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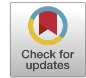
Rumen metagenome reveals the mechanism of mitigation methane emissions by unsaturated fatty acid while maintaining the performance of dairy cows



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ABSTRACT

Dietary fat content can reduce the methane production of dairy cows; however, the relevance fatty acid (FA) composition has towards this inhibitory effect is debatable. Furthermore, in-depth studies elucidating the effects of unsaturated fatty acids (UFA) on rumen function and the mechanism of reducing methane (CH_4) production are lacking. This study exposed 10 Holstein cows with the same parity, similar milk yield to two total mixed rations: low unsaturated FA (LUFA) and high unsaturated FA (HUFA) with similar fat content. The LUFA group mainly added fat powder (C16:0 > 90%), and the HUFA group mainly replaced fat powder with extruded flaxseed. The experiment lasted 26 d, the last 5 d of which, gas exchange in respiratory chambers was conducted to measure gas emissions. We found that an increase in the UFA in diet did not affect milk production ($P > 0.05$) and could align the profile of milk FAs more closely with modern human nutritional requirements. Furthermore, we found that increasing the UFA content in the diet lead to a decrease in the abundance of *Methanobrevibacter* in the rumen (|linear discriminant analysis [LDA] score| > 2 and $P < 0.05$), which resulted in a decrease in the relative abundance of multiple enzymes (EC:1.2.7.12, EC:2.3.1.101, EC:3.5.4.27, EC:1.5.98.1, EC:1.5.98.2, EC:6.2.1.1, EC:2.1.1.86 and EC:2.8.4.1) during methanogenesis ($P < 0.05$). Compared with the LUFA group, the pathway of CH_4 metabolism was inhibited in the HUFA group (|LDA| > 2 and $P < 0.05$), which ultimately decreased CH_4 production ($P < 0.05$). Our results illustrated the mechanism involving decreased CH_4 production when fed a UFA diet in dairy cows. We believe that our study provides new evidence to explore CH_4 emission reduction measures for dairy cows.

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1. Introduction

Methane (CH_4) is 28 times more potent as a greenhouse gas than carbon dioxide (CO_2), and it accounts for 14% of global greenhouse gas emissions (IPCC, 2014). Enteric CH_4 emissions from livestock production have been documented to be 98.7 million tonnes in 2016 (Ornelas et al., 2019). Methane emissions from ruminants are mainly produced through gastrointestinal fermentation and primarily originate in the rumen. Therefore, a reduction in CH_4 emissions from ruminants is mainly achieved through a reduction in rumen CH_4 production. Reducing CH_4 production in ruminants through nutritional means is more advantageous than breeding because it produces immediate results. Increasing the dietary fat content is one of the most effective nutritional strategies to reduce CH_4 emissions in ruminants (Rabiee et al., 2012). Some studies showed that CH_4 production decreases in dairy cows as the fat content of the diet increases (Alvarez-Hess et al., 2019; Brask et al., 2013). However, the relation between the inhibitory effect of fat on

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rumen CH_4 and the composition of fatty acids (FA) is debatable. Some studies showed that the reduction in CH_4 production in the rumen by fat is positively correlated to the degree of unsaturation across the FAs (Giger-Reverdin et al., 2003). In contrast, some researchers believed that dietary fat reduced rumen CH_4 emissions in a way unrelated to the FA profile (Grainger and Beauchemin, 2011).

Typically, the dietary fat content of high-yield dairy cows is approximately 5%–6%, with almost 3% of the fat arising from forage grass and grains, the remainder is supplemented with fat powder, which contains over 90% of the FA that comprise the 6% fat present in the milk (Bionaz et al., 2020). Dietary fat in the rumen undergoes two processes: fat hydrolysis and biohydrogenation. Fat is hydrolysed in the rumen to produce FAs, which are rapidly hydrogenated (Lourenço et al., 2010). A study revealed that the rumen fermentation reduces by approximately 50% when the fat content in the diet is over 10%, especially when more unsaturated fatty acids (UFA) are supplemented in the diet (Jenkins, 1993). This phenomenon could be attributed to the fact that numerous FAs adhere to the feed surface, which hinders the microbes from being able to hydrolyse the feed substrate (Jenkins, 1993). Most methanogens rely on the coupling of methanogenesis with membrane potential generation for ATP production (Müller et al., 1986). However, FAs can potentially disrupt the electron transport chain by binding to electron carriers or altering membrane integrity, while also interfering with oxidative phosphorylation by decreasing membrane potential and the proton gradient (Yoon et al., 2018), meanwhile, UFA undergo biohydrogenation in the rumen, resulting in hydrogen consumption (Jenkins et al., 2008). Therefore, these two factors may be the reason UFA reduce CH_4 emissions from the rumen. In some of our in vitro previous studies, changes in FA composition and UFA ratio in the diet were also found to significantly reduce CH_4 production in the rumen (Sun et al., 2022; Yang et al., 2022). Furthermore, the addition of UFA (soybean oil or flaxseed oil) to the diets of dairy cows and beef cattle reduced daily CH_4 production in vivo (Jordan et al., 2006; Martin et al., 2008). Consequently, some previous in vivo studies explored the effect of the percentage of dietary UFA on CH_4 emission but did not keep fat levels consistent (Beauchemin et al., 2009; Brask et al., 2013; Martin et al., 2016). These studies focused on effects of UFA on the milk production, rumen fermentation, CH_4 production, etc. of dairy cows. In vivo studies elucidating the effects of UFA on rumen microorganisms, rumen function, and the mechanism behind the reduced CH_4 production, with similar fat contents are lacking. Thus, the purpose of this study is to determine whether UFA can reduce the CH_4 production in the rumen without affecting the production performance of dairy cows and explore the underlying mechanisms of how UFA reduce CH_4 production.

