

Article

Effects of Increasing Corn Grain Inclusion and Reducing Hay Proportion on Growth Performance, Methane Emissions, Rumen Fermentation, and Microbial Diversity in Winter-Housed Yaks

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Abstract

The expansion of ruminant production has increased methane (CH₄) emissions, highlighting the need for nutritional strategies that improve productivity while mitigating environmental impacts. Yaks, generally considered low CH₄ producers, are increasingly raised under intensive winter-housed systems on the Qinghai–Xizang Plateau, highlighting the need to assess how dietary concentrate-to-forage (C:F) ratios affect both CH₄ emissions and growth performance. This study investigated the effects of three dietary C:F ratios [L-C (48:52), M-C (60:40), H-C (72:28)] on growth performance, ruminal fermentation, microbial diversity ($n = 6$ per group) and CH₄ emission ($n = 3$ per group) in winter-housed yaks. The results indicated that average daily gain (ADG) was significantly higher in M-C and H-C, while the feed-to-gain ratio (F/G) was significantly lower in M-C and H-C than in L-C ($p < 0.05$). Total CH₄ production (g/day) did not differ among treatments ($p > 0.05$), while CH₄ yield per unit body weight gain (CH₄/BWG) was significantly reduced in M-C and H-C ($p < 0.05$). The protozoal count was significantly lower in H-C, and the proportions of isobutyrate and isovalerate were significantly higher in H-C and M-C compared with L-C ($p < 0.05$). 16S rRNA gene sequencing revealed that increasing the C:F ratio reduced the relative abundance of the archaeal genus *Methanobrevibacter*, while *Thermogymnomonas* exhibited a significant increase ($p < 0.05$). Collectively, these findings indicate that increasing the C:F ratio in winter-housed yaks improves growth efficiency and lowers CH₄/kg BWG, with the M-C group showing the most favorable balance between productivity and environmental sustainability.

Keywords: yaks; concentrate-to-forage ratio; rumen metabolism; CH₄ production; 16S rRNA



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1. Introduction

Global population projections indicate an 80% probability of reaching 9.6 to 12.3 billion by the end of the 21st century [1], intensifying food security pressure and driving the expansion of ruminant livestock production for meat and dairy supply [2]. This expansion has triggered a sharp rise in CH₄ emissions [3], a key greenhouse gas with a 100-year

global warming potential of 27 for non-fossil CH₄ and 30 for fossil CH₄, according to the IPCC Sixth Assessment Report (AR6) [4,5]. Livestock-derived CH₄ accounts for ~44% of agricultural greenhouse gas emissions, over 90% from rumen enteric fermentation [6], with rumen methanogenic archaea (mainly *Methanobrevibacter*) contributing ~18% of total anthropogenic CH₄ [7]; 78% of rumen archaea produce CH₄ via the hydrogenotrophic pathway using fermentation-derived H₂ and CO₂ [8–10]. Enteric CH₄ also causes 2–12% metabolizable energy loss [11], making its mitigation urgent for climate governance and production efficiency improvement.

Dietary nutritional regulation, especially optimizing the concentrate-to-forage (C:F) ratio, is a safe and efficient strategy to reduce ruminant CH₄ emissions while maintaining productivity [12]. High-concentrate diets suppress methanogenesis by promoting propionate production, which competitively consumes H₂ (a key methanogen substrate) [13,14]. Existing studies confirm that adjusting the C:F ratio, typically by increasing readily fermentable carbohydrates such as corn, barley, or wheat, alters rumen methanogenic communities and improves performance in sheep and goats [15,16], but systematic research on plateau endemic yaks (*Bos grunniens*) and the regulatory mechanism of the C:F ratio on their CH₄ emissions and rumen microbiota remains lacking.

Yaks, the dominant endemic livestock on the Qinghai–Xizang Plateau, support local animal husbandry and herder livelihoods [17]. Though individual CH₄ emissions are lower than other ruminants, this difference is attributed to multiple factors: their lower voluntary feed intake under grazing conditions, distinctive rumen microbiome composition, lower relative abundance of *Methanobrevibacter*, and long-term adaptation to low-quality forages on the Qinghai–Xizang Plateau [18]. Optimizing dietary nutrition to develop low-carbon feeding strategies—reducing CH₄ emissions, improving energy utilization, and enhancing growth performance—is the core direction for sustainable yak farming. The SF₆ tracer technique, suitable for both grazing and confined systems with relatively low equipment and operational costs compared to respiration chambers, and with the capacity to measure multiple animals simultaneously [19], has been used to quantify CH₄ emissions in yaks [20]. To elucidate microbial mechanisms, 16S rRNA gene sequencing was applied to characterize rumen bacterial and archaeal communities. Preliminary research shows concentrate supplementation reduces CH₄ emissions in grazing yaks and significantly alters the ruminal microbial diversity of housed yaks [21,22] but targeted studies on C:F ratio effects on winter-housed yaks are absent.

This study tested the hypothesis that increasing the dietary C:F ratio (by increasing corn grain inclusion and reducing oat hay proportion) reduces CH₄ emissions and improves growth performance in winter-housed yaks. The objective was to evaluate the effects of three C:F ratios on growth performance, ruminal CH₄ emission, fermentation, and microbial diversity.

2. Materials and Methods

2.1. Animal Feeding Experiments and Management

The experiment was conducted at the Digestive Metabolism Base of the Qinghai Academy of Animal Sciences and Veterinary (October to February, the winter season). The experiment involved 18 castrated male yaks (124.47 ± 3.02 kg, 18–24 months) in good physical condition and health status; growth performance and rumen metabolism data were collected from all six yaks per group ($n = 6$). Yaks were housed in individual pens (3 m × 2 m). The average indoor temperature was −8 °C (range: −15 °C to 0 °C), relative humidity 55 ± 10%, and natural photoperiod (approximately 10 h light:14 h dark). Temperature and humidity were recorded daily at 08:00, 14:00, and 20:00 using a digital thermo-hygrometer. The diet for each group was formulated to adjust the C:F ratios [L-C

(C:F = 48:52), M-C (C:F = 60:40), H-C (C:F = 72:28)]. The total duration of the experiment was 157 days, first 7 days for adaptation period, after 150 days for data collection. The dietary composition of the experimental diets is shown in Table 1. All yaks were kept in individual pens; 4.30 kg of feed was offered daily (the feeding restriction is to ensure that each yak receives the same amount of diet individually per day). During the entire experimental period, yaks had access to drinking ad libitum.

