivated, no formal identification or voucher specimens were required. This study did not involve wild plant species, and no special permissions were necessary. All experimental procedures involving these plants comply with institutional, national, and international guidelines. The research aligns with local agricultural practices and adheres to relevant legislation. These collected samples were dried at 65 °C for 24 h to obtain air-dried samples. The air-dried samples were manually cut into 3–5 cm fragments with scissors, ground using a grinder to pass through a 1 mm sieve to ensure uniformity and similar particle size of the samples. Subsequently, the samples were mixed according to the experimental groups and packed in sealed plastic bags for nutrient analysis and in vitro rumen fermentation.

ANKOM^{RF} Gas Production System Calibration and Fermentation bottle preparation: The ANKOM^{RF} Gas Production System (Model RF16) is designed to measure the kinetics of a microbial fermentation in an automated fashion by monitoring the gas pressure within multiple Modules and remotely recording the data in computer spreadsheets. The pressure recording module should first be tested for interaction with the computer to ensure sufficient charge of rechargeable battery (RF16) and proper functioning of the exhaust valve. One gram of substrate sample for in vitro rumen fermentation was weighed per group into nylon bags (previously weighed) with a pore size of 48 μm and dimensions of 3×4 cm, and placed into 250 ml fermentation bottles (4×6=24). Additionally, three blank fermentation bottles were prepared similarly and along with the pressure recording module, placed in a 39 °C constant temperature oscillating incubator for storage and later use.

Buffer solution preparation: Rumen buffer solution was prepared following the method by Goering and Van Soest [52]. First, the following solutions were prepared: resaruzin 0.1% (w/v) solution: 0.1 g resaruzin dissolved in 100 ml H_2O; in vitro buffer solution: 4.0 g NH_4HCO_3, 35.0 g $NaHCO_3$, Bring volume to 1 L using Distilled Water; in vitro micromineral solution: 13.2 g $CaCl_2{\cdot}2H_2O$, 10.0 g $MnCl_2{\cdot}4H_2O$, 1.0 g $CoCl_2{\cdot}6H_2O$, 8.0 g $FeCl_3{\cdot}6H_2O$, Bring volume to 100 ml using Distilled Water; in vitro constant macromineral solution: 5.7 g Na_2HPO_4 anhydrous, 6.2 g KH_2PO_4 anhydrous, 0.6 g $MgSO_4{\cdot}7H_2O$, Bring volume to 1 L using Distilled Water; reducing solution: 625 mg Cysteine·HCL, 4 ml 1 N NaOH, 625 mg $Na_2S{\cdot}9H_2O$, Bring volume to 100 ml using Distilled Water. Then, 2 g of trypticase was mixed with 400 ml distilled water and 0.1 ml micromineral solution, stirred to dissolve, followed by addition of 200 ml

buffer solution, 200 ml macromineral solution, and 1 ml of resaruzin solution, mixed together to form the final buffer solution. To remove oxygen from the buffer solution, 2 ml of reducing solution was added until the color of the buffer changed from red to colorless. The buffer solution was stored for later use in a 39 °C constant temperature water bath [52].

Rumen fluid was collected from the rumens of 6 donor yaks selected for this study following their slaughter at 4:00 AM. Fresh rumen digesta from each yak was filtered through four layers of cheesecloth and immediately transferred into pre-warmed, sealed insulated bottles maintained at 39 °C. The fluid was thoroughly mixed to ensure uniformity before being transported to the laboratory. Upon arrival, the rumen fluid was swiftly transferred into sealable glass bottles and placed in a 39 °C constant-temperature water bath to maintain a stable temperature. Inoculation was performed within 5 h of the fluid's collection to ensure the freshness and integrity of the sample [53].

In vitro fermentation and sampling

In vitro fermentation: Using a 100 ml syringe, 60 ml of buffer and 30 ml of rumen inoculum were transferred into 250 ml fermentation bottles containing 1 g of fermentation substrate (FRN, FRM, FRH, or FR). During this process, all operations were conducted rapidly, and continuous CO_2 flushing was employed to remove air from the headspace, ensuring an anaerobic environment was maintained throughout. Additionally, three fermentation bottles without substrate were used as controls for calibration analysis and blank gas. The fermentation bottles were immediately sealed with the pressure recording module of the ANKOM^{RF} gas production system, and aluminum foil gas bags were connected at the exhaust valve. The entire fermentation setup (fermentation bottles + pressure recording module) was placed in a 39 °C constant temperature oscillating incubator for 48 h of fermentation. This process was completed within 1 h.

Sample Collection: Aluminum foil gas bags connected to the exhaust valve of the pressure recording module were replaced at 12, 24, and 48 h for gas composition analysis. At the end of the 48-h fermentation period, nylon bags containing substrate from each fermentation bottle were collected, washed with cold running water until clear, and used for determination of dry matter disappearance (DM) of fermentation substrates. The pH of the filtrate from each fermentation bottle was measured using a pH meter. Fifteen milliliters of filtrate samples from each fermentation bottle were collected in cryovials for VFA analysis, and an additional 2 ml of filtrate samples were collected for microbial analysis. Samples for VFA analysis were frozen at −20 °C, while samples for microbial analysis were frozen at −80 °C.

Gas production calculation and CH_4 gas analysis

Gas production calculation: Gas production at each stage was calculated using the following formula:

$$n = p \times \left(\frac{V}{RT} \right)$$

In the equation, n represents the gas production (in mol), p represents the pressure inside the fermentation bottle (in kPa), V represents the headspace volume of the fermentation bottle (in L), T represents the temperature inside the fermentation bottle (in K), and R represents the gas constant (8.314472 L kPa K^{-1} mol^{-1}).