This study used two total mixed ration (TMR) diets with different levels of UFA while maintaining the same fat levels in dairy cows. The apparent performance of UFA was evaluated in dairy cows, together with the effects of UFA on rumen microbes, rumen function, and the mechanism of mitigation of CH_4 using high-throughput sequencing technology. We hypothesised that diets containing high UFA concentrations would reduce methane production in dairy cows by affecting the composition of the rumen microbiota and rumen function. This study is anticipated to provide an important understanding of how UFA decrease CH_4 emissions and provides a theoretical basis for exploring CH_4 mitigation in dairy cows.

2. Materials and methods

2.1. Animal ethics statement

All procedures were approved by the Ethical Committee of the College of Animal Science and Technology of China Agriculture University (approval number: AW61902202-1-3). All applicable institutional and national guidelines for the care and use of animals were followed.

2.2. Experimental design, and diets

The experiments were performed at Zhongdi Dairy Farm in Shunyi District, Beijing, China. Ten lactating Holstein cows with similar days in milk (209 ± 9.96 d) and weight (718.8 ± 38.86 kg) during the second lactation period were selected from the dairy farm and randomly divided into two groups at the start of the experiment. The two groups were fed two diets with different percentages of UFA (low UFA: LUFA = 35.5% ± 0.21%, n = 5; high UFA: HUFA = 80.60% ± 0.50%, n = 5) with similar fat content (59.5 ± 0.1 g /kg DM) replacing the fat powder (Wilmar Oil Technology Co., Ltd., Tianjin, China; C16:0 > 90%) with an equal amount of fat from extruded flaxseed in the TMR. The two diets had similar energy levels ($NE_{L\ at}$7.60 ± 0.09 MJ /kg DM) and crude protein (CP) levels (159.3 ± 0.28 g /kg DM), which were achieved by decreasing soybean meal, corn flour, and soybean hulls of total mixed ration (TMR) in the HUFA group. The diets of both groups were in accordance with those of lactation dairy cows in the late stage of the nutrient requirement of dairy cattle (NRC, 2001). The experiment was conducted over 26 d, the last 5 d of which, gas exchange in respiratory chambers was conducted, to measure gas emissions. Cows were fed a single trough during the entire experimental period. The cows were offered two TMR (Table 1) twice a day at 07:30 and 14:30 for ad libitum intake, with free access to water after milking at 07:00, 14:00, and 19:00. In respiratory chambers, cows were offered TMR twice daily at 07:00 and 18:00 for ad libitum intake, with free access to water after milking at 06:30 and 17:30.

2.3. Production performance, milk sampling, and analysis

Feed intake was recorded daily throughout the experiment using an automatic feed trough feeding system (Roughage Intake Control System, RIC, Insentec B.V., Marknesse, The Netherlands). Feed samples were collected twice weekly and dried at 55 °C for 72 h in a forced-draft oven to calculate the dry matter intake (DMI) for each cow. The dried TMR samples were ground in a feedstuff mill (KRT-34, KunJie, Beijing, China) through a 1-mm screen for the subsequent determination of nutrients. The TMR were analysed for DM content according to the method of the Association of Official Analytical Chemists, 930.15 (AOAC, 2012). Organic matter (OM), CP, and ether extract (EE) were measured using the following methods: 924.05, 984.12, and 920.39 (AOAC, 2012), respectively. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined using the Van Soest et al.'s method (1991). Heat-stable alpha amylase and anhydrous sodium sulphite were used for NDF analysis, and sulfuric acid and hexadecyl ammonium bromide were used for ADF analysis. The FA content in the diets was determined according to a method (Loor and Herbein, 2001). The FA methyl esters were extracted from the feed and determined using gas chromatography (GC) with a fused quartz capillary column (DB-23, 60.0 m × 0.25 mm × 0.25 μm, US) and a 6890N GC system (Agilent Technologies) equipped with a flame ionisation detector. The FA profile of the diets is shown in Table 2.

Milk production was recorded daily throughout the experiment. Milk samples were collected after milking for three consecutive days (days 19–21). Energy-corrected milk (ECM) and 3.5% fat-corrected milk (3.5% FCM) were calculated according to a previous method (Erdman, 2011; Jaunja et al., 1991). A subsample was stored at 4 °C with bronopol as preservative before analysis for milk composition, including milk protein, milk fat, milk lactose, and milk

Table 1
Ingredients and nutrient composition (g/kg, DM basis).