Table 1. Dietary composition and nutrient content of the diets fed to yaks during the experiment.

Items	Concentrate-to-Forage		
	L-C (C:F = 48:52)	M-C (C:F = 60:40)	H-C (C:F = 72:28)
Ingredients, % of DM			
Oat hay	52.00	40.00	28.00
Corn	22.00	33.00	44.00
Cottonseed meal	20.00	20.00	20.00
Rapeseed	2.00	3.00	4.00
Premix ¹	1.00	1.00	1.00
Bentonite	2.00	2.00	2.00
Salt	0.50	0.50	0.50
CaHPO ₄	0.50	0.50	0.50
Nutrient composition, % of DM			
Dry Matter	89.57	89.42	89.46
Crude protein	15.36	15.24	15.12
Ether extract	3.28	3.72	4.12
Neutral detergent fiber	35.94	28.75	24.01
Acid detergent fiber	22.16	18.88	15.60
Ca	0.89	0.85	0.89
P	0.24	0.30	0.30
ME ² (MJ/kg)	10.19	10.77	11.28

¹ Composition of the cattle premix: Vitamin A, 1000 KIU/kg; copper, 800 mg/kg; Vitamin D3, 300 KIU/kg; Zn, 3800 mg/kg; Vitamin E, 1500 IU/kg; Mn, 3200 mg/kg; Biotin, 1000 µg/kg; I2, 90 mg/kg; Folic acid, 10 mg/kg; Se, 70 mg/kg; Nicotinic acid, 3000 mg/kg; Fe, 3600 mg/kg; water ≤ 10%. ² ME, metabolizable energy, calculated value, ME = Σ (ingredient ME × proportion in DM); ingredient ME values from GB/T 47364-2026 [23].

2.2. Experiments for the Determination of CH₄ Yield

2.2.1. The Fabrication of SF₆ Permeation Tubes and the Determination of Permeation Rates

Large-sized permeation tubes for yaks were used, and the SF₆ gas was filled at the Qinghai Academy of Animal Husbandry and Veterinary Medicine. The filling process was completed in liquid nitrogen. Each permeation tube was filled with at least 2 g of SF₆ gas. The tubes were continuously incubated in a constant-temperature chamber at 39 °C, with the chamber maintained in a dry condition. The tubes were weighed every 3 days for a period of 1 month to achieve a stable SF₆ release rate. As shown in Table 2, all regression coefficients (R²) reached above 0.997, indicating good regression effects and ensuring high accuracy in the experiment. In addition to the nine permeation tubes used in the yaks (three per dietary group), three extra tubes (labeled Con) were kept in the constant-temperature chamber as controls to monitor any drift in permeation rates over time. These control tubes were not administered to animals.

Table 2. Regression equation of SF₆ permeation rates.

Permeation Tubes	Regression Equation	R ²
1 (L-C)	$y = -0.003x + 215.1$	0.999
2 (L-C)	$y = -0.003x + 222.2$	0.997
3 (L-C)	$y = -0.004x + 265.3$	0.998
4 (M-C)	$y = -0.004x + 249.8$	0.999
5 (M-C)	$y = -0.003x + 220.2$	0.998
6 (M-C)	$y = -0.003x + 195.1$	0.999
7 (H-C)	$y = -0.003x + 217.8$	0.999
8 (H-C)	$y = -0.004x + 224.5$	0.998
9 (H-C)	$y = -0.003x + 223.7$	0.997
10 (Con)	$y = -0.005x + 295.8$	0.999
11 (Con)	$y = -0.004x + 225.4$	0.998
12 (Con)	$y = -0.004x + 245.2$	0.997

L-C (C:F = 48:52), M-C (C:F = 60:40), H-C (C:F = 72:28). Con: control tubes kept in the incubator (not administered to yaks) for quality assurance of permeation rate stability.

2.2.2. The Experimental Procedure for Measuring CH₄ Yield

During days 80–90 of the experiment, CH₄ emissions from three randomly selected yaks ($n = 3$) per group were measured using the SF₆ tracer technique. Steady-release SF₆ permeation tubes were orally administered into the rumen, with daily checks for retention and physiological responses, and expelled tubes reinserted during the 3-day acclimation. Yaks were then habituated to gas-collection halters and PVC yokes for 5 days, with behavioral adaptation recorded. Prior to sampling, PVC canisters were evacuated to −70 kPa and validated for pressure stability, then used for 24 h continuous gas collection. After sampling, valid canisters (pressure ≥ -50 kPa) were purged with high-purity nitrogen; following equilibration, five 5 mL replicates were taken for analysis, alongside ambient air background samples.

2.3. Collection of Rumen Fluid

During the 10-day CH₄ emission measurement, rumen liquor was collected on the last day. Before the morning feeding, an oroesophageal tube was inserted and rumen liquor collected. The first 30 mL of rumen liquor was discarded to prevent saliva contamination. Then continued to collect 100 mL of rumen fluid and pH of the rumen fluid was recorded using a portable pH meter (PHS-25, Jinan GuangYao medical equipment Co., Ltd., Jinan, China). The 100 mL of the rumen fluid was filtered through two layers of gauze, then transferred into an anaerobic bottle and stored at 4 °C for protozoal counting [24]. The remaining rumen fluid is filtered through four layers of gauze, dispensed into sterile centrifuge tubes, placed in liquid nitrogen, and stored at −80 °C. Rumen fluid collected from each yak over three consecutive days was pooled and homogenized for subsequent analysis of rumen-fermentation parameters and 16S rRNA high-throughput sequencing.

