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Article Title (within 20 words without abbreviations)	Reducing methane emissions in Tibetan sheep at high altitudes through indoor feeding of a low-fibre total mixed pellet diet
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Author	Mengjiao Sun1, 2, Hangqi Han1, Xiaoxue Zhang1, Huiyun Liu1, He Ding1, Tana1, Xiaozhen Liu1, Xiaoqing Zhang1, 3*
Affiliation	1Grassland Research of Chinese Academy of Agricultural Sciences, Hohhot 010010, China 2College of Prataculture, Qingdao Agricultural University, Qingdao 266109, China 3Northern Agriculture and Livestock Husbandry Technology Innovation Center, Hohhot 010010, China
ORCID (for more information, please visit https://orcid.org)	Mengjiao Sun (https://orcid.org/0009-0009-4173-6333) Hangqi Han (https://orcid.org/0009-0002-8079-0147) Xiaoxue Zhang (https://orcid.org/0009-0009-3023-0150) Huiyun Liu (https://orcid.org/0009-0009-5422-0918) He Ding (https://orcid.org/0000-0003-2245-3847) Tana (https://orcid.org/0000-0003-4906-0103) Xiaozhen Liu (https://orcid.org/0000-0003-4208-0961) Xiaoqing Zhang (https://orcid.org/0000-0002-3334-282X)
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Authors' contributions Please specify the authors' role using this form.	Conceptualization: Xiaoqing Zhang. Data curation: Mengjiao Sun, Huiyun Liu. Formal analysis: Mengjiao Sun. Methodology: Xiaoxue Zhang, Xiaozhen Liu. Software: Mengjiao Sun, Hangqi Han, Xiaoxue Zhang. Validation: Mengjiao Sun, Xiaoqing Zhang. Investigation: Hangqi Han, He Ding, Tana. Writing - original draft: Mengjiao Sun. Writing - review & editing: Mengjiao Sun, Hangqi Han, Xiaoxue Zhang, Huiyun Liu, He Ding, Tana, Xiaozhen Liu, Xiaoqing Zhang.
Ethics approval and consent to participate	All animal experimental procedures were conducted in strict accordance with the approved protocol (Protocol Number: 2023001) of the Animal Care and Use Committee of the Institute of Grassland Research, Chinese Academy of Agricultural Sciences, in Hohhot, China. The experiments were conducted in full compliance with the guidelines issued by the Chinese Science and Technology Committee.

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Xiaoqing Zhang
Email address – this is where your proofs will be sent	zhangxiaoqing@caas.cn
Secondary Email address	-
Address	Grassland Research of Chinese Academy of Agricultural Sciences, Hohhot 010010, China
Cell phone number	+86-15648108199
Office phone number	0471-4926884
Fax number	-

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(Unstructured) Abstract (up to 350 words)

This study aimed to the effects of low-fibre total mixed diets on ruminal microorganisms and the potential mechanisms of methane (CH_4) reduction in high-altitude regions remain understudied. In this study, 18 Tibetan sheep of the same age and weight were divided into two groups at random: the grazing group (GF, control) and the house-feeding group (HF). The GF group grazed on natural pasture, while the HF group was fed a low-fibre total mixed pellet diet indoors. The experiment lasted 78 days, and CH_4 emissions were measured via gas exchange in CH_4 emissions monitoring cage during the final three days. The results showed that compared to the GF group, the HF group had significantly higher dry matter intake and average daily gain ($p < 0.01$), and the animals in the HF group experienced an hour delay in the onset of peak CH_4 emissions and had significantly lower ($p < 0.05$) CH_4 emissions per unit of intake and per unit of weight gain. Additionally, the total volatile fatty acid concentration and molar proportion of propionic acid were significantly higher ($p < 0.01$) in the HF group than in the GF group. Rumen metagenome analysis revealed a significant reduction ($p < 0.05$) in rumen microbial diversity in the HF group. Regarding the bacterial composition, the relative abundances of *Prevotella* and *Ruminococcus* were significantly higher ($p < 0.05$), while the abundance of *Butyrivibrio* was significantly lower ($p < 0.05$) in the HF group. Regarding archaeal composition, the relative abundances of the *Methanobrevibacter* genus and *Methanobrevibacter millerae* were significantly higher ($p < 0.05$) in the HF group, whereas the relative abundance of unclassified_*f_Candidatus Methanomethylophilaceae* was significantly lower ($p < 0.05$). Furthermore, the relative abundances of several enzymes involved in methanogenesis (EC: 2.1.1.90, EC: 2.1.1.246, EC: 2.1.1.247, EC: 2.1.1.248, EC: 1.8.7.3, EC: 1.8.98.1, EC: 1.8.98.4, EC: 1.8.98.5 and EC: 1.8.98.6) were significantly reduced ($p < 0.05$) in the HF group. Overall, feeding low-fibre total mixed pellets indoors could reduce CH_4 emissions by modulating the rumen microbiota. This study's findings provided a valuable reference for optimising feeding management in high-altitude regions to mitigate CH_4 emissions from ruminants.

Keywords (3 to 6):

High-altitude region, Tibetan sheep, Grazing, Low-fibre total mixed pellet diet, Rumen metagenome,

Methane emissions

Introduction

Methane (CH_4) is the second most significant greenhouse gas after carbon dioxide (CO_2). Its global warming potential is 28 times that of CO_2 [1], and it accounts for approximately 20% of global greenhouse gas emissions [2]. In recent years, reducing CH_4 emissions has become a critical focus of global climate governance. China's central government introduced the CH_4 Emissions Control Action Plan in 2023, which is now being implemented nationwide, including in Tibet. Globally, CH_4 emissions account for 40%–45% of greenhouse gas emissions from ruminant livestock, in which leads to a loss of 2%–12% of dietary energy [3–4]. By the end of 2023, China's ruminant livestock population had reached 750 million [5]. Higher CH_4 emissions are typically associated with increased livestock production inputs. Therefore, reducing CH_4 emissions is crucial for environmental sustainability and improving animal production efficiency.

In the intricate digestive process of ruminants, CH_4 is an unavoidable metabolic by-product of rumen microbial fermentation. It is mainly produced through synergistic interactions between fibre-degrading microbiota and methanogenic bacteria within the rumen ecosystem. Rumen microorganisms break down plant fibres into nutrients such as volatile fatty acids (VFAs), hydrogen (H_2), and carbon dioxide (CO_2) through synergistic metabolism, in which methanogens synthesise CH_4 from H_2 and CO_2 [6]. This microbially mediated CH_4 production mechanism makes ruminants a primary source of greenhouse gas emissions from livestock production. The Qinghai-Tibetan Plateau, the world's most elevated pastureland, has fostered unique rumen microbiome characteristics in its endemic yak and Tibetan sheep species, shaped by extreme hypoxic conditions. These plateau-adapted microbes may influence CH_4 emissions by regulating metabolic pathways.

Gangba sheep are mainly raised in Kamba County, Xizang Autonomous Region, China. These exceptional local Tibetan sheep have adapted to the challenging environment of the Qinghai-Tibetan

Plateau, which boasts an average elevation of 4,700 m above sea level. Currently, the Tibetan sheep population is estimated at around 30 million, and is vital for the maintenance and economic prosperity of nomadic communities throughout the Tibetan Plateau [7]. Gangba sheep generally graze year-round with no supplementation and receive only a small amount of maize during extremely cold winters. This grazing practice results in low productivity and greater CH_4-associated energy loss. Pastureland is also under constant degradation pressure owing to overgrazing. To maximise animal productivity and promote grassland restoration, traditional grazing strategies are gradually being replaced by housing and semi-housing systems [8]. Compared with traditional grazing, housing and feeding on total mixed diets enable animals to consume more highly digestible, lower-fibre diets, leading to increased live weight. Extensive prior research has predominantly centered around the influence of dietary fibre content on animal CH_4 emissions [9–10]. Santander et al. [11] reported that a reduction inneutral detergent fibre (NDF) content from 54.3% to 49.1% in a forage-based diet resulted in a maximum 8%decreased in CH_4 yield (g/kg dry matter intake) among Angus steers. A diet containing reduced levels of NDF can reduce CH_4 emissions compared with a diet having elevated NDF concentrations. Naturally grazing animals rely on a high-fibre diet from pasture, which inevitably influences CH_4 production. As expected, on the Qinghai-Tibetan Plateau, Ding et al. [12] found that yaks housed and fed on total mixed diets produced less CH_4 than those grazing on natural winter alpine meadows. However, current research remains incomplete concerning the possible effects of grazing and housing systems on CH_4 production and the underlying mechanisms influencing CH_4 emissions in Tibetan sheep. Therefore, this trial aims to assess the effect of housing-fed Tibetan sheep on a low-fibre total mixed pellet diet on dry matter intake, growth performance, and CH_4 emissions, as well as the potential mechanism for reducing CH_4 production by altering the rumen microbiota in a high-altitude region. The hypothesis is that compared with naturally grazing Tibetan sheep, those fed a low-fibre total mixed pellet diet indoors will produce lower CH_4 emissions and exhibit improved growth performance. This study provides a foundation for understanding the key mechanisms behind CH_4 reduction through housing feeding, with positive implications for the sustainable management of livestock systems in harsh high-altitude environments.