Item	Groups	
	LUFA	HUFA
Ingredients		
Corn silage	319.4	319.2
Alfalfa hay	223.2	223.0
Steam-flaked corn	86.1	86.0
Corn flour	98.4	89.8
Soybean meal	106.9	80.4
Soybean hulls	44.1	32.1
Whole cottonseeds	22.2	22.1
Corn gluten meal	22.6	22.6
Fat powder	26.2	–
Extruded flaxseed	–	73.7
Yeast culture	1.3	1.3
Sodium bicarbonate	7.3	7.3
Premix[1]	24.1	24.1
Molasses	18.3	18.3
Nutrient levels		
DM	487.1	482.8
OM	916.5	922.0
CP	159.5	159.1
NDF	262.4	260.3
ADF	168.9	166.1
EE	59.4	59.6
NE_L[2], MJ/kg	7.66	7.53

HUFA = high unsaturated fatty acid; LUFA = low unsaturated fatty acid; DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; EE = ether extract; NE_L = net energy for lactation.
[1] Additives per kilogram of premix: 440,000 IU vitamin A, 110,000 IU vitamin D_3, 4000 IU vitamin E, 400 mg niacin, 152 g Ca, 41 g P, 2970 mg Zn, 1140 mg Mn, 750 mg Cu, 36 mg Se, and 30 mg I.
[2] NE_L was the calculated value according to NRC (2001).

urea nitrogen (MUN) by an automated near-infrared milk analyser (CombiFoss FT+; Foss Electric, Hillerød, Denmark) at Beijing Dairy Cow Centre (Beijing, China). To analyse milk FA, the lipids were extracted and methylated as described in a previous study (Chouinard et al., 1997). Briefly, milk fat was extracted from freeze-dried milk solids using the Mojonnier technique with a single extraction and evaporation in the presence of nitrogen. This was followed by transesterification with sodium methoxide, and then FA content was analysed. Butter oil (10 mg) was placed in a 2-mL container and dissolved in 1 mL of hexane, with 20 μL of 0.5 mol/L Na of methanol solution added. Then, the resulting solution was gently shaken in a water bath set at 65 °C for 3.5 min, heated for 1 min without shaking, and then cooled to room temperature. Furthermore, 100 to 150 mg of silica gel was added and shaken vigorously before allowing the silica gel to settle. The liquid was aspirated and analysed using GC as described above.

Table 2
Diet fatty acid composition (g/100 g of fatty acid).

Item	Groups	
	LUFA	HUFA
C10:0	0.13	0.11
C12:0	0.60	0.34
C14:0	1.23	0.26
C15:0	0.17	0.10
C16:0	56.55	13.10
C16:1	0.20	0.20
C17:0	0.16	0.15
C18:0	4.50	3.82
C18:1n9c	9.14	18.89
C18:2n6c	20.99	28.83
C18:3n3	5.02	32.04
C20:0	0.33	0.42
C20:1	0.13	0.27
C21:0	0.02	0.05
C22:0	0.34	0.43
C23:0	0.14	0.16
C24:0	0.36	0.47
ΣUFA[1]	35.48	80.23
Σω-6UFA/Σω-3UFA[2]	4.19	0.89

HUFA = high unsaturated fatty acid; LUFA = low unsaturated fatty acid; UFA = unsaturated fatty acids.
[1] ΣUFA = the proportion of total unsaturated fatty acids.
[2] Σω-6UFA/Σω-3UFA = the ratio of total n-6 unsaturated fatty acids to ω-3 unsaturated fatty acids.

2.4. Methane and carbon dioxide emission

Cows were moved to two separate open-circuit respiration chambers in the last five days of the experiment to measure CH_4 and CO_2 emissions. The length, width, and height of the airtight chambers were 5.93 m × 2.34 m × 2.4 m, respectively. The air in the chamber flowed out at a fixed rate and a low negative pressure was maintained to prevent the air from entering the room through a ventilation duct and to prevent the loss of CH_4 and CO_2. The average air-flux velocity in the chambers was maintained at 48 ± 1 m^3/h, the temperature was maintained at 18 ± 1 °C, and the relative humidity was maintained at 50%–60%. Gas samples were drawn using a membrane pump (80 L/h; KNF Neuburger Laboport, Freiburg, Germany) to enter the gas analysis system through a polytetrafluoroethylene pipe, and the two chambers shared a gas analysis and data acquisition system. The analyses of the entrance air and exhaust air samples were performed alternately, and the CH_4 and CO_2 concentrations of the entrance air and exhaust air in each chamber were continuously measured every 320 s using an infrared gas analyser (Siemens Ultramat 6E, Munich, Germany). The measuring range of CH_4 was 0 to 1000 parts per million (ppm) and that of CO_2 was 0 to 10,000 ppm. The analyser was calibrated using pure N_2 at zero CH_4 and CO_2 concentrations and two subsequent certified mixed sample gases (one containing a concentration of 300 ppm CH_4 and 3000 ppm CO_2, and another containing 700 ppm CH_4 and 7000 ppm CO_2) as a span gas before measuring the CH_4 and CO_2 concentrations from the samples. The amount of CH_4 and CO_2 entering and leaving the chamber was calculated by determining the concentration of CH_4 and CO_2 and the airflow at the entrance and exhaust. The CH_4 and CO_2 emissions of each cow were calculated by the difference in CH_4 and CO_2 leaving and entering the chamber. Before the experiment, the CO_2 recovery rates of the two respiration chambers were measured to be (100.65 ± 0.54)% and (101.52 ± 0.58)%, respectively. The cows were allowed to adapt to the environment in the first three days of entering the open-circuit respiration chamber, and the last two days were used to collect the CH_4 and CO_2 emissions. Cows were considered adapted when they fed normally, lay down, and rested.