2.4. Sample Analysis

2.4.1. Feed Sample Analysis

The feed samples were dried at 65 °C for 48 h until reaching a constant weight, then ground to pass through a 40-mesh sieve, and stored in self-sealing bags. The samples were then dried in an oven at 105 °C for 4 h, cooled, and weighed to determine Dry Matter (DM); ether extract (EE) was analyzed using a Soxhlet extractor; crude protein (CP) was determined using the Kjeldahl method; ash was determined by calcination in a muffle furnace at 550 °C; Calcium (Ca) and phosphorus (P) were determined using atomic absorption spectrophotometry and colorimetric method [25]; neutral detergent fiber

(NDF) and acid detergent fiber (ADF) were measured using an automatic fiber analyzer (ANKOM A200i Semi-Automatic Fiber Analyzer, New York, NY, USA), following the method described by Van Soest [26], with heat-stable α -amylase used for NDF analysis.

2.4.2. CH₄ and SF₆ Analysis

The analysis of CH₄ and SF₆ was conducted using a gas chromatograph (Beijing Famous, GC 3420, Beifen-Ruili Analytical Instrument (Group) Co., Ltd., Beijing, China) with a packed Porapak column. For CH₄ analysis, the column temperature was set to 80 °C; Flame Ionization Detector (FID, Agilent Technologies, Santa Clara, CA, USA) was used, with the auxiliary chamber (AUX) temperature at 250 °C; the injection port temperature was 150 °C; and the tail blow pressure was 0.2 MPa. The CH₄ standard gas concentration was 40.8 ppm. For SF₆ analysis, the column temperature was set to 100 °C; an Electron Capture Detector (ECD, Agilent Technologies, Santa Clara, CA, USA) was used, with the auxiliary chamber (AUX) temperature at 300 °C; the injection port temperature remained at 150 °C; and the tail blow pressure was also 0.2 MPa. The SF₆ standard gas concentration was 99 ppt. A manual injection volume of 500 μ L was used, and the analysis calculation was performed using the single-point calibration method.

2.4.3. CH₄ Yield Calculation

To calculate the CH₄ yield rate from the rumen of yaks, the samples were first measured at least six times to ensure stable data. The daily CH₄ emissions were calculated using the following formula [27]:

$$\text{CH}_4\text{Q (g/d)} = (\text{CH}_4\text{M} - \text{CH}_4\text{B}) / (\text{SF}_6\text{M} - \text{SF}_6\text{B}) \times \text{SF}_6\text{Q} \times (\text{MWCH}_4 / \text{MWSF}_6) \quad (1)$$

CH₄Q and SF₆Q: the daily emission of CH₄ and the permeation rate of SF₆ (g/d); CH₄M and SF₆M: the concentrations of CH₄ and SF₆ measured in the yoke (μ g/m³); CH₄B and SF₆B: the concentrations of CH₄ and SF₆ measured in the background samples of the air (μ g/m³); and MWCH₄ and MWSF₆: the molecular weights of CH₄ and SF₆.

2.5. Counting of Ruminal Protozoa

Rumen fluid (1 mL) was mixed with 2 mL of methyl green formalin–sodium chloride (MFS) staining solution and refrigerated at 4 °C until analysis. Protozoa quantification was performed using a microscope at 10 $\times$ 10 magnification. The counting chamber was divided into 25 large squares, each containing 16 small squares. To ensure representative sampling, protozoa were counted in five diagonally positioned squares: upper left, lower left, upper right, lower right, and center. For consistency, protozoa touching the upper or left borders of a square were included in the count for that square, while those overlapping the lower or right borders were excluded and assigned to adjacent squares. This standardized approach minimized edge-related counting errors [28].

2.6. Rumen-Fermentation Analysis

The collected rumen fluid was analyzed for fermentation metabolites, 1 mL of rumen fluid was collected and stabilized with 0.2 mL of a mixed solution of metaphosphoric acid and crotonic acid with a concentration of 25%, mixed well, then placed it in a freezer at −20 °C. After 12 h, it was transferred to a refrigerator at 4 °C to thaw. Centrifuged at 12,000 r/min for 10 min, we aspirated the supernatant and filtered it through a water system, then took 0.2 mL of the sample and placed it in a sample vial. The content of VFA was determined using a high-performance gas chromatograph (Agilent, GC 7890A, Agilent Technologies, Santa Clara, CA, USA). The parameters for the gas chromatograph were set as follows: injector temperature at 220 °C; a FUSED SILICA capillary column

(PerkinElmer, Waltham, MA, USA) was used, with the column temperature programmed at 40 °C for 4 min, then raised from 40 to 110 °C at 40 °C/min, followed by 110 °C for 2 min, and finally increased from 110 to 150 °C at 40 °C/min, held at 150 °C for 5 min. A flame ionization detector was employed with nitrogen as the carrier gas, and the detector temperature was set at 250 °C. The NH₃-N had been measured using the phenol–sodium hypochlorite colorimetric method. The microbial protein (MCP) had been determined using the Coomassie Brilliant Blue colorimetric method according to Bradford [29], with bovine serum albumin as the standard.

2.7. Rumen Microbiota Analysis

Total microbial DNA was extracted using the E.Z.N.A. Soil DNA kit (Omega, D5625-01, Omega Bio-tek, Inc., Norcross, GA, USA). The concentration and purity of the genomic DNA were detected using a spectrophotometer. Samples with a purity ratio of D260/280 between 1.8 and 2.0 were considered qualified and were stored in a –80 °C freezer.