Materials and Methods

Animal ethics statement

All animal experimental procedures in this study were performed in strict accordance with the protocol approved by the Animal Ethics Committee of the Grassland Research, Chinese Academy of Agricultural Sciences (Protocol no. 2023001). The experiments were carried out in full compliance with the guidelines issued by the Chinese Science and Technology Committee.

Study site

The experiment was conducted between April and June 2023 on a local farm in Kamba County (88°08'20"–88°56'47"E, 27°56'32"–28°45'27"N), situated at an elevation of 4,700 m above sea level, in Xizang Autonomous Region, China. Kamba County is situated within the alpine landform of the Himalayas and has approximately 24,348 hm^2 of grassland. With an average annual temperature of 1.5°C and annual rainfall ranging from 280 to 300 mm, the regional climate is temperate and semi-arid. The rainy season typically occurs from July to September, and the frost-free period lasts about 60 days. The dominant forage species in the experimental area mainly include *Festuca wallichanica*, *Artemisia minor*, *Iris collettii*, *Kobresia capillifolia* and *Kobresia deasyi*.

Animal and experimental design

In this study, there were 18 male Gangba lambs and they were randomly assigned to two equal groups, which ensured similar body weight (BW, 14.48 ± 0.26 kg) and age (approximately one year old). The two treatment groups (n = 9) were: the grazing group (GF, control) and the house-fed group (HF). Lambs of the GF group grazed under the local traditional natural management system from 10:00 to 20:00 h without any supplementation. In contrast, lambs of the HF group were housed in individual pens and administered a low-fibre total mixed pellet diet. The low-fibre total mixed pellet diet was created based on the Chinese Feed Standard for Meat-Producing Sheep (NY/T816–2004) [13]. The dietary composition, expressed on a dry matter basis, included 480 g/kg concentrate and 52 g/kg oat hay. The HF group was fed twice a day at 10:00 h and 20:00 h. Throughout the trial period, water was freely accessible to all lambs. The nutritional component of the pellet diet is presented in Table 1.

The study lasted for 78-day included an initial 8-day adaptation period followed by a 70-day experimental period. The pellet diet provided to the HF group was adjusted according to the lambs' intake from the previous day during the adaptation period. The feed consumed and refused by each lamb was weighed daily, with the difference used to calculate individual daily intake in the HF group. Alkanes (C_{32}-alkane) were applied to assess pasture dry matter intake (DMI) in GF group during the spring months (April, May, and June), following the method previously described by Zhang et al. [14]. During the experimental period, the BW was weighed early in the morning at 15 days intervals, and the average daily gain (ADG) of the sheep in each group was calculated.

Rumen fluid sample preparation

On the final morning of the feeding trial, six sheep were randomly selected from each group, and approximately 50 mL of rumen fluid was obtained from each sheep using an oral stomach tube. To minimise saliva contamination, the initial 100–200 mL of rumen fluid was removed prior to sample processing. The remaining fluid samples were then subjected to filtration using a four-layer cheese-cloth. The extracts were centrifuged at 12,000 × *g* for a 10-minute. The resultant supernatants were carefully collected and allocated to 5-mL frozen tubes, and preserved at –80°C in liquid nitrogen for future metagenomic sequencing. In addition, a separate 5-mL aliquot of rumen fluid was collected from each sheep and analysed to VFA profile using a gas chromatograph (Agilent 6850, Agilent Technologies Inc., Santa Clara, CA, USA).

Determination of CH_4 emissions

At trial completion, nine sheep with similar body conditions were randomly selected from the GF and HF groups to measure CH_4 emissions. Three sheep from each group were rotated into the homemade CH_4 emissions monitoring cage daily for three consecutive days. During the experimental period, the monitored ambient temperature was 19.78–23.80°C, and the actual atmospheric pressure was 59.49–59.60 kPa. The CH_4 emissions monitoring cage was designed as a cubic structure composed of a metal framework, and its top and four sides were covered with nylon fabric, ensuring a semi-enclosed environment for accurate gas measurement. The bottom of the monitoring cage was left open with ventilation gaps to facilitate gas exchange and maintain a relatively comfortable environment for the animals. A portable gas detector (MS600, Yiyuntian Electronics Co., Ltd., Shenzhen, China) was suspended from the center of the

monitoring cage, positioned near the top to avoid contact with the sheep's head, allowing for continuous measurement of CH_4 concentration. The size of the monitoring cage was 1.5 m × 1.5 m × 2.0 m (4.5 m^3), and its space was enough for individual sheep. The animals were placed in the monitoring cage from 20:00 h to 11:00 h the following day for continuous monitoring of daily emissions. The detailed methodology was described in study of Zhao et al. [15]. The instrument could measure from 0–100% Vol, with an allowable error of < ± 1% (F.S) and a minimum reading of 0.001%. Calibrate the instrument using eight CH_4 standard gases of 0.50%, 0.40%, 0.20%, 0.10%, 0.05%, 0.04%, 0.03% and 0.02%, respectively. When the measured data on standard gases were < 0.40%, correction was required using the regression model of y = 1.1223 x + 0.0192 ($R^2 = 0.995$; $p < 0.001$).

A 15-hour trapezoidal area accumulation method was employed to quantify total CH_4 emissions. The concentration curve was constructed by plotting monitoring time (in 5-minute intervals) on the x-axis against real-time CH_4 concentration (%) on the y-axis, with sequential trapezoidal units derived from the dataset.

The total CH_4 concentration was calculated using the following equation:

$$\text{Total } CH_4 \text{ concentration (\%)} = \sum_{i=1}^{n} \left[\frac{(C_i + C_{i+1})}{2} \right] \times \Delta t$$

where C_i and C_{i+1} represent the initial and final concentrations of each trapezoidal segment, and Δt denotes the monitoring interval (0.083 h, equivalent to 5 min). The daily total CH_4 emissions (g) from the ovine herds were calculated using the following equation:

$$\text{Total } CH_4 \text{ emissions (g)} = \frac{\text{Total } CH_4 \text{ concentration (\%)} \times \rho\ CH_4\left(g/m^3\right) \times V(m^3)}{100}$$

where ρ CH_4 (g/m^3) represents the CH_4 mass concentration, and V denotes the volume of the CH_4 emissions monitoring cage (4.5 m^3). The ρ CH_4 (g/m^3) was determined using the following equation:

$$\rho\ CH_4\ (g/m^3) = \frac{16\ g/mol}{V\ air} \times 1000\ L/m^3$$

where 16 g/mol is the molar mass of CH_4, and V air (L/mol) represents the molar volume of air, which is calculated as follows:

$$V\ air\ (L/mol) = \frac{29\ g/mol}{\rho\ air \times 1000\ g/kg}$$

where 29 g/mol is the molar mass of air, and ρ air (kg/m^3) represents the air density, which is calculated as follows:

$$\rho \text{ air (kg/m}^3\text{)} = 1.293 \times \frac{\text{P}}{101325 \text{ Pa}} \times \frac{273.15 \text{ K}}{\text{T} + 273.15 \text{ K}}$$

where P (Pa) represents the actual pressure, 101,325 Pa is the standard atmospheric pressure, 273.15 K is the absolute temperature, and T (°C) is the temperature inside the CH_4 emissions monitoring cage.