2.5. Rumen fluid sample and analysis

Rumen fluid samples were obtained before morning feeding for three consecutive days (days 19-21) by oral intubation. The first 50 mL of the rumen fluid sample was discarded to avoid contamination of the sample by cow saliva, and the samples were collected in four 2-mL cryopreservation tubes which were stored at −80 °C. The rumen fluid obtained on day 21 was also analysed for metagenomic determination. Furthermore, the remaining samples were stored at −20 °C for the determination of subsequent fermentation parameters. Volatile fatty acids (VFA) were determined in the rumen fluid using GC (6890 N; Agilent Technologies, Avondale, PA, USA) (Zhang and Yang, 2011), and ammonia nitrogen (NH_3–N) in the rumen fluid was measured using spectrophotometry, as previously described (Verdouw et al., 1978).

2.6. DNA extraction, metagenome sequencing, and annotation of function and taxonomy

Total genomic DNA was extracted from the rumen contents using the repeat bead-beating plus column method (Yu and Morrison, 2004). DNA purity and integrity were analysed by 1% agarose gel electrophoresis and measured using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Two samples (one from the LUFA group and one from the HUFA group) were discarded because of low DNA quantity after quantifying the number of DNA samples. The genomic DNA of the qualified DNA samples was broken into 300-bp fragments using a Covaris ultrasonic crusher, and a library was prepared by end repair, A-tail, sequencing junction, purification, and PCR amplification. The metagenome libraries were sequenced on an Illumina NovaSeq platform at Allwegene Technology Co., Ltd., Beijing, China. Then, Illumina Analysis Pipeline Version 2.6. software was used for image analysis, basic calling, and error estimation. Quality control for each dataset was performed using Trimmomatic (Bolger et al., 2014). The reads were filtered if they contained the adapter sequence, a N (uncertain base) ratio greater than 1%, or a content of low-quality bases (quality score < 20) above 50%. Fragments with read lengths less than 150 bp were filtered after quality control. The filtered reads were assembled de novo for each sample using MEGAHIT (Li et al., 2015) and contigs below 500 bp were filtered out. Contigs were annotated using Prodigal software (Hyatt et al., 2012) to predict open reading frames, and CD-HIT software (Li et al., 2001) was used to construct a non-redundant gene set. Rumen metagenome sequences were deposited into the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number PRJNA932960.

Sequences were classified into different taxonomic groups using the DIAMOND tool (Buchfink et al., 2015) tool against the NCBI Reference Sequence (RefSeq) database after quality control. Bowtie (Langmead et al., 2009) was used to compare the sequencing data with the non-redundant gene set, and the abundance of genes was determined in different samples. Taxonomic profiles were obtained for domains, phyla, classes, orders, families, genera, and species, and the relative abundances were calculated. The contigs were annotated against the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000) using DIAMOND with an E value of 1×10^{-5}. Carbohydrate-active enzymes (CAZymes) database annotation was performed using USEARCH (Edgar, 2010). KEGG Orthology (ko), KEGG enzymes, and CAZymes were normalised to count per million (cpm) reads.

2.7. Statistical analysis

This experiment adopted a completely randomised design. According to the results of the power analysis, each group was to have a power of 80.99% with a 0.05 significance level (α) based on the CH_4 emissions per kilogram of DMI from dairy cows. Intake, milk yield, milk composition, milk FA profile, gas emission, and rumen fermentation parameters were compared using the t-test with SAS version 9.4 software (SAS Institute Inc., Cary, NC, USA). The model for the above data was $Y_{ij} = \mu + Trt_i + \varepsilon_{ij}$, where Y_{ij} is the dependent variable; μ is the overall mean; Trt_i is the fixed effect of the treatment; ε_{ij} is the random error. Statistical significance was established at $P < 0.05$ meaning a significant difference. Means and standard errors are shown in the tables and figures. The alpha diversity indexes were calculated using the “vegan” and “picante” packages in R. The KEGG enzymes, the abundance and composition of CAZymes, and alpha diversity indexes were compared using the Wilcoxon rank-sum test, with the FDR adjusted P-value < 0.05 considered statistically significant. Histograms of intake, milk yield, milk composition, gas emission, and rumen fermentation parameters, differential KEGG enzymes, differential CAZymes, and violin charts of alpha diversity indexes were visualised using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

The rumen microbial phyla, genera, species, different CAZymes and abundance of KEGG pathways were compared using linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011), and significant differences were examined using an |LDA score| > 2 and P-value < 0.05. The histogram of differential microorganisms and the bubble chart of differential KEGG pathways was visualised using “ggplot” packages in R. Correlation analysis between dominant genera, species, and different KEGG pathways was performed using Spearman's rank correlation, with a P-value < 0.05 (Spearman's rank correlation coefficient) considered significant. Calculation of the correlation coefficient, P-value, and visualisation of the correlation heatmap using “corrplot” package was performed in R.