Qualified DNA samples were sent to Sheng Gong Biotechnology (Shanghai) for 16S rRNA amplicon sequencing. Bacterial V3–V4 hypervariable regions were amplified with primers 341F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Archaeal V4–V5 regions were amplified using 519F (5'-CAGCMGCCGCGGTAA-3') and 915R (5'-GTGCTCCCCCGCCAATTCCT-3'). Purification of PCR amplification products was carried out using Agencourt AMPure XP magnetic beads. Using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to assess the fragment size range and concentration of the library. The libraries that pass the quality control are sequenced based on the size of the insert fragments. Sequencing was performed on the Illumina NovaSeq 6000 platform (2 × 250 bp paired-end) using the NovaSeq 6000 Reagent Kit v1.5 (Illumina Inc., San Diego, CA, USA). For denoising and clustering, we used DADA2 to generate amplicon sequence variants (ASVs) (100% identity). Chimeras were removed using the 'consensus' method in DADA2 [30,31]. Rarefaction was performed at 30,000 reads per sample for bacteria and 25,000 reads per sample for archaea. Singletons were removed from the sequence variant table prior to downstream analysis.

2.8. Statistical Analysis

The original data on yak growth performance, CH₄ production, and fermentation parameters were organized using Excel 2020. Growth performance was measured on all six yaks per group ($n = 6$); CH₄ measurements were performed on three yaks per group ($n = 3$). One-way ANOVA was performed with SPSS 24.0, followed by Tukey's HSD test for post hoc comparisons. Before performing one-way ANOVA on growth and fermentation parameters, normality was checked using the Shapiro–Wilk test, and homoscedasticity was verified using Levene's test. All data met these assumptions. Microbial data derived from 16S rRNA gene high-throughput sequencing were analyzed using the Kruskal–Wallis H test (a nonparametric method) using ALDEx2 with CLR transformation to assess intergroup differences, with p -values adjusted for false discovery rate (FDR) via the Benjamini–Hochberg (BH) method. Data are expressed as mean and SEM, where $p < 0.05$ indicates statistical significance and $0.05 < p < 0.10$ suggests a tendency toward significance. Figures were generated using R v4.1.0 and GraphPad Prism v8.0. Spearman's correlation test was applied to evaluate associations among yak growth performance, rumen-fermentation parameters, and microbial taxa.

3. Results

3.1. Growth Performance of Yaks Fed Diets with Different C:F Ratios

The M-C and H-C groups showed significantly greater BWG and ADG compared to the L-C yaks (Table 3, $p < 0.05$), with no significant difference between the M-C and H-C groups. The F/G was also better in the M-C and H-C groups ($p < 0.05$), indicating that increasing the C:F ratio improved growth performance.

Table 3. Effects of three diets with different C:F ratios on growth performance in yaks.

Items ¹	C:F			SEM	p-Value
	L-C	M-C	H-C		
DMI (kg)	4.30	4.30	4.30	—	—
IBW (kg)	125.33	124.42	123.67	3.017	0.978
FBW (kg)	163.35	182.60	178.75	3.967	0.106
BWG (kg)	38.02 ^b	58.18 ^a	55.08 ^a	2.835	0.002
ADG (kg/d)	0.25 ^b	0.39 ^a	0.37 ^a	0.019	0.002
F/G	17.27 ^a	11.25 ^b	12.13 ^b	0.815	0.001

The different superscripts in the same row indicate significant difference ($p < 0.05$). L-C (C:F = 48:52), M-C (C:F = 60:40), H-C (C:F = 72:28). ¹ DMI, dry matter intake; IBW, initial body weight; FBW, final body weight; BWG, body weight gain; ADG, average daily gain; F/G, feed to gain ratio.

3.2. Methane Production of Yaks Fed Diets with Different C:F Ratios

CH₄ variables were measured on $n = 3$ yaks per group. Increasing the C:F ratio effectively reduced the CH₄/BWG (Table 4, $p < 0.05$), with the lowest CH₄ yield observed in the M-C group. However, CH₄ production g/d and CH₄ yield g/kg DMI did not differ significantly among the groups. The lack of statistical significance for CH₄ g/d and CH₄/kg DMI ($p = 0.355$) should be interpreted with caution due to the limited power from the small sample size.

Table 4. Effects of three diets with different C:F ratios on methane production in yaks ($n = 3$).

Items ¹	C:F			SEM	p-Value
	L-C	M-C	H-C		
CH ₄ g/d	78.13	62.90	63.91	4.460	0.355
CH ₄ g/kg DMI	18.17	14.63	14.86	1.037	0.355
CH ₄ g/kg BWG	312.52 ^a	161.29 ^b	172.74 ^b	21.796	0.001

The different superscripts in the same row indicate significant difference ($p < 0.05$). L-C (C:F = 48:52), M-C (C:F = 60:40), H-C (C:F = 72:28). ¹ CH₄ g/d: CH₄ yield per day; CH₄ g/kg DMI: CH₄ yield per unit DMI; CH₄ g/kg BWG: CH₄ yield per unit BWG.

3.3. Rumen Fermentation of Yaks Fed Diets with Different C:F Ratios

There was no significant difference (Table 5, $p > 0.05$) in pH among the groups. Protozoa counts were significantly lower in the H-C group ($p < 0.05$) as compared to other treatments. Isobutyrate and isovalerate concentrations were also significantly higher ($p < 0.05$) in the M-C and H-C groups compared to the L-C group, while acetate, butyrate and valerate were not influenced ($p > 0.05$) by varying levels of the C:F ratio. Compared to the L-C group, the M-C and H-C groups showed an increasing trend in MCP ($p = 0.056$) and propionate ($p = 0.061$), and a decreasing trend in the acetate-to-propionate ratio ($p = 0.073$).

Table 5. Effects of three diets with different C:F ratios on rumen fermentation in yaks.

Items	C:F			SEM	<i>p</i> -Value
	L-C	M-C	H-C		
Rumen-fermentation parameter					
pH	7.31	7.36	7.40	0.108	0.961
NH ₃ -N (mg/100 mL)	7.69	6.59	7.11	0.666	0.839
MCP (mg/mL)	0.93	1.46	1.25	0.097	0.056
Protozoa (×10 ⁵ n/mL)	2.42 ^a	2.17 ^{ab}	1.33 ^b	0.210	0.041
TVFA (mmol/L)	39.29	33.64	28.28	2.385	0.172
Proportion of TVFA (%)					
Acetate	70.06	69.32	71.66	1.053	0.460
Propionate	14.90	16.44	16.26	0.807	0.061
Isobutyrate	0.90 ^b	1.45 ^a	1.88 ^a	0.154	0.003
Butyrate	11.85	8.75	6.26	1.123	0.111
Isovalerate	1.42 ^b	2.92 ^a	2.90 ^a	0.262	0.001
Valerate	0.86	1.12	1.04	0.055	0.149
Acetate/Propionate	4.70	4.22	4.41	0.327	0.073

The different superscripts in the same row indicate significant difference ($p < 0.05$). L-C (C:F = 48:52), M-C (C:F = 60:40), H-C (C:F = 72:28).