Rumen metagenome sequencing

Total genomic DNA was extracted from rumen contents using the Mag-Bind Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA). After DNA extraction, the genomic DNA was assessed for concentration and purity, and its integrity was verified via 1% agarose gel electrophoresis. Subsequently, the DNA was fragmented to an average length of approximately 400 bp using a Covaris M220 ultrasonicator (Gene Company Limited, China). A paired-end sequencing library was constructed using the NEXTFLEX Rapid DNA-Seq Kit (Bioo Scientific, Austin, TX, USA). Metagenomic sequencing was performed on the Illumina NovaSeq 6000 platform (Meiji Biomedical Technology Co., Ltd., Shanghai, China). Bioinformatics analysis was subsequently conducted using the Majorbio Cloud Platform (www.majorbio.com). Fastp was employed for sequence quality trimming, with low-quality reads (length < 50 bp, quality value < 20, or containing N bases) removed [16]. Metagenomic data were assembled using MEGAHIT [17], and contigs ≥ 300 bp were selected as the final assembly output. Open reading frames were predicted from each assembled contig using Prodigal [18], and a non-redundant gene catalogue was subsequently constructed with CD-HIT [19]. The rumen metagenome sequences have been deposited in the sequence read archive (SRA) of the National Centre for Biotechnology Information under accession number PRJNA1126439. Representative sequences from the non-redundant gene catalogue were aligned to the NR database using DIAMOND [20] with an e-value cutoff of $1 \times e^{-5}$ for taxonomic annotation. Protein sequences were similarly aligned against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using DIAMOND, and the same e-value threshold of $1 \times e^{-5}$ was applied to obtain functional annotations.

Statistical analysis

Data on DMI, BW, ADG, CH_4 emissions, and rumen fermentation characteristics were analysed using SPSS

27.0 software. An independent samples t-test was performed, with statistical significance set at $p < 0.05$. Alpha diversity of microbial communities was assessed using the ACE index, Chao1 index, Simpson index, and Shannon index. Beta diversity was evaluated via examination of the microbial community structural variation using unweighted UniFrac distance metrics combined with principal coordinate analysis (PCoA). The bacterial composition was analysed using a community histogram and the Wilcoxon rank-sum test at the phylum and genus levels, while archaeal composition was assessed at the phylum, genus, and species levels. Differences in KEGG enzyme gene abundance and composition were evaluated using the Wilcoxon rank-sum test, with statistical significance set at $p < 0.05$. Rumen microbiota from the phylum to genus or species levels were analysed using LDA Effect Size (LEfSe), with significance defined as $|LDA| > 4$ and $p < 0.05$. According to the Majorbio Cloud Platform, differential abundance testing and visual analysis of the relative gene abundance of enzymes in the ko00680 CH_4 metabolic pathway were conducted. Differential analysis was performed using the Wilcoxon rank-sum test with a two-tailed p-value threshold of $p < 0.05$. The FDR (False Discovery Rate) correction method was applied to control for multiple testing, and only FDR-significant results were considered for further interpretation.

Results

Feed intake and growth performance of sheep in the GF and HF groups

As shown in Table 2, compared to the GF group, the HF group had significantly higher DMI, BW and ADG ($p < 0.01$).

Differences in sheep CH_4 emissions of sheep in the GF and HF groups

A clear difference in CH_4 emissions was observed between the two groups (Fig. 1). Regarding dynamic changes, CH_4 emissions in the HF group increased rapidly from 20:00 to 22:00 h, reached the highest daily peak, and then gradually declined from 03:00 to 11:00 h. In the GF group, CH_4 emissions rose sharply from 20:00 to 21:00 h and reached the daily peak, followed by a gradual decline from 00:00 to 11:00 h. Daily CH_4 emissions (g/day) did not differ significantly between the two groups (Fig. 2A). However, CH_4 yield (g/kg dry matter intake), CH_4 emissions per kilogram of BW (g/kg BW), and CH_4 emissions per gram of average daily gain (g/g ADG) were significantly lower ($p < 0.05$) in the HF group than in the GF group

(Figs. 2B and 2D).

Rumen fermentation profiles

Rumen fermentation profiles differed significantly between the GF and HF groups (Table 3). Compared with the GF group, the HF group exhibited a higher total VFA concentration and a higher molar proportion of propionic acid ($p < 0.001$; $p = 0.003$), while the molar proportion of acetic acid and the acetic acid/ propionic acid ratio were lower ($p = 0.039$; $p = 0.002$).

Diversity analysis of rumen microorganisms from sheep in the GF and HF groups

The HF group exhibited significantly lower alpha diversity in ruminal microbiota, as indicated by the Ace and Chao1 indices, compared with the GF group ($p < 0.05$; Figs. 3A and 3B). However, the Shannon and Simpson indices did not differ significantly between the two treatments (Figs. 3C and 3D). PCoA based on Bray-Curtis distances revealed a clear separation between the rumen microbial communities of the HF and GF groups (Fig. 3E).

Analysis of rumen bacterial composition and differential flora in the GF and HF groups

Among the top 10% of rumen bacterial phyla, the dominant phyla in both groups were Bacteroidetes (46.79% vs. 48.12%) and Firmicutes (46.47% vs. 44.67%) (Fig. 4A). Compared with the GF group, the HF group exhibited significantly higher relative abundances of Actinobacteria and Fibrobacteres ($p < 0.05$; Fig. 4B), whereas the relative abundances of Spirochaetes, unclassified_d_Bacteria, Kiritimatiellaeota, and Proteobacteria were significantly lower ($p < 0.05$).

Among the top 15% of rumen bacterial genera, *Prevotella*, unclassified*_f_Lachnospiraceae*, unclassified*_f_Muribaculaceae*, unclassified*_o_Eubacteriales*, and *Ruminococcus* exhibited significantly higher ($p < 0.05$; Figs. 4C and 4D) relative abundances in the HF group compared to the GF group. The HF group exhibited significantly lower ($p < 0.05$) relative abundances of unclassified*_o_Bacteroidales*, *Butyrivibrio*, unclassified*_f_Bacteroidaceae*, unclassified*_f_Selenomonadaceae*, and *Treponema* than the GF group.

Furthermore, LEfSe analysis revealed a distinction in bacterial communities between the two groups (Fig. 4E) and identified a total of 16 differential bacteria. The abundances of the phylum Actinobacteria, as well as *Prevotella*, unclassified*_f_Muribaculacea*e, unclassified*_f_Lachnospiraceae*, and *Ruminococcus*, were

significantly higher in the HF group (|LDA| > 4, $p < 0.05$). In contrast, the abundances of unclassified_*o_Bacteroidales*, *Butyrivibrio*, unclassified_*f_Bacteroidaceae*, and unclassified_*f_Selenomonadaceae* were significantly higher in the GF group ($p < 0.05$).

Analysis of rumen archaeal composition and differential flora in the GF and HF groups

Compared with the GF group, the HF group exhibited a significantly higher ($p < 0.05$) relative abundance of the dominant rumen archaeal phylum, Euryarchaeota, but significantly lower ($p < 0.05$) relative abundances of unclassified_d_Archaea and Candidatus Thermoplasmatota (Figs. 5A and 5B). At the genus level, within the top 10% of rumen archaeal genera, the dominant genera *Methanobrevibacter* and *Methanomicrobium* exhibited significantly higher ($p < 0.05$; Figs. 5C and 5D) relative abundances in the HF group compared to the GF group. The HF group exhibited significantly lower ($p < 0.05$) abundances of unclassified_*d_Archaea*, unclassified_*f_Candidatus Methanomethylophilaceae*, unclassified_*o_Methanobacteriales*, *Methanobacterium*, unclassified_*c_Thermoplasmata*, and unclassified_*f_Methanobacteriaceae* than the GF group. At the species level, within the top 10% of rumen archaeal species, the dominant species *Methanobrevibacter* and *Methanobrevibacter millerae* exhibited significantly higher ($p < 0.05$) relative abundances in the HF group compared to the GF group. However, the HF group exhibited significantly lower ($p < 0.05$) relative abundances of *Candidatus Methanomethylophilaceae archaeon*, *Methanobrevibacter ruminantium*, *Methanobacteriales archaeon HGW-Methanobacteriales*-1, and *Methanosphaera stadtmanae* than the GF group (Figs. 5E and 5F).