3. Results

3.1. Intake, milk yield, milk composition, and milk FA profile

In Table 3, DMI (P < 0.001), organic matter intake (OMI, $P = 0.002$), crude protein intake (CPI, $P = 0.004$), neutral detergent fibre intake (NDFI, $P < 0.001$), and acid detergent fibre intake (ADFI, P < 0.001) were lower in the HUFA group than that in the LUFA group. Milk yield ($P = 0.913$) and feed efficiency ($P = 0.330$) did not change with an increase in UFA; however, ECM ($P = 0.011$) and 3.5% FCM ($P < 0.001$) decreased in the HUFA group compared to in the LUFA group. A significant decrease was observed in milk fat (P = 0.011) and milk fat yield (P = 0.011) in the HUFA group compared to in the LUFA group. Furthermore, milk protein yield

Table 3
Intake, milk yield and composition in LUFA and HUFA groups.

Item	Groups		SEM	P-value
	LUFA	HUFA		
Intake, kg/d				
DMI	22.17[a]	20.10[b]	0.406	<0.001
OMI	19.99[a]	18.08[b]	0.309	0.002
CPI	3.41[a]	3.12[b]	0.052	0.004
NDFI	5.72[a]	5.11[b]	0.090	<0.001
ADFI	3.68[a]	3.18[b]	0.060	<0.001
EEI	1.23	1.17	0.018	0.096
Milk yield, kg/d				
MY	25.15	25.31	0.728	0.913
3.5% FCM[1]	32.22[a]	26.67[b]	0.836	<0.001
ECM[2]	31.74[a]	27.42[b]	0.813	0.011
Feed efficiency[3]	1.44	1.39	0.025	0.330
Milk composition, %				
Fat	5.39[a]	3.81[b]	0.117	<0.001
Protein	3.73[a]	3.60[b]	0.032	0.042
Lactose	4.85	4.81	0.042	0.674
MUN, mg/dL	16.69	15.18	0.777	0.340
Milk composition yield, kg/d				
Fat yield	1.34[a]	0.95[b]	0.038	<0.001
Protein yield	0.93	0.92	0.029	0.845
Lactose yield	1.23	1.23	0.042	0.998

LUFA = low unsaturated fatty acid; HUFA = high unsaturated fatty acid; SEM = standard error of the mean; DMI = dry matter intake; OMI = organic matter intake; CPI = crude protein intake; NDFI = neutral detergent fiber intake; ADFI = acid detergent fiber intake; EEI = ether extract intake; MY = milk yield; FCM = fat corrected milk; ECM = energy corrected milk; MUN = milk urea nitrogen.

[a,b] Within the same row, values with different small letter superscripts mean significant difference (P < 0.05), while with the same or no letter superscripts mean no significant difference (P > 0.05).

[1] 3.5% FCM = 0.432 × milk yield + 16.216 × fat yield.

[2] ECM = 12.96 × fat yield + 7.04 × protein yield + 0.3246 × milk yield.

[3] Feed efficiency = ECM/DMI.

(P = 0.845), milk lactose (P = 0.674), and milk lactose yield (P = 0.998) were unaffected by the UFA content (P > 0.05).

The milk FA profile is shown in Table 4. The concentrations of C16:0 (P < 0.001), C16:1 (P = 0.031), and C20:3n6 (P = 0.003) were higher in the LUFA group than in the HUFA group (41.66% vs. 26.07%, 2.44% vs. 1.63%, and 0.18% vs. 0.11%, respectively), and the ratio of ω-6 to ω-3 polyunsaturated FA (PUFA) was higher in the LUFA group (5.68% vs. 2.97%, P < 0.001). In contrast, the concentrations of C13:0 (P = 0.011), C18:0 (P = 0.020), C18:1n9c (P = 0.004), C18:2n6c (P = 0.003), C18:3n3 (P < 0.001), CLA-c9t11 (P = 0.001), CLA-t10c12 (P = 0.004), C20:0 (P = 0.039), C20:1 (P < 0.001), ΣUFA (P < 0.001), ΣCLA (P = 0.001), Σω-3 PUFA (P < 0.001), and Σω-6 PUFA (P = 0.005) were higher in the HUFA group than in the LUFA group by 25.00%, 42.68%, 44.24%, 55.17%, 220.45%, 129.17%, 200.00%, 42.86%, 180.00%, 39.06%, 146.15%, 184.91%, and 44.40%, respectively. No differences were found in the concentrations of the other FAs between the LUFA and HUFA groups.

Table 4
Milk fatty acid profile (g/100 g of fatty acid).