3.4. Rumen Microbial Diversity of Yaks Fed Diets with Different C:F Ratios

According to Figure 1A, a total of 1201 common ASVs were found in the rumen of the three groups of yaks, with 4574 (L-C), 3920 (M-C), and 3998 (H-C) unique ASVs. It was found that the three groups shared 739 ASVs, along with their own unique ASVs, which were 1484 (L-C), 1080 (H-C), and 887 (M-C) respectively (Figure 1B).

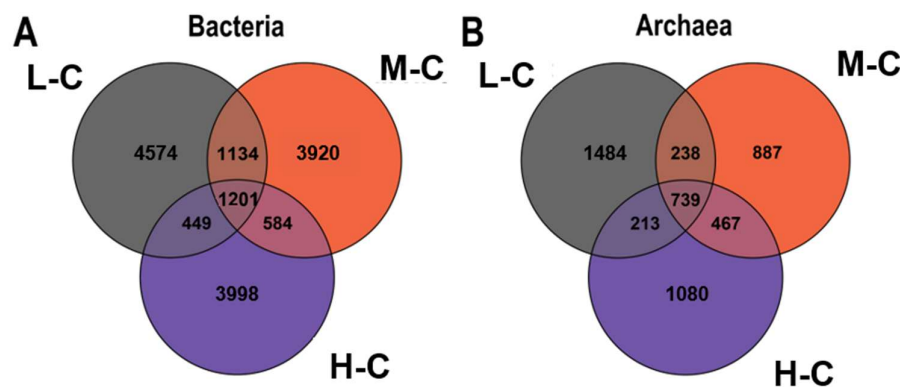


Figure 1. Effects of three diets with different C:F ratios on rumen microbial ASVs distribution in yaks. (A) Bacterial ASV distribution, (B) Archaeal ASV distribution.

There was no difference in bacterial α -diversity among the three groups (Figure 2A), but there were differences in archaeal α -diversity (Figure 2C). The Simpson index of the L-C group was significantly lower than those of the M-C and H-C groups ($p < 0.05$). The similarity or differences in the composition of microbial communities among samples were investigated using the PCoA algorithm, and the PERMANOVA (Adonis) test was utilized for statistical examination and identification. Significant differences in rumen archaeal communities were found among the three groups (Figure 2D).

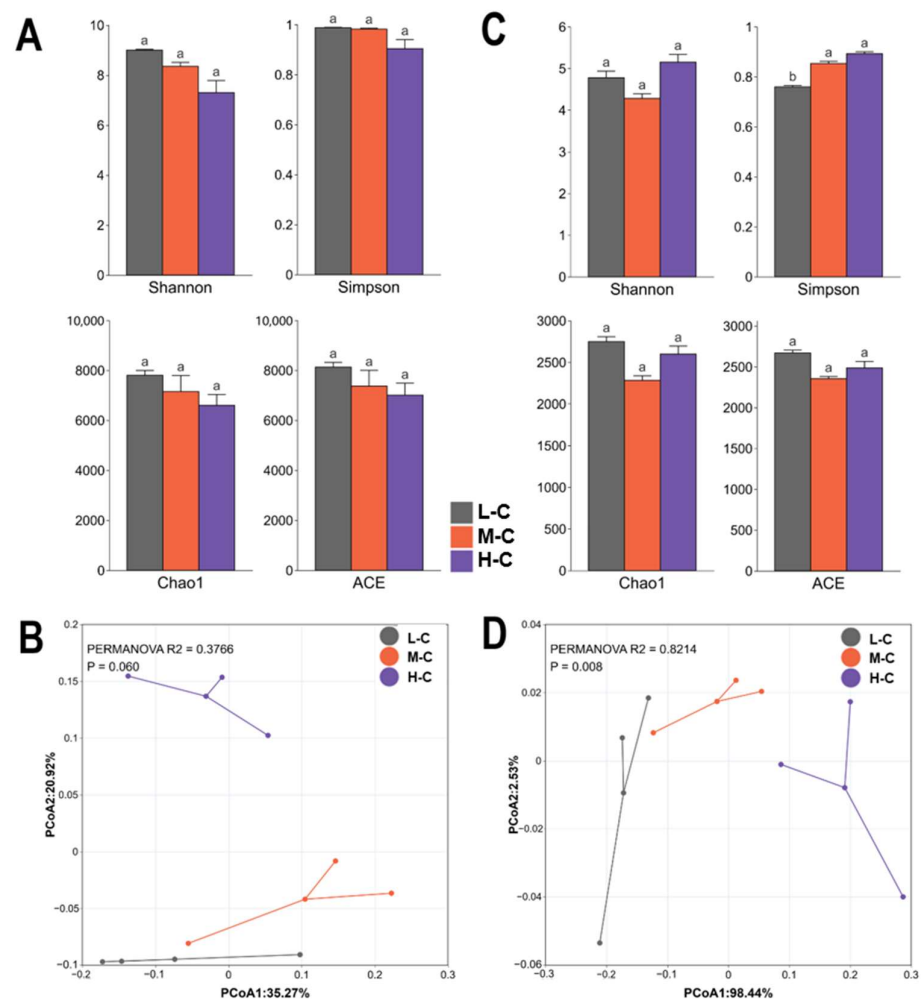


Figure 2. Effects of three diets with different C:F ratios on rumen microbial α -diversity and β -diversity in yaks. (A,C) α -diversity indices (Shannon, Simpson, Chao1, ACE) for bacteria and archaea, (B,D) β -diversity analysis (PCoA) for bacteria and archaea. The different superscripts letters indicate significant difference ($p < 0.05$).