According to Avogadro's law, at 273.15 K and 101.325 kPa (standard conditions), 1 psi equals 6.894757293 kPa (psi is the unit of pressure measurement in the ANKOM^{RF} gas production system), and 1 mol of gas occupies 22.4 L. Therefore, gas measured in mol can be converted into gas measured in ml using the following formula:

$$N = n \times 22.4 \times 1000$$

In the equation, N represents the gas volume in ml, n represents the gas volume in mol, and *1000* represents the conversion constant from L to ml. The actual volume of each fermentation bottle exceeds its rated volume. According to the calibration values provided by the manufacturer of the ANKOM^{RF} gas production system, the actual volume of a 250 ml fermentation bottle is 310 ml. Therefore, in this study, the headspace volume V inside the fermentation bottle is 220 ml.

CH_4 gas analysis was conducted using a gas chromatograph equipped with a dual thermal conductivity detector (PerkinElmer Clarus 580). CH_4 gas concentrations collected at 12 h, 24 h, and 48 h of fermentation were measured. Samples were introduced into the instrument's inlet via gas bags, filled into the syringe, and automatically injected through a six-port valve for analysis according to the programmed method. The chromatographic column and detector temperatures were maintained at 60 °C and 200 °C, respectively. The flow rates of the detection and reference flow paths were both set to 40 ml min^{-1}, with no splitting of the injection. A 13X chromatographic column with dimensions of 3 m × 3 mm was utilized for CH_4 detection, with a quantification loop volume of 0.5 ml. Hydrogen gas was used as the carrier gas, and the cumulative runtime was set to 11 min to obtain CH_4 concentration data.

Analysis of fermentation substrates and rumen fluid samples

The determination of dry matter (DM) of fermentation substrates was determined by the drying method. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the procedure described in the Van Soest fiber analysis method [54]. Crude protein (CP) content and crude ash (Ash) content were measured through the method adopted by AOAC [55].

For DNA extraction and amplicon sequencing of rumen fluid samples, the OMEGA DNA kit (M5635-02) (Omega Bio-Tek, Norcross, GA, USA) was used to extract genomic DNA from rumen fluid samples, which were then stored at −20 °C before further analysis. The quantity and quality of extracted DNA were assessed using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. PCR amplification was performed using 25μL of Q5® High-Fidelity DNA Polymerase (New England Biolabs, Hitchin, UK). The target regions for bacterial amplification were the V3-V4 regions of the 16S rRNA gene, with primers 338F (5'- ACTCCTACGGGAGGCAGCA-3') and 806R (5'- GGACTACHVGGGTWTCTAAT-3'), while for archaea amplification, the V8-V9 regions of the 16S rRNA gene were targeted using primers 1106F (5'-TTWAGT CAGGCAACGAGC-3') and 1378R (5'-TGTGCAAGG AGCAGGGAC-3'). Library construction was performed using the TruSeq Nano DNA LT Library Prep Kit (Illumina, San Diego, CA, USA), and qualified libraries were subjected to paired-end sequencing (2×250 bp) using the Illumina NovaSeq 6000 SP Reagent Kit (500 cycles) on an Illumina NovaSeq machine.

The determination of dry matter disappearance rate (DMdr) of fermentation substrates post-fermentation involved the following steps: the initial weight of substrates was recorded as m_0, nylon bags were washed and dried at 60 °C for 48 h to obtain constant weight, and the weight of residual substrates (m_1) was recorded. The DMdr of fermentation substrates was calculated as (m_0—m_1) / 100. VFA were determined using capillary gas chromatography. Rumen fluid samples were thawed and transferred to 10 ml centrifuge tubes, centrifuged at 10,000 rpm for 5 min, and 1 ml of supernatant was transferred to a PE tube and mixed with 200 μl of 5% metaphosphoric acid using a vortex mixer. After centrifugation at 12,000 rpm for 10 min, the supernatant was collected and analyzed using a gas chromatograph (Agilent 7890A).

Data processing and analysis

Nutrient levels of substrates, substrate DMdr data, rumen fermentation parameter data, and gas emission data were analyzed using one-way analysis of variance (ANOVA). When differences were found among experimental groups, multiple comparisons were performed using Duncan's test. Raw paired-end sequencing data of microbial communities were filtered and analyzed using QIIME2 (https://docs.qiime2.org/2019.4/tutorials/) [56]. The Dada2 method was employed for primer trimming, quality control, denoising, merging, and chimera removal, resulting in the generation of the final ASV table [57]. Kruskal–Wallis test and PERMANOVA analysis were conducted to statistically analyze 16S rRNA sequencing data of rumen fluid microbial communities. Differential microbial community analysis was performed using the Random Forests algorithm. Pearson correlation coefficient analysis was utilized to explore the relationships among gas production, substrate nutrients, substrate DMdr, fermentation parameters, and rumen microbial communities. Data were processed and statistically analyzed using Excel 2021, SPSS 24.0, and R software v3.6.1. Data visualization was performed using Origin 2021 and Genescloud (https://www.genescloud.cn). Adobe Illustrator 2020 was employed for figure layout.

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Authors' contributions

Z.Q. performed experiment and drafted the manuscript. G.T, W.X, W.L, W.Y, L.S, L.H, and Z.N. helped collect samples. The corresponding authors X.S. designed this study and helped in writing the manuscript. All authors had read and approved the published version of the manuscript.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. All raw sequences for this study can be found in the NCBI Sequence Read Archive under BioProject PRJNA1097211 with the accession https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1097211.

Declarations

Ethics approval and consent to participate

This experimental protocol was reviewed and approved by the Institution of Animal Care and Ethics Committee of the Northwest Institute of Plateau Biology, Chinese Academy of Sciences (NWIPB20160302). Farmers who own these animals are aware of the research effort and strongly support it.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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