Item	Groups		SEM	P-value
	LUFA	HUFA		
C6:0	1.13	1.12	0.075	0.980
C8:0	0.87	0.85	0.067	0.892
C10:0	2.32	2.15	0.196	0.700
C12:0	3.10	3.18	0.187	0.850
C13:0	0.08[b]	0.10[a]	0.005	0.011
C14:0	9.22	10.16	0.416	0.289
C14:1	0.97	0.92	0.105	0.827
C15:0	0.90	0.92	0.044	0.801
C16:0	41.66[a]	26.07[b]	2.704	<0.001
C16:1	2.44[a]	1.63[b]	0.199	0.031
C17:0	0.57	0.60	0.024	0.542
C18:0	9.49[b]	13.54[a]	0.943	0.020
C18:1n9c	22.92[b]	33.06[a]	2.078	0.004
C18:2n6c	2.61[b]	4.05[a]	0.288	0.003
C18:3n3	0.44[b]	1.41[a]	0.172	<0.001
CLA-c9t11	0.24[b]	0.55[a]	0.060	0.001
CLA-t10c12	0.03[b]	0.09[a]	0.012	0.004
C20:0	0.14[b]	0.20[a]	0.015	0.039
C20:1	0.05[b]	0.14[a]	0.016	<0.001
C21:0	0.05	0.05	0.008	0.897
C20:2	0.05	0.6	0.006	0.228
C20:3n6	0.18[a]	0.11[b]	0.013	0.003
C20:4n6	0.25	0.23	0.011	0.389
C22:0	0.06	0.07	0.003	0.242
C20:5n3	0.04	0.05	0.005	0.242
C22:1n9	0.09	0.14	0.015	0.078
C23:0	0.05	0.05	0.003	0.580
C24:0	0.05	0.05	0.002	0.571
C22:6n3	0.05	0.06	0.004	0.502
ΣUFA[1]	30.08[b]	41.83[a]	2.309	<0.001
ΣCLA[2]	0.26[b]	0.64[a]	0.007	0.001
Σω-3PUFA[3]	0.53[b]	1.51[a]	0.174	<0.001
Σω-6PUFA[4]	3.04[b]	4.39[a]	0.281	0.005
ω-6PUFA/ω-3PUFA[5]	5.68[a]	2.97[b]	0.481	<0.001

LUFA = low unsaturated fatty acid; HUFA = high unsaturated fatty acid; SEM = standard error of the mean; CLA = conjugated linoleic acid; UFA = unsaturated fatty acids; PUFA = polyunsaturated fatty acids.
[a,b] Within the same row, values with different small letter superscripts mean significant difference (P < 0.05), while with the same or no letter superscripts mean no significant difference (P > 0.05).
[1] ΣUFA = The content of the sum of unsaturated fatty acids.
[2] ΣCLA = The content of the sum conjugated linoleic acid.
[3] Σω-3PUFA = The content of the sum of ω-3 polyunsaturated fatty acids.
[4] Σω-6PUFA = The content of the sum of n-6 polyunsaturated fatty acids.
[5] ω-6PUFA/ω-3PUFA = The ratio of total ω-6 polyunsaturated fatty acids to total ω-3 polyunsaturated fatty acids.

3.2. Methane and carbon dioxide emission

The methane and carbon dioxide emissions are shown in Table 5. Compared to the LUFA group, CH_4 (P < 0.001) and CO_2 (P = 0.011) emissions decreased by 21.60% and 12.48%, respectively, in the HUFA group. Furthermore, the CH_4 yield per kg of DMI (P = 0.017) and OMI (P = 0.015) decreased in the HUFA group by 15.17% and 15.65%, respectively. However, no difference was observed in the CO_2 yield per kg of DMI (P = 0.435), CO_2 yield per kg of OMI (P = 0.390), and the CH_4 (P = 0.112) and CO_2 (P = 0.897) yield per kg of ECM between the LUFA and HUFA groups.

3.3. Rumen fermentation parameters

In Table 6, the rumen fermentation parameter illustrates that an increase in UFA concentration in the diet did not affect the concentrations of total volatile fatty acids (TVFA, P = 0.826), acetate (P = 0.891), propionate (P = 0.528), butyrate (P = 0.723), and valerate (P = 0.408) in the rumen; however, the ratio of acetate to propionate decreased in the HUFA group (P = 0.004). Simultaneously, the concentrations of iso-butyrate (P = 0.002), iso-valerate (P = 0.001), and NH_3—N (P < 0.001) decreased in the HUFA group.

3.4. Profiles of the rumen microbiome and taxonomic differences

The Shannon's index for the bacteria in the HUFA group was lower than in the LUFA group (Fig. 1A, P < 0.05). The dominant bacterial phylum included Bacteroidetes (59.10% ± 8.28%), Firmicutes (35.28% ± 7.60%), Proteobacteria (1.74% ± 1.07%), and Fibrobacteres (1.38% ± 0.60%) (Fig. 1A). The dominant bacterial genera are *Prevotella* (58.25% ± 8.07%), followed by *Succiniclasticum* (5.81% ± 1.38%), *Clostridium* (4.44% ± 2.24%), *Bacteroides* (3.71% ± 0.45%), *Butyrivibrio* (3.55% ± 1.27%), *Ruminococcus* (3.42% ± 0.95%), and *Fibrobacter* (3.06% ± 1.18%) (Fig. 1A). The dominant bacterial species included *Succiniclasticum_ruminis* (8.89% ± 1.40%), *Prevotella_ruminicola* (6.83% ± 1.68%), *Prevotella_sp_tc2-28* (4.95% ± 2.17%), *Prevotella_sp_tf2-5* (4.30% ± 1.99%), *Prevotella_sp_ne3005* (3.61% ± 0.71%), and *Clostridiales_bacterium* (3.44% ± 1.28%) (Fig. 1A). Differential analysis of bacteria showed that the abundance of *Bacteroidetes* was enriched in the HUFA group cows, whereas that of *Spirochaetes* and 12 *Candidatus* phyla were enriched in the LUFA group cows (Fig. S1A, |LDA| > 2 and P < 0.05). The *Parabacteroides* genus was enriched in the HUFA group cows, whereas *Clostridium*, *Butyrivibrio*, *Treponema*, *Coprobacillus*, *Bacillus*, and *Acholeplasma* were enriched in the LUFA group cows (Fig. S2A, |LDA| > 2 and P < 0.05). In total, 39