3.5. Rumen Microbial Distribution of Yaks Fed Diets with Different C:F Ratios

Figure 3 showed the relative abundance of bacteria and archaea at the phylum and genus levels. In the bacterial community, the dominant phyla across the three groups were Firmicutes, Bacteroidota, and Proteobacteria. As the C:F ratio increased, the relative abundance of Firmicutes decreased, while the relative abundance of Bacteroidota and Proteobacteria increased (Figure 4A, $p < 0.05$). At the bacterial genus level, there were significant differences between the three groups: *Ruminobacter*, *Succiniclasticum*, *Paraprevotella*, *Pseudoflavonifractor*, *Ornithobacterium*, *Pseudobutyrvibrio* and *Bilophila* ($p < 0.05$).

Euryarchaeota was the largest component of rumen archaeal at the phylum level (Figure 3B). *Methanobrevibacter* and *Thermogymnomonas* were the two main components of rumen archaeal at the genus level, accounting for more than 98.7% of the total archaea (Figure 3D). *Methanobrevibacter* was the predominant methanogen genus in yak rumen, with its relative abundance decreasing as the dietary C:F ratio increased (L-C, 98.36%; M-C, 83.46%; H-C, 62.02%), with a significant difference between pairwise comparisons (Figure 4B, $p < 0.05$). On the contrary, the relative abundance of *Thermogymnomonas* increased with increasing dietary C:F ratio (L-C, 1.61%; M-C, 16.07%; H-C, 36.71%), with a significant difference between pairwise comparisons (Figure 4B, $p < 0.05$).

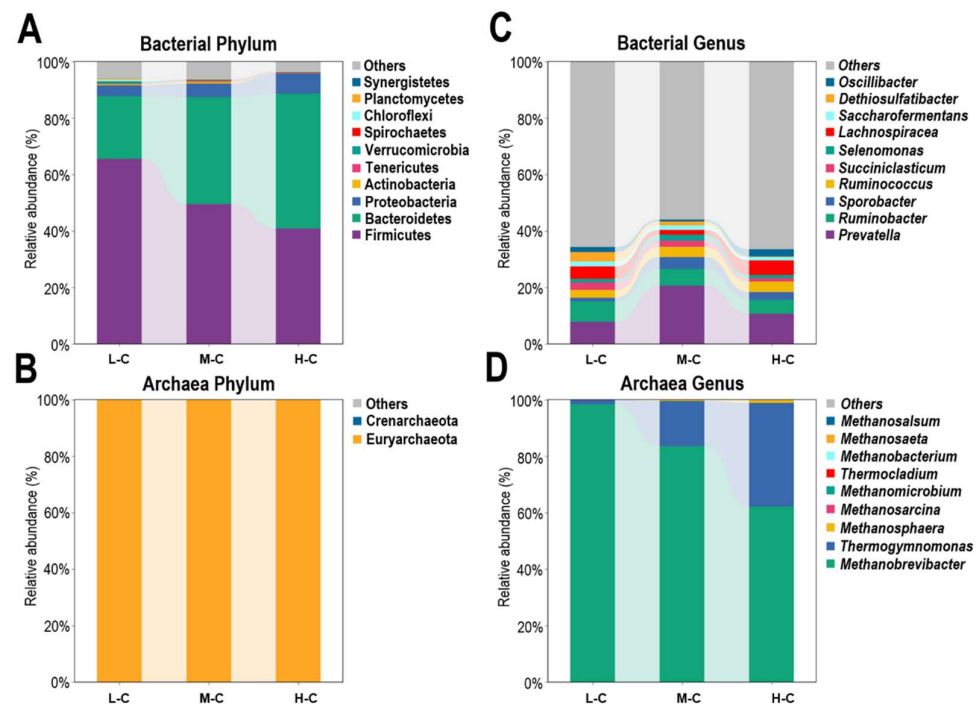


Figure 3. Effects of three diets with different C:F ratios on rumen microbial distribution in yaks. (A,B) bacterial and archaeal phyla, (C,D) bacterial and archaeal genera.

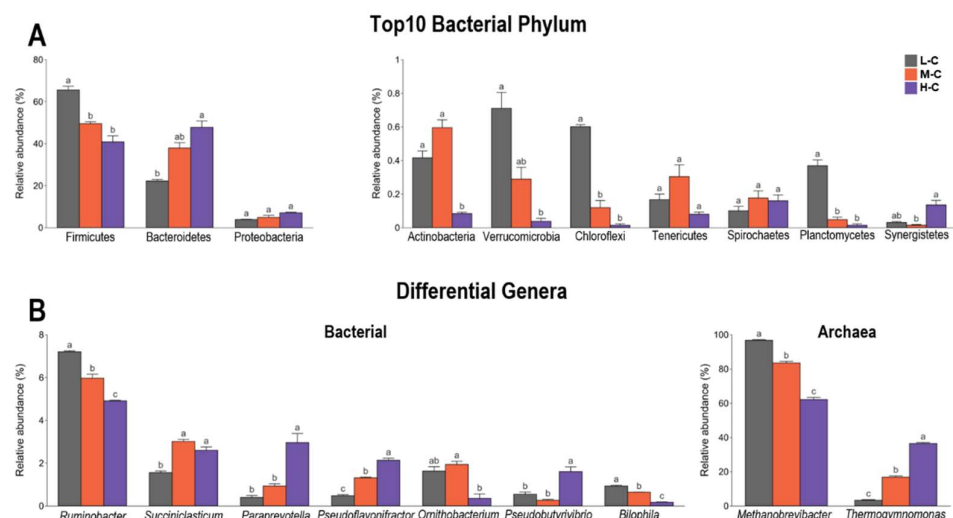


Figure 4. Effects of three diets with different C:F ratios on rumen microbial community structure in yaks. (A) Relative abundance changes at the bacterial phylum level (Top 10 Bacterial Phylum). (B) Relative abundance changes in differentially abundant genera of bacteria and archaea. The different superscripts letters indicate significant difference ($p < 0.05$).