A total of 24 differential archaeal taxa were identified between the two groups (Fig. 5G). The abundances of the archaeal phylum Euryarchaeota, the genus *Methanobrevibacter*, and the species *Methanobrevibacter* sp. and *Methanobrevibacter millerae* were significantly higher in the HF group (|LDA| > 4, $p < 0.05$). In contrast, the abundances of the phylum *Candidatus Thermoplasmatota*, as well as four genera—unclassified_*d_Archaea*, unclassified_*f_Candidatus Methanomethylophilaceae*, unclassified_*o_Methanobacteriales*, and *Methanobacterium*—along with four species—*archaeon*, *Candidatus Methanomethylophilaceae archaeon*, *Methanobrevibacter ruminantium*, and *Methanobacteriales archaeon HGW-Methanobacteriales*-1—were significantly higher in the GF group.

Analysis of differences in rumen CH_4 metabolism pathways between the GF and HF groups

Further analysis of the KEGG CH_4 metabolic pathway (Fig. 6) revealed three complete methanogenic pathways annotated in this experiment: the hydrogenotrophic methanogenesis pathway (M00567), the methylotrophic methanogenesis pathway (M00356 and M00563), and the acetoclastic methanogenesis pathway (M00357). The relative gene abundance of enzymes involved in the hydrogenotrophic pathway for reducing CO_2 to 5-Methyl-THMPT (EC:2.3.1.101 and EC:3.5.4.27) was significantly higher ($p < 0.05$) in the HF group than in the GF group (Fig. 6A). When catalysing the conversion of methyl compounds to Methyl-CoM, the relative gene abundance of trimethylamine methyltransferase (EC: 2.1.1.250) was significantly higher ($p < 0.05$) in the HF group. However, the relative gene abundances of enzymes EC: 2.1.1.90, EC: 2.1.1.246, EC: 2.1.1.247, and EC: 2.1.1.248 were significantly lower ($p < 0.05$) in the HF group compared with the GF group (Fig. 6B). The gene abundance of phosphate acetyltransferase (EC:2.3.1.8), which catalyses the conversion of acetate to 5-Methyl-THMPT, was higher ($p < 0.05$) in the HF group (Fig. 6C). However, the relative gene abundance of enzymes EC: 1.8.7.3, EC: 1.8.98.1, EC: 1.8.98.4, EC: 1.8.98.5 and EC: 1.8.98.6, involved in the core steps of methanogenesis, was significantly higher ($p < 0.05$) in the GF group (Fig. 6D).

Discussion

Methane (CH_4) emissions in ruminants mainly result from the fermentation of rumen carbohydrates, which produce CH_4 precursors. The generated CH_4 is then released into the environment through breathing, belching, flatulence, and faeces [3, 21]. As reported in study of Savian et al. [22] and Hammond et al. [23], the CH_4 emissions of grazing or ryegrass feeding sheep (BW, 24.0 and 51.4 kg) ranged from 12.2 to 25.6 g/d, employed the SF_6 tracer gas technique and open-circuit respiration chamber, respectively. In comparison, the 15 hours CH_4 emissions (15.26–15.28 g/d) of sheep measured in this experiment using the homemade monitoring cage were in the range of both, essentially reflecting the actual daytime CH_4 emissions of lower BW sheep (15.99–29.32 kg). In terms of daily emissions patterns, previous studies have shown that peak daily CH_4 emissions in sheep typically occured about within two hours after feeding [24–25]. In this study, the HF group exhibited a similar pattern, with peak CH_4 emissions occurring two hours

post-feeding (at 22:00 h). However, this peak was delayed by one hour compared with the GF group, which reached its peak CH_4 emissions at 21:00 h, one hour after feeding. The delayed CH_4 emissions peak in the HF group might be attributed to a shortened rumen retention time, likely owing to increased intake and reduced particle size. A higher intake of the low-fibre total mixed pellet diet could accelerate the flow rate through the gastrointestinal tract and consequently limit the time during which CH_4 is produced within the rumen [3, 26]. A reduced opportunity for rumen microorganisms to act on the diet is suggested by the shorter retention time in the HF group [26]. Consequently, the slower supply of substrates for CH_4 metabolism in the rumen might have contributed to the delayed peak in daily CH_4 emissions observed in the HF group.

The production of H_2 and CO_2 during ruminal fermentation plays a crucial role in rumen CH_4 production [27–28]. Congio et al. [29] suggested that feed intake was the most effective parameter for assessing CH_4 emissions. Similarly, Ding et al. [12] found that CH_4 emissions per kilogram of DMI were significantly reduced by 25.53% in yaks fed a mixed diet (60% oat hay + 40% concentrate) indoors compared with those grazing on alpine meadows dominated by herbage, sedge, and grass. Likewise, da Cunha et al. [30] found that grazing animals with lower feed intake and poor nutritional quality exhibited higher CH_4 emissions per kilogram of DMI and per gram of weight gain. Consistent with these findings, compared with the GF group, the HF group, which was fed a low-fibre diet, exhibited significantly lower CH_4 emissions per unit of DMI (by 29.16%), per unit of BW (by 60.82%), and per unit of average daily gain (by 91.84%). The reduction in CH_4 production was attributed to lower rumen microbial diversity in the HF group [31]. The diverse natural forage species in the GF group promoted the colonisation of various microorganisms. In contrast, the simpler composition of the low-fibre total mixed pellet diet in the HF group likely reduced rumen microbial diversity. Similarly, Shabat et al. [31] observed that low-CH_4-yield cows had lower microbiota diversity than high-CH_4-yield cows. The rumen microbiomes of low-CH_4-yield animals consist of fewer but more dominant taxa, which participate in a more limited range of metabolic pathways [32]. These microbiomes generate metabolites that more efficiently align with the energetic requirements of the host [32]. In summary, a reduction in CH_4 emissions is typically associated with lower rumen microbial diversity.

Alternatively, the reduced CH_4 yield in the HF group was mainly attributed to the low-fibre total mixed pellet diet consumed by these sheep. As demonstrated in this study, the NFC content of the HF group's diet

was 1.92 times higher than that of the high-fibre natural grass diet grazed by the GF group (46.36% vs. 24.17%). Consequently, the low-fibre total mixed pellet diet not only significantly improved feed intake and weight gain, but also supplied a substantial amount of fermentation substrate and effectively promoted the proliferation of *Prevotella* within the Bacteroidetes phylum. *Prevotella* rapidly colonised the favourable ecological niche within the rumen microbiota and enhanced the degradation and utilisation of starch and plant cell wall polysaccharides. The molar proportion of propionic acid in the rumen of HF group increased significantly as a result of these effects. Martínez-Álvaro et al. [33] reported that increased propionic acid production reduced CH_4 emissions by competing with methanogenesis for H_2 during fermentation. Furthermore, the abundance of *Butyrivibrio* was significantly higher in the GF animals, consistent with the observations of Grilli et al. [34], who also found an increased abundance of *Butyrivibrio* in high-fibre diets. *Butyrivibrio* is recognised as a biomarker of CH_4 emissions and plays a crucial role in releasing substrates that enhance CH_4 production [35]. In this trial, the significantly reduced levels of *Butyrivibrio* in the HF animals was likely due to the low fibre content of the total mixed pellet diet. Moreover, this reduction contributed to lower rumen CH_4 production in HF sheep. Analysis of the CH_4 metabolic pathway in KEGG indicated that the relative gene abundance of enzymes including EC: 1.8.7.3, EC: 1.8.98.1, EC: 1.8.98.4, EC: 1.8.98.5 and EC: 1.8.98.6 were significantly reduced in the HF group than the GF animals, which catalyze the reduction of the heterodisulfide CoM-S-S-CoB and ferredoxin by oxidizing H_2 [36–37]. The reason for this phenomenon was probably the limited availability of H_2, which could constrain the rate of ruminal methanogenesis under certain conditions [38]. These findings indicated that the HF group provided much less substrate H_2 than the GF group, which resulted in lower CH_4 yields. Therefore, the rumen microbial H_2 metabolism process was more efficient in the HF group than the methanogenic process. Overall, these results confirmed that a low-fibre total mixed pellet diet was beneficial for reducing CH_4 emissions from ruminants.