Table 5
Methane and carbon dioxide yield in LUFA and HUFA groups.

Item	Groups		SEM	P-value
	LUFA	HUFA		
CH_4, g/d	478.36[a]	375.18[b]	8.610	<0.001
CO_2, g/d	18,881.89[a]	16,526.31[b]	352.521	0.011
CH_4, g/kg of DMI	22.15[a]	18.79[b]	0.775	0.017
CO_2, g/kg of DMI	898.62	851.30	27.850	0.435
CH_4, g/kg of OMI	24.16[a]	20.38[b]	0.812	0.015
CO_2, g/kg of OMI	980.49	923.31	28.910	0.390
CH_4, g/kg of ECM	15.49	14.35	0.340	0.112
CO_2, g/kg of ECM	625.76	629.15	12.304	0.897

LUFA = low unsaturated fatty acid; HUFA = high unsaturated fatty acid; SEM = standard error of the mean; DMI = dry matter intake; OMI = organic matter intake; ECM = energy corrected milk.
[a,b] Within the same row, values with different small letter superscripts mean significant difference (P < 0.05), while with the same or no letter superscripts mean no significant difference (P > 0.05).

Table 6
Rumen fermentation parameters in LUFA and HUFA groups.

Item	Groups		SEM	*P*-value
	LUFA	HUFA		
TVFA, mmol/L	79.80	81.36	3.428	0.826
Acetate, mmol/L	52.84	54.45	2.144	0.891
Propionate, mmol/L	13.93	14.90	0.744	0.528
Butyrate, mmol/L	9.35	9.02	0.446	0.723
Iso-butyrate, mmol/L	1.44[a]	0.93[b]	0.036	0.002
Valerate, mmol/L	0.93	0.86	0.042	0.408
Iso-valerate, mmol/L	1.60[a]	1.25[b]	0.057	0.001
Acetate to propionate ratio	3.83[a]	3.47[b]	0.065	0.004
N—NH$_3$, mg/dL	21.83[a]	16.94[b]	0.769	<0.001

LUFA = low unsaturated fatty acid; HUFA = high unsaturated fatty acid; SEM = standard error of the mean; TVFA = total volatile fatty acids; N—NH$_3$ = N-ammonia.
[a,b] Within the same row, values with different small letter superscripts mean significant difference ($P < 0.05$), while with the same or no letter superscripts mean no significant difference ($P > 0.05$).

species were enriched in the LUFA group cows, including 17 *Clostridium* sp., seven *Treponema* sp., four *Butyrivibrio* sp., three *Mycoplasma* sp., two *Firmicute* sp., and six other species. However, nine species showed enrichment in the HUFA group cows: seven *Prevotella* sp., one *Ruminococcus* sp., and one *Selenomonas* sp. (Fig. 2A, |LDA| > 2 and $P < 0.05$).

The abundance of the dominant archaeal phylum *Euryarchaeota* (99.30% ± 0.21%) was higher in the LUFA group cows, whereas the abundances of two *Candidatus* sp. and one *Crenarchaeota* were higher in the HUFA group cows (Fig. S1B, |LDA| > 2 and $P < 0.05$). The abundance of the dominant archaeal genus *Methanobrevibacter* (96.80% ± 0.88%) was higher in the LUFA group cows (Fig. S2B, |LDA| > 2 and $P < 0.05$). The abundance of *Methanobrevibacter_sp_YE315* was higher in the LUFA group cows, whereas abundances of four species were higher in the HUFA group cows (Fig. 2B, |LDA| > 2 and $P < 0.05$) among the differential archaeal species.

3.5. Rumen functional profiles, differential functions, and correlation between the rumen microbiome and functions

The functions of the rumen microbiome were measured using KEGG and genes encoding CAZymes. A total of 582 genes encoded CAZymes (Table S1), including 22 auxiliary activities (AA, 5.09%), 79 carbohydrate-binding modules (CBM, 11.43%), 16 carbohydrate esterases (CE, 11.04%), 277 glycoside hydrolases (GH, 41.95%), 102 glycosyl transferases (GT, 25.48%), and 86 polysaccharide lyases (PL, 5.01%). The difference analysis did not reveal differences in the abundance of the total CAZymes, AA, CBM, CE, GH, GT, and PL gene abundance between the LUFA and HUFA groups (Fig. 3A, $P > 0.05$). Further analysis showed that the level of four CAZymes (CBM57, CBM58, GT45, and GT66) was higher in the LUFA group, whereas 18 CAZyme levels were higher in the HUFA group: GH43_5, GH43_2, GH66, GH43, GH43_29, GH43_19, GH26, GH105, GH35, GH73, GH115, GH51, GH43_10, and GH28 (Fig. 3B, |LDA| > 2 and $P < 0.05$).