3.6. Spearman Correlation Analysis

The heatmap in Figure 5 presented the results of a Spearman correlation analysis that had been conducted between various bacterial and archaeal genera in the rumen of yaks and different phenotypic traits, including ADG, F/G, CH₄/BWG, rumen pH, protozoa, propionate, isobutyrate and isovalerate. *Methanobrevibacter* was found to be positively correlated with protozoa, F/G and CH₄/BWG, but negatively correlated with ADG, propionate, isovalerate and isobutyrate. In contrast, *Thermogymnomonas* exhibited a pattern opposite to that of *Methanobrevibacter*.

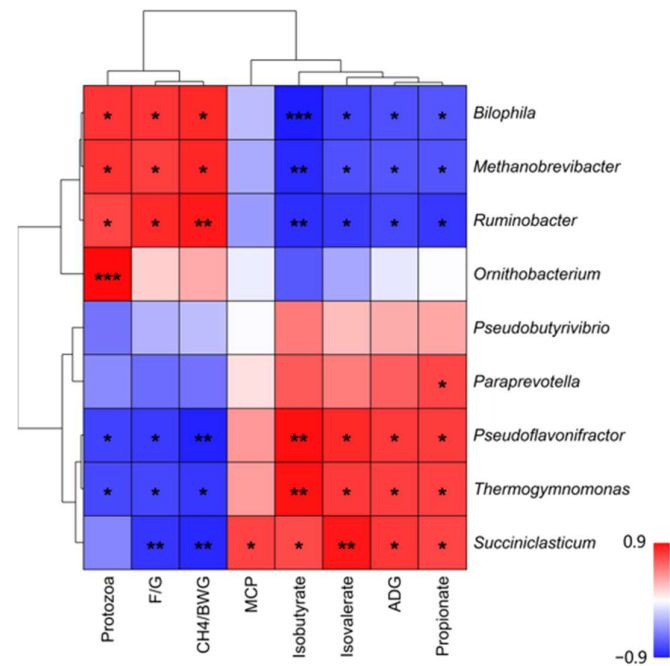


Figure 5. Spearman correlation analysis between bacterial and archaeal genera and fermentation parameters, growth performance in yaks. Correlations with $p < 0.05$ after false discovery rate (FDR) correction using the Benjamini–Hochberg method are shown with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4. Discussion

In the present study, increasing the dietary concentrate-to-forage (C:F) ratio from 48:52 (L-C) to 60:40 (M-C) or 72:28 (H-C) resulted in a numerical decrease in CH₄ emission per unit of dry matter intake (CH₄/DMI), although this reduction did not reach statistical significance, which may be attributed to the relatively small sample size (three yaks per group for CH₄ measurement) and individual variation. With such limited power, moderate but biologically meaningful differences may not reach statistical significance, larger-scale studies are warranted to confirm these trends. Nevertheless, the decreasing trend is consistent with a previous housing study in yaks, where CH₄/DMI values were 23.2, 17.0, and 14.4 g/kg DMI for C:F ratios of 100:0, 40:60, and 60:40, respectively [20]. More importantly, we found that CH₄ emission per unit of body weight gain (CH₄/BWG) was significantly lower in the M-C and H-C groups compared with the L-C group, with the M-C group showing the lowest value (161.29 vs. 312.52 g CH₄/kg BWG in L-C), which is a more relevant indicator than CH₄/DMI for assessing the environmental cost of animal production as it integrates both emission intensity and growth efficiency. The pronounced reduction in CH₄/BWG resulted from the combination of a moderate decrease in daily CH₄ production and a substantial increase in body weight gain; furthermore, similar findings have been reported in grazing and confined yaks [32], confirming that concentrate supplementation improves feed efficiency and simultaneously lowers methane intensity. Additionally, all experimental diets contained rapeseed (2–4% of DM), and dietary inclusion of rapeseed has been shown to reduce enteric CH₄ emissions, likely due to its moderate lipid content and potential effects on rumen protozoa and methanogens [33]; consistent with this, our previous work demonstrated that increasing rapeseed levels in yak diets decreases CH₄ yield [34], so the observed methane mitigation in the M-C and H-C groups may also be partially attributable to the rapeseed component, although the main driver is considered to be the increased C:F ratio.

Ruminal pH, $\text{NH}_3\text{-N}$, and VFA concentrations are key indicators of microbial community stability and nutrient metabolism. In this study, rumen pH did not differ significantly among dietary groups and remained relatively high (>7.30), which is above the typical range of 5.3–7.0 reported for ruminants [22]. This can be explained by the sampling time: rumen fluid was collected before the morning feeding (fasting condition), when feed from the previous day had been almost completely digested. After overnight metabolism, little substrate remained for microbial fermentation, reducing VFA production and thus preventing a drop in pH [35]. The absence of significant differences in pH across groups further supports that pre-feeding sampling minimized postprandial fermentation effects [36,37]. These findings confirm that our experimental conditions were appropriate for baseline rumen-fermentation assessment, which is essential for testing our hypothesis that increasing the C:F ratio alters rumen metabolism and reduces CH_4 emission.

High-concentrate diets modulate methanogenesis by shifting rumen fermentation toward propionate production. Starch and sugars in high-concentrate feeds are rapidly fermented and preferentially metabolized via the propionate pathway (succinate or pyruvate pathway), which consumes hydrogen (H_2) [38]. Since methanogenic archaea require H_2 as a substrate for CH_4 production ($\text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$), the competitive use of H_2 by propionate synthesis directly reduces substrate availability for methanogenesis [39]. In our study, although the increase in propionate was only a trend ($p = 0.061$), the direction supports this mechanism. Moreover, the branched-chain volatile fatty acids (BCVFAs) isobutyrate and isovalerate were significantly higher in the M-C and H-C groups compared with L-C. BCVFAs promote rumen microbial growth and nitrogen retention, and previous research has shown that isobutyrate supplementation linearly decreases methane production in Simmental bulls [40]. Additionally, protozoa do not produce CH_4 directly but maintain symbiotic relationships with methanogens, supplying H_2 and a favorable microenvironment [41,42]. High-concentrate diets, being lower in NDF, limit protozoal proliferation, which in turn reduces methanogen-associated H_2 supply. In our study, the H-C group had significantly lower protozoal counts than the L-C group, consistent with the trend of lower CH_4 production. These results collectively support our hypothesis that increasing the C:F ratio reduces CH_4 emission per unit of body weight gain (CH_4/BWG), as observed in the M-C and H-C groups.