Ruminococcus is a primary cellulolytic bacterium that produces substrates for CH_4 synthesis and promotes ruminal CH_4 production [6]. Theoretically, a higher dietary fibre content should lead to greater *Ruminococcus* proliferation. However, in this trial, a higher abundance of *Ruminococcus* was observed in the HF group despite lower CH_4 production. This outcome was likely due to the high-quality total mixed

pellet diet in the HF group, which stimulated *Ruminococcus* proliferation. Nonetheless, both the findings of this trial and those of Girija et al. [39] indicated that *Ruminococcus* accounted for only a small proportion (0.89%–3.33%) of the rumen microbiota. Hence, the fermentation rate of *Ruminococcus* on the substrate was much lower than the utilisation rate of the highly abundant *Prevotella*. Aguilar-Marin et al. [40] also observed a higher abundance of *Ruminococcus* in low-CH_4-emitting buffaloes compared with high-CH_4-emitting buffaloes, which may indicate a microbiome with a higher fermentative capacity. Further analysis of the KEGG CH_4 metabolic pathway (Fig. 6C) revealed a significantly higher gene abundance of phosphate acetyltransferase (EC:2.3.1.8), which catalysed the conversion of acetylphosphate to acetyl-CoA in the acetate methanogenesis pathway, in the HF group. This finding aligned with Wallace et al. [41], who reported that the gene abundance of EC:2.3.1.8 was higher in low-CH_4-emitting cattle than in high-CH_4-emitting cattle. This finding thus suggested that EC:2.3.1.8 might contribute to lower CH_4 emissions in ruminants. In addition, the low proportion of *Ruminococcus* in the rumen appeared to have minimal impact on CH_4 emissions.

Methanogens are the sole known producers of CH_4 in the rumen and convert H_2, CO_2, acetic acid, and other compounds into CH_4 [6]. *Methanobrevibacter*, the most dominant methanogen, is associated with the hydrogenotrophic methanogenesis pathway [42]. The increased abundance of *Methanobrevibacter*, particularly *Methanobrevibacter millerae*, and the relative gene abundance of key enzymes involved in the hydrogenotrophic pathway have been associated with enhanced CH_4 production [43–44]. In this study, daily CH_4 emissions (g/d) were slightly higher in the HF sheep than in the GF sheep, although the difference was not statistically significant. This trend was aligned with higher abundances of *Methanobrevibacter*, particularly *Methanobrevibacter millerae*, and the relative gene abundance of key enzymes involved in the hydrogenotrophic pathway, likely owing to the HF sheep consuming a high-quality total mixed pellet diet that provided sufficient nutrients. Compared with the grazing group, the drylot group exhibited higher counts of *Methanobrevibacter* and relative gene abundance of key enzymes involved in the hydrogenotrophic pathway in the rumen. This finding was consistent with the findings of Zhang et al. [45], who conducted a feeding trial using the same experimental design at a similar location (Nima, Tibet, China; altitude > 4800 m) and during the same season (spring) as this study. Zhang et al. attributed this difference

to the considerably higher concentration of fermentable carbohydrates in the drylot diet. In contrast, grazing goats were provided with a comparatively lower energy-density diet when raised in extreme alpine environments. Although *Methanobrevibacter* abundance was higher, CH_4 production efficiency may have been limited by the availability of H_2. As previously discussed, the HF group provided less substrate H_2 than the GF group, which led to a significantly lower CH_4 yield (g/kg dry matter intake), as well as reduced CH_4 emissions per kilogram of BW and per gram of ADG. These findings indicated that CH_4 yield was not only dependent on methanogens but was also closely related to the availability of substrate H_2.

In this trial, the abundance of unclassified *f_Candidatus Methanomethylophilaceae* decreased by 89.63% in the HF sheep compared with the GF group. Correspondingly, the relative gene abundance of enzymes involved in the methylotrophic pathway was significantly lower in the HF group, which suggested that methylotrophic methanogenesis was less active and not favourable for CH_4 production. In summary, a reduction in the gene abundance of enzymes in the methylotrophic methanogenesis pathway could contribute to decreased CH_4 production.

Conclusion

In conclusion, this study demonstrated that a low-fibre total mixed pellet diet effectively reduced ruminal CH_4 production through microbial modulation. This diet improved feed intake and growth performance of Tibetan sheep and increased the relative abundance of *Prevotella*, while inhibiting the proliferation of *Butyrivibrio*, unclassified *f_Candidatus Methanomethylophilaceae* and decreasing the relative abundance of key methanogenic enzymes, including EC: 2.1.1.90, EC: 2.1.1.246, EC: 2.1.1.247, EC: 2.1.1.248, EC: 1.8.7.3, EC: 1.8.98.1, EC: 1.8.98.4, EC: 1.8.98.5 and EC: 1.8.98.6, thereby inhibiting the methanogenic pathway and ultimately reducing CH_4 production per unit of feed intake and per unit of weight gain. The results are beneficial for the sustainable development of green and low-carbon livestock production on the Qinghai-Tibetan Plateau.

Acknowledgements

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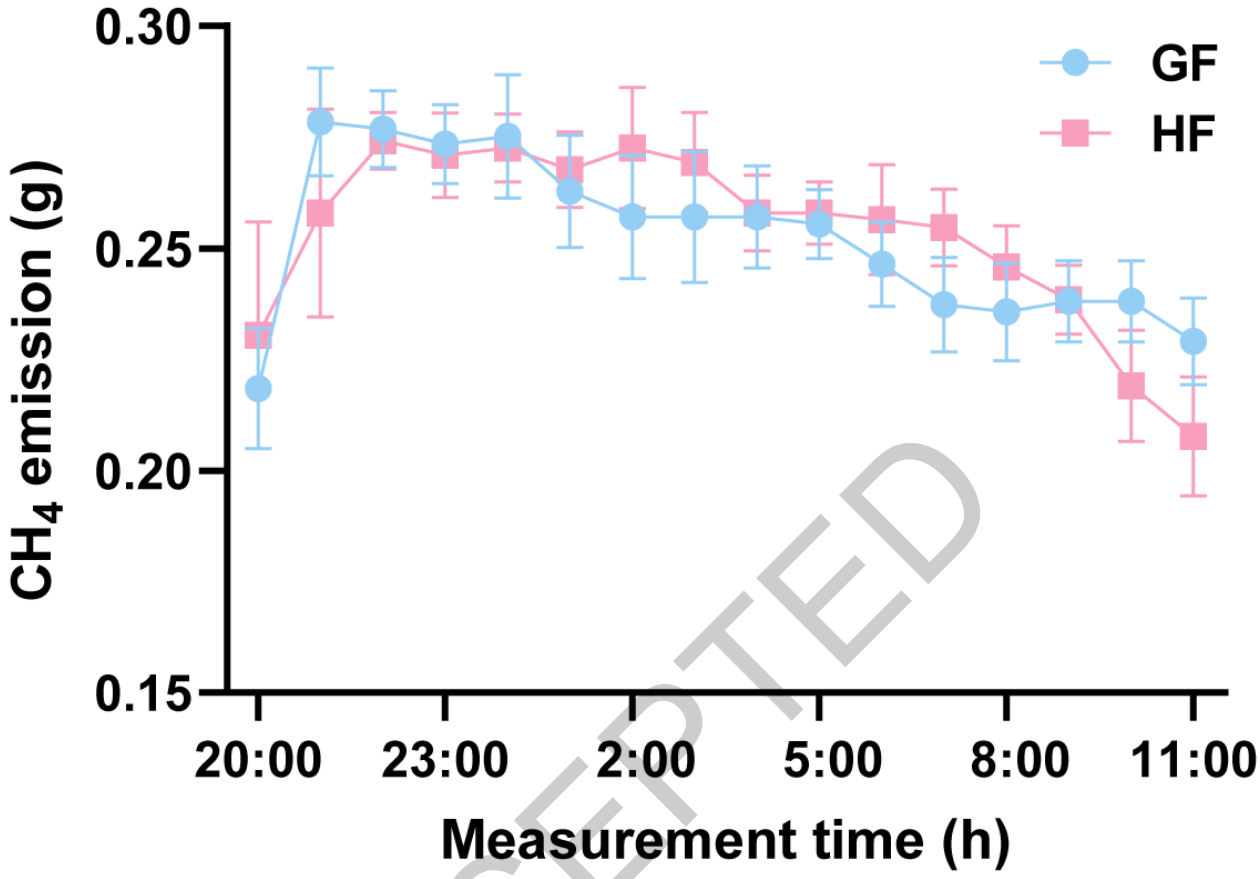
536 **Tables and Figures**



537

538 **Fig. 1.** Dynamic changes in CH_4 emissions from the GF and HF groups sheep. GF, natural grazing; HF,

539 house feeding.