The structure and function of the rumen archaeal community are directly linked to CH_4 emissions. *Methanobrevibacter* is the predominant methanogenic genus in ruminants [43], and our high-throughput sequencing data confirmed that *Methanobrevibacter* and *Thermogymnomonas* together accounted for $>98.7\%$ of total archaea, consistent with previous yak studies [44]. With increasing C:F ratio, the relative abundance of *Methanobrevibacter* decreased (from 98.36% in L-C to 83.46% in M-C and 62.02% in H-C), while *Thermogymnomonas* increased correspondingly. Spearman correlation analysis showed that *Methanobrevibacter* was positively correlated with CH_4/BWG , F/G, and protozoal counts, and negatively correlated with ADG, propionate, isobutyrate, and isovalerate. *Thermogymnomonas* exhibited the opposite pattern; the remaining *Methanobrevibacter* population may have upregulated per-cell activity to compensate for reduced numbers. Additionally, the concurrent increase in *Thermogymnomonas* (from 1.61% to 36.71%) may have contributed to methanogenesis via alternative pathways that are not yet fully understood. Finally, the limited statistical power associated with $n = 3$ per group for CH_4 measurements restricts the ability to detect moderate differences, and future studies with larger sample sizes and functional assays are needed to clarify these relationships. These findings strongly support our hypothesis that the dietary C:F ratio shapes the rumen archaeal community in a way that reduces methanogenic activity and improves growth efficiency [45]. Although the specific role of *Thermogymnomonas* in yak rumen has not been previously reported, its inverse relationship

with *Methanobrevibacter* and positive correlation with growth-related parameters suggest it may be a beneficial or compensatory taxon under high-concentrate feeding, possibly reflecting yaks' unique adaptation to high-altitude environments [46]. Overall, the reduced *Methanobrevibacter* abundance in the M-C and H-C groups contributed to lower CH₄/BWG, improved ADG, and reduced F/G, confirming that a moderate C:F ratio (60:40) is optimal for balancing productivity and environmental sustainability in winter-housed yaks (Figure 6).

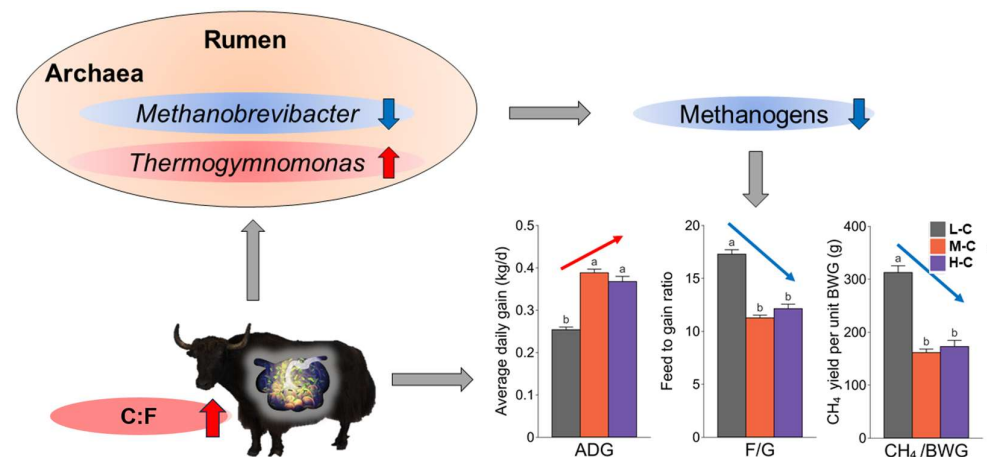


Figure 6. Increasing the C:F ratio in yak diets reduces CH₄ production per unit of body weight gain and enhances growth performance. Red arrows: increase/positive effect; blue arrows: decrease/suppressive effect. The different superscripts letters indicate significant difference ($p < 0.05$).

5. Conclusions

Increasing corn grain inclusion and reducing hay proportion in winter-housed yaks significantly reduced CH₄/BWG, improved ADG and lowered F/G, with the most favorable effects observed in the M-C group. Elevated C:F ratios significantly decreased the relative abundance of the archaeal genus *Methanobrevibacter* while increasing that of *Thermogymnomonas* in the rumen.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The corresponding author may provide supporting data for this research upon reasonable demand.

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

Abbreviations

The following abbreviations are used in this manuscript:

ADG	Average daily gain
CH ₄	Methane
C:F	Concentrate-to-forage
SF ₆	Sulfur hexafluoride
BWG	Body weight gain
CO ₂	Carbon dioxide
GWP	Global warming potential
VFA	Volatile fatty acid
H ₂	Hydrogen
DM	Dry matter
EE	Ether extract
CP	Crude protein
NDF	Neutral detergent fiber
ADF	Acid detergent fiber
MCP	Microbial protein
FDR	False discovery rate
DMI	Dry matter intake
IBW	Initial body weight
FBW	Final body weight
F/G	Feed to gain ratio
BCVFA	Branched-chain volatile fatty acid
MFS	Methylgreen-formalin-sodium chloride
ASVs	Amplicon sequence variants
PCoA	Principal coordinate analysis
PERMANOVA	Permutational multivariate analysis of variance
NH ₃ -N	Ammonia nitrogen
ECD	Electron capture detector
FID	Flame ionization detector
BH	Benjamini–Hochberg

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