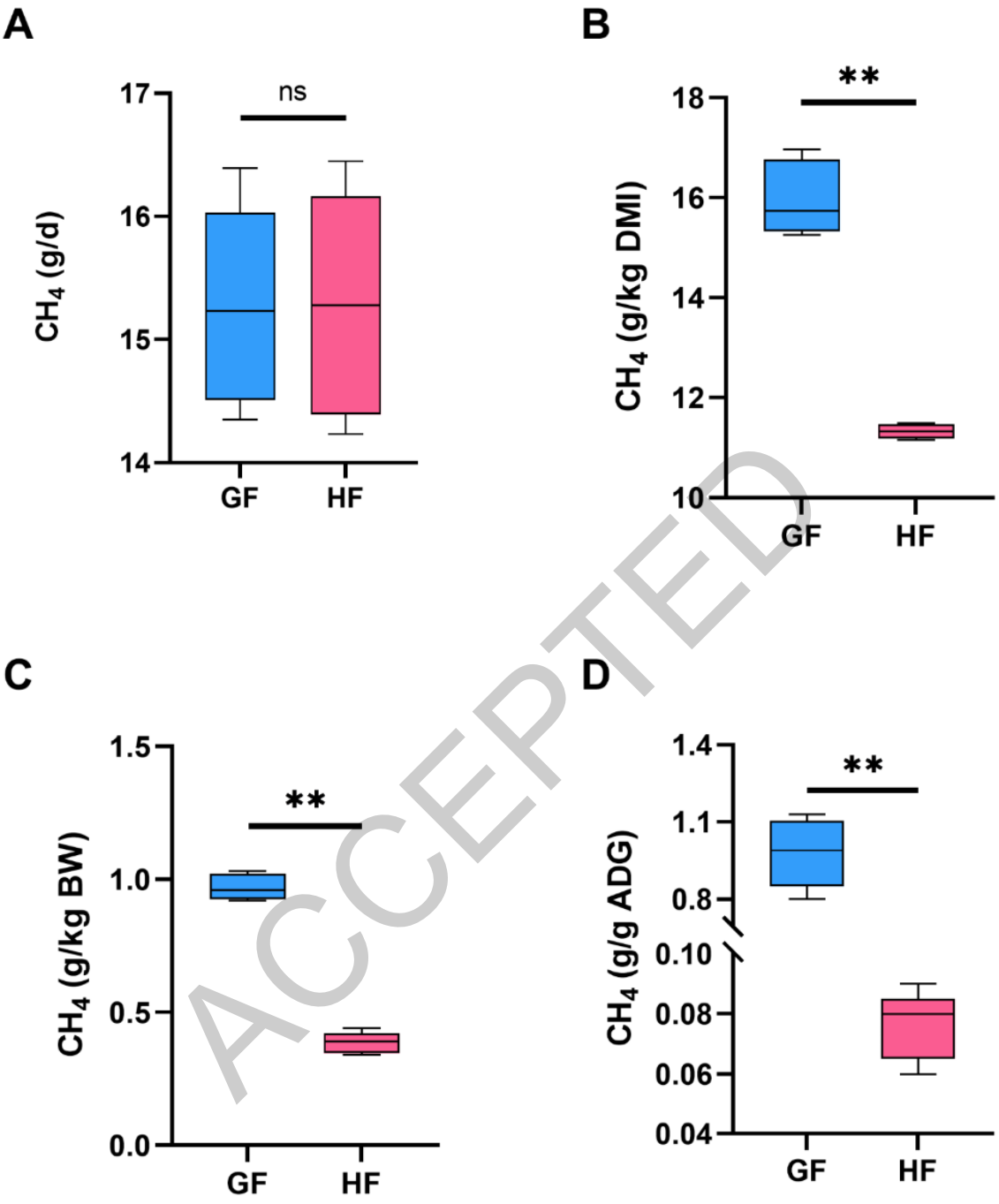
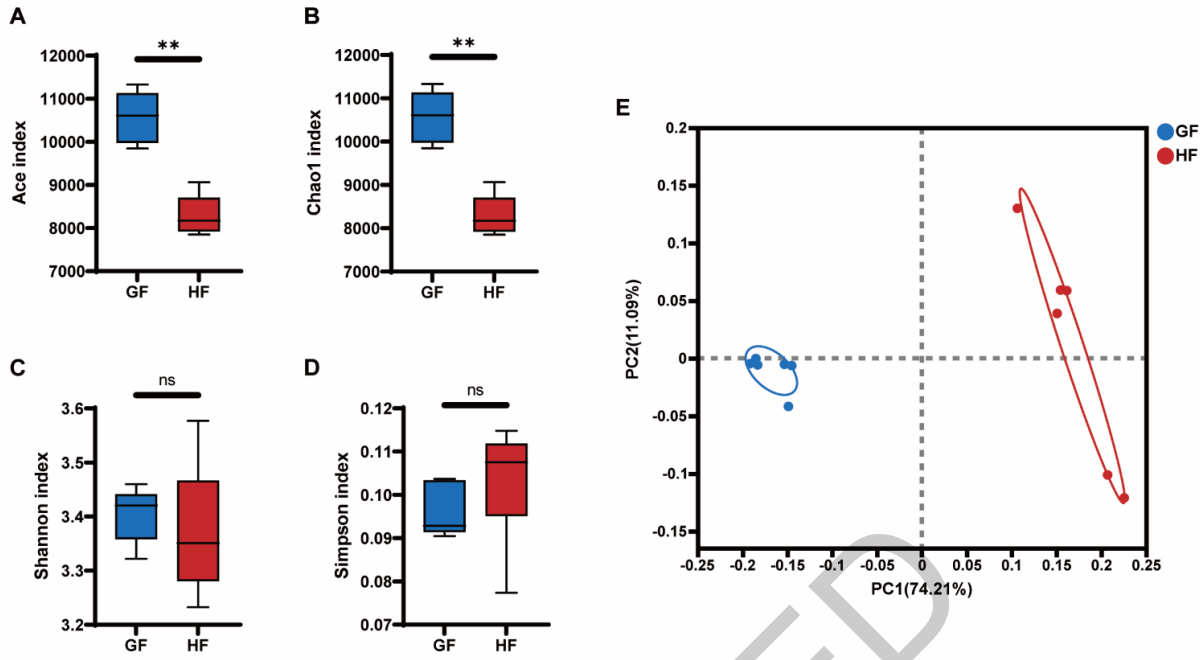


Fig. 2. CH_4 yield of the GF and HF groups sheep. (A) Daily CH_4 emissions (g/d). (B) CH_4 yield (g/kg dry matter intake). (C) CH_4 emissions per kilogram of body weight (g/kg BW). (D) CH_4 emissions per gram of average daily gain (g/g ADG). GF, natural grazing; HF, house feeding. $^{ns}p > 0.05$, $*p < 0.05$, $**p < 0.01$.



544

545 **Fig. 3.** Diversity of rumen microorganisms of the GF and HF groups sheep. (A) The alpha diversity of the
546 Ace index of rumen microorganisms. (B) The alpha diversity of the Chao1 of rumen microorganisms. (C)
547 The alpha diversity of the Shannon index of rumen microorganisms. (D) The alpha diversity of the Simpson
548 index of rumen microorganisms. (E) Principal co-ordinates analysis (PCoA) score plot of rumen
549 microorganisms. GF, natural grazing; HF, house feeding. $^{ns}p > 0.05$, $*p < 0.05$, $**p < 0.01$.

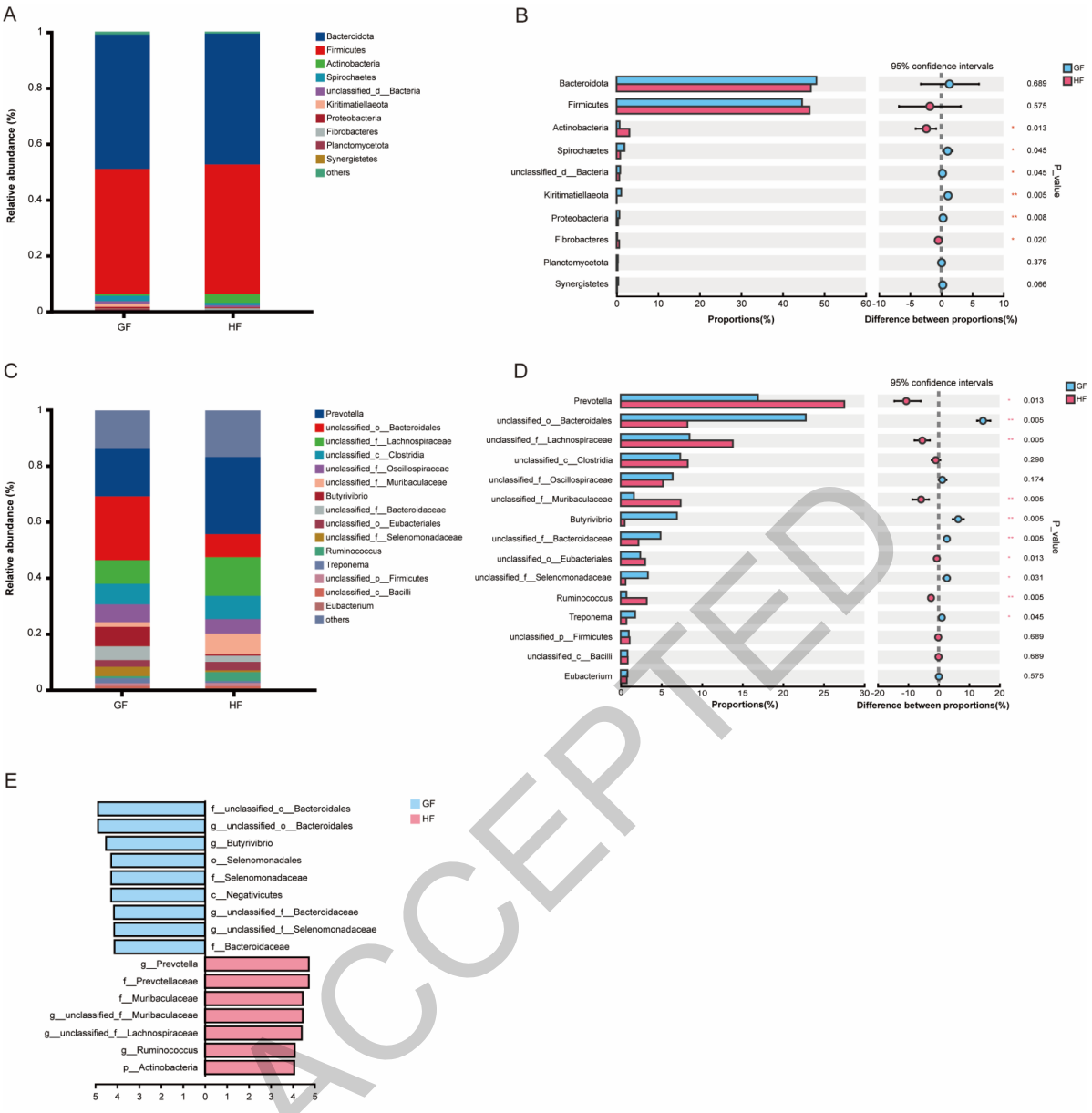


Fig. 4. Composition and differential of rumen bacteria between the GF and HF groups sheep. (A) Relative abundances of rumen bacteria at the phylum level. (B) The differences in relative abundance of dominant bacteria at the phylum level were assessed by the Wilcoxon rank-sum test. (C) Relative abundances of microbiota communities at the genus level. (D) The differences in relative abundance of dominant bacteria at the genus level were assessed by the Wilcoxon rank-sum test. (E) Significantly different bacterial. GF, natural grazing; HF, house feeding. *$p < 0.05$, **$p < 0.01$.

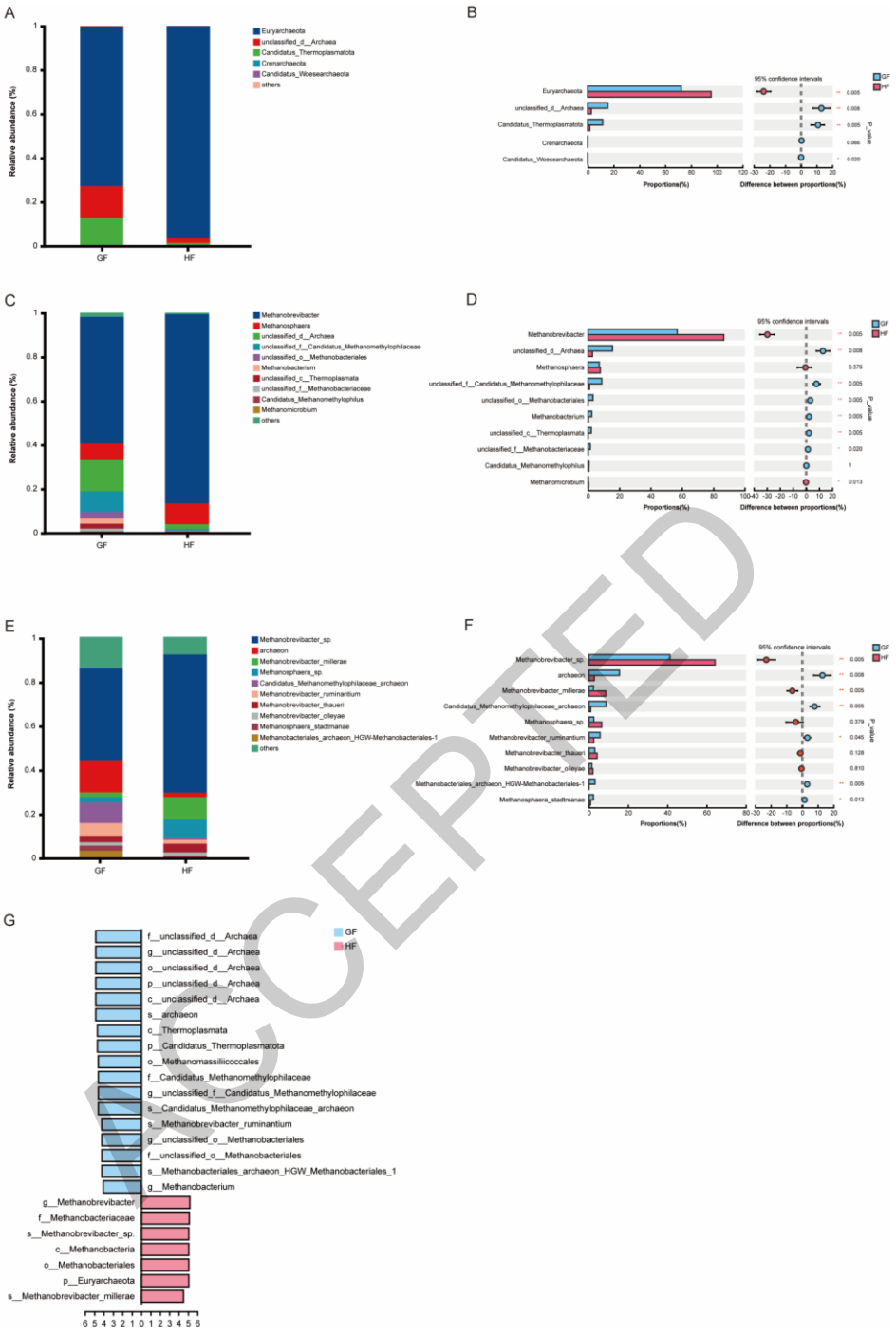


Fig. 5. Composition and differential of rumen archaea between the GF and HF groups sheep. (A) Relative abundances of rumen bacteria at the phylum level. (B) The differences in relative abundance of dominant bacteria at the phylum level were assessed by the Wilcoxon rank-sum test. (C) Relative abundances of microbiota communities at the genus level. (D) The differences in relative abundance of dominant bacteria at the genus level were assessed by the Wilcoxon rank-sum test. (E) Relative abundances of microbiota communities at the species level. (F) The differences in relative abundance of dominant bacteria at the species level were assessed by the Wilcoxon rank-sum test. (G) Significantly different bacterial. GF, natural grazing; HF, house feeding. *$p < 0.05$, **$p < 0.01$.

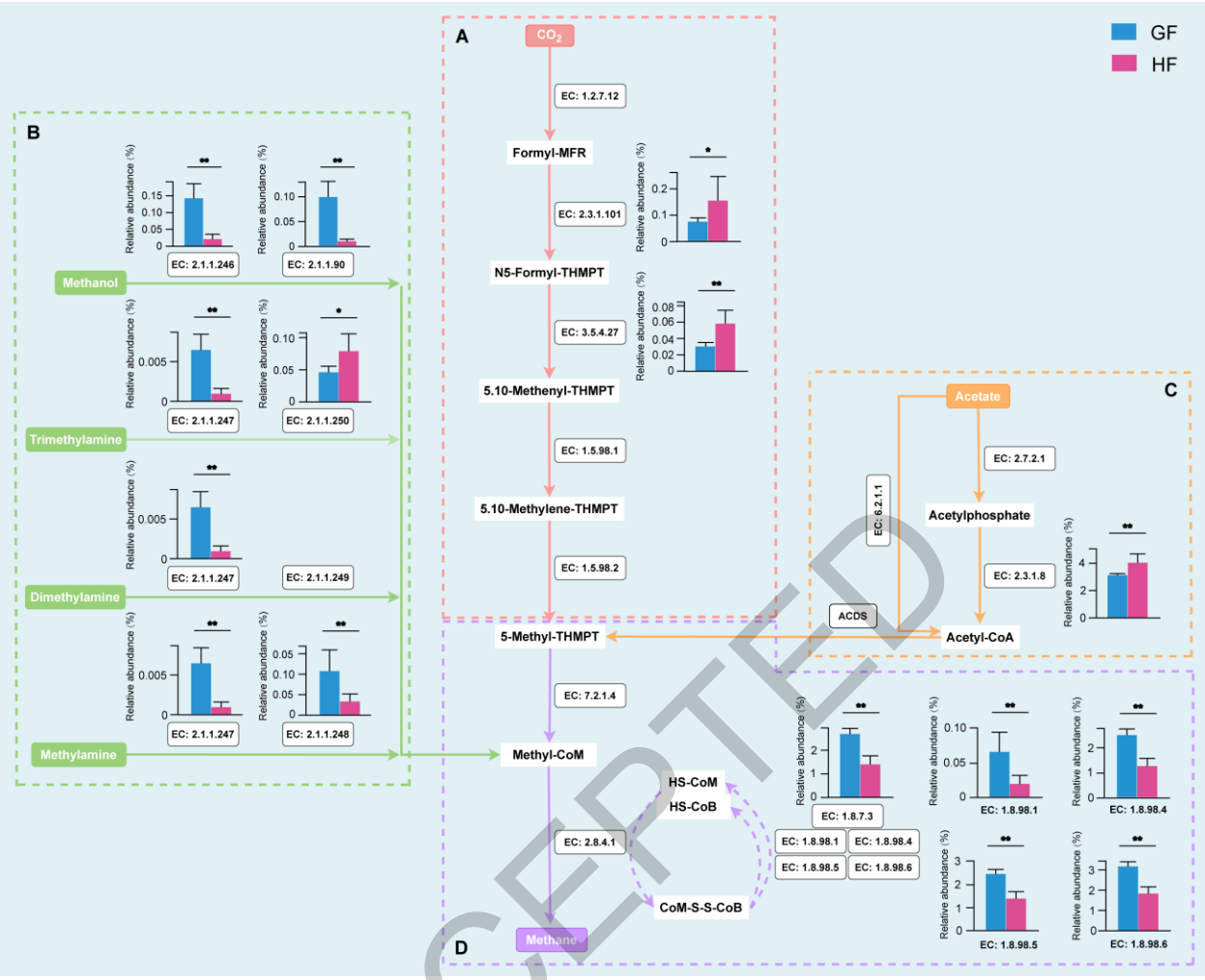


Fig. 6. The methanogenesis pathways and relative gene abundances of related enzymes between the GF and HF groups. (A) Hydrogenotrophic methanogenesis pathway (KEGG pathway entry MD: M00567). (B) Methylotrophic methanogenesis pathway (KEGG pathway entry MD: M00356 and M00563). (C) Acetoclastic methanogenesis pathway (KEGG pathway entry MD: M00357). (D) Core steps in the methanogenesis pathway. KEGG, Kyoto Encyclopaedia of Genes and Genomes; THMPT, tetrahydromethanopterin; EC: 2.3.1.101, formylmethanofuran-tetrahydromethanopterin N-formyltransferase; EC:3.5.4.27, methenyltetrahydromethanopterin cyclohydrolase; EC:2.1.1.246, coenzyme M methyltransferase; EC:2.1.1.90, methanol-5-hydroxybenzimidazolylcobamide Co-methyltransferase; EC:2.1.1.247 coenzyme M methyltransferase; EC:2.1.1.250, trimethylamine-corrinoid protein Co-methyltransferase; EC:2.1.1.248, methylamine-corrinoid protein Co-methyltransferase; EC:2.3.1.8, phosphate acetyltransferase; EC: 1.8.7.3, CoB, CoM: ferredoxin oxidoreductase; EC: 1.8.98.1, coenzyme B: coenzyme M: methanophenazine oxidoreductase; EC: 1.8.98.4, CoB, CoM, ferredoxin:coenzyme F420 oxidoreductase; EC: 1.8.98.5, CoB, CoM, ferredoxin: H_2 oxidoreductase; EC: 1.8.98.6, coenzyme B, coenzyme M, ferredoxin:formate oxidoreductase. GF, natural grazing; HF, house feeding. The Wilcoxon rank-sum test tested the significance of the relative gene abundances of enzymes, $*p < 0.05$, $**p < 0.01$.

583 **Table 1.** Ingredients and nutrient composition of natural mixed forage and low-fibre total mixed pellet diet

584 (dry matter [DM] basis)

Item	Groups	
	Natural mixed forage[3]	low-fibre total mixed pellet diet
Ingredients (%)		
Oat green hay	-	52.00
Corn	-	28.00
Soybean meal	-	13.00
Wheat bran	-	5.00
Limestone powder	-	0.50
Premix[1]	-	1.00
NaCl	-	0.50
Total	-	100.00
Nutrient levels		
Digested energy (MJ/kg)	-	12.69
CP (%)	21.73	13.34
EE (%)	3.13	3.24
NDF (%)	42.53	38.64
ADF (%)	25.06	21.53
Ash (%)	8.44	8.42
NFC[2] (%)	24.17	46.36

585 [1)]The premix provides per kilogram of diet: 940 IU vitamin A, 20 IU vitamin E, 200 mg S, 25 mg Fe, 40

586 mg Zn, 8 mg Cu, 0.3 mg I, 40 mg Mn, 0.2 mg Se, 0.1 mg Co.

587 [2)]NFC (%) = 100 - (CP + EE + NDF + ash), while other nutrient levels were measured values.

588 [3)]Natural mixed forage was collected in June.

589 GF, natural grazing; HF, house feeding; CP, crude protein; EE, ether extract; NDF, neutral detergent fibre;

590 ADF, acid detergent fibre; Ash, crude ash; NFC, non-fibre carbohydrate.

591

592 **Table 2.** Feed intake and growth performance of sheep in the GF and HF groups sheep

Item	Groups		SEM	*p*-value
	GF	HF		
Dry matter intake (kg/d)	0.68[b]	1.03[a]	0.066	0.006
Body weight (kg)	15.99[b]	29.32[a]	1.811	< 0.001
Average daily gain (g/d)	19.01[b]	204.21[a]	24.672	< 0.001

593 [a,b] Within the same row, values with different small letter superscripts mean significant difference ($p <$

594 0.05), while with the same or no letter superscripts mean no significant difference ($p > 0.05$).

595 GF, natural grazing; HF, house feeding.

596 **Table 3.** Rumen fermentation parameters in GF and HF groups sheep

Item	Groups		SEM	*p*-value
	GF	HF		
TVFA (mmol/L)	36.12[b]	57.93[a]	4.94	< 0.001
Acetic acid (%)	73.55[a]	67.04[b]	1.74	0.039
Propionic acid (%)	17.58[b]	22.25[a]	1.09	0.003
Acetic acid/ Propionic acid	4.19[a]	3.02[b]	0.27	0.002
Butyric acid (%)	4.58	4.34	0.25	0.679
Valeric acid (%)	0.63	0.79	0.06	0.250

597 [a,b] Within the same row, values with different small letter superscripts mean significant difference ($p <$

598 0.05), while with the same or no letter superscripts mean no significant difference ($p > 0.05$).

599 GF, natural grazing; HF, house feeding; SEM, standard error of the mean; TVFA, total volatile fatty acids.