The KEGG profiles identified 350 third-level pathways (Table S2). These pathways belonged to six first-level categories, including "metabolism" (43.27% ± 3.72%), "human diseases" (19.88% ± 3.49%), "genetic information processing" (18.60% ± 0.87%), "cellular processes" (7.88% ± 0.22%), "environmental information processing" (6.20% ± 0.25%), and "organismal systems" (4.16% ± 0.47%). Forty-five categories were observed at the second level. The most abundant categories included "translation" (8.68% ± 0.62%), "amino acid

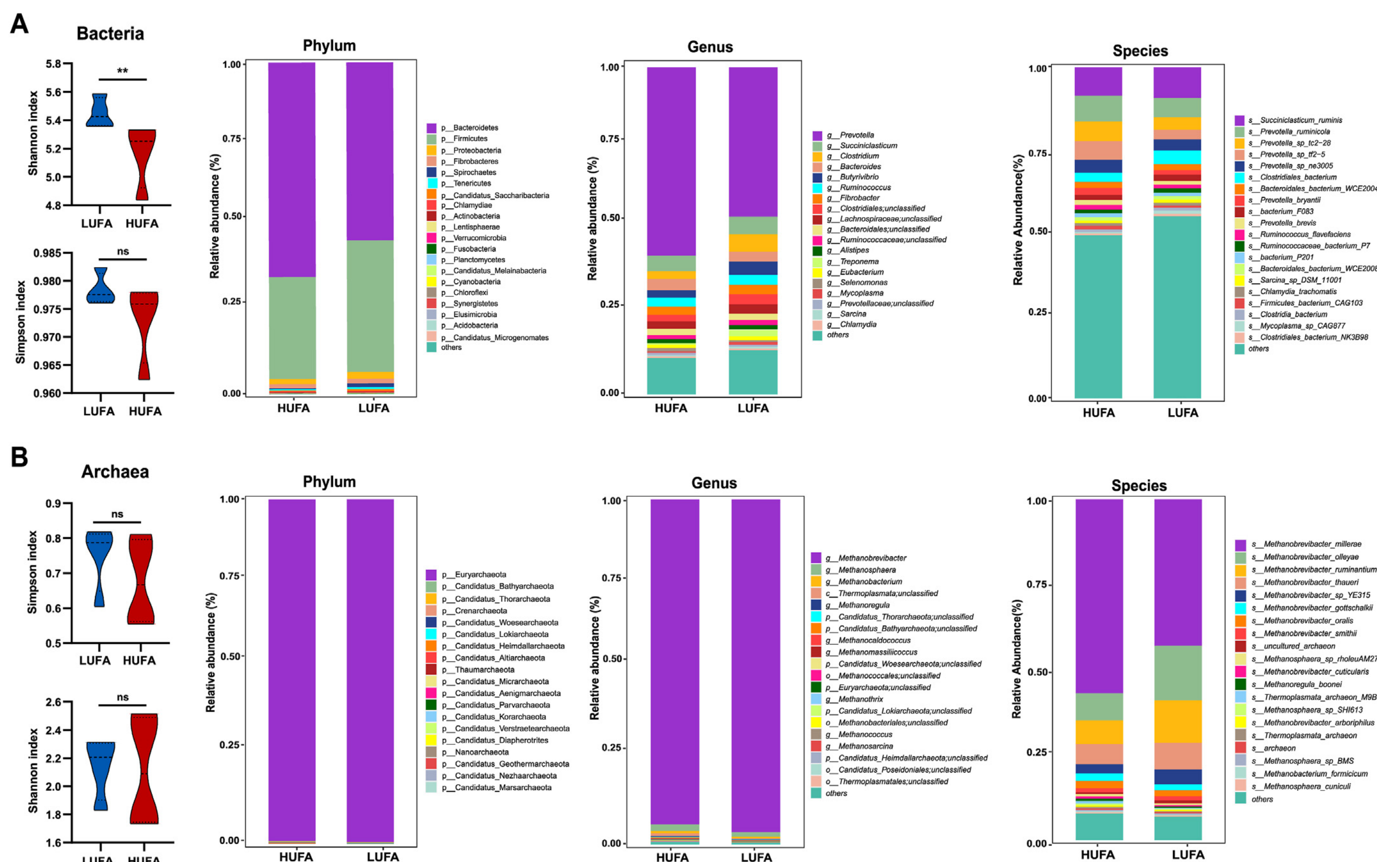

Fig. 1. Alpha diversity and composition of rumen microorganisms. (A) Alpha diversity and composition of bacteria. (B) Alpha diversity and composition of archaea. LUFA = low unsaturated fatty acid; HUFA = high unsaturated fatty acid. Significance of the alpha diversity indexes were tested by Wilcoxon rank-sum test, [ns] $P > 0.05$, *$P < 0.05$, **$P < 0.01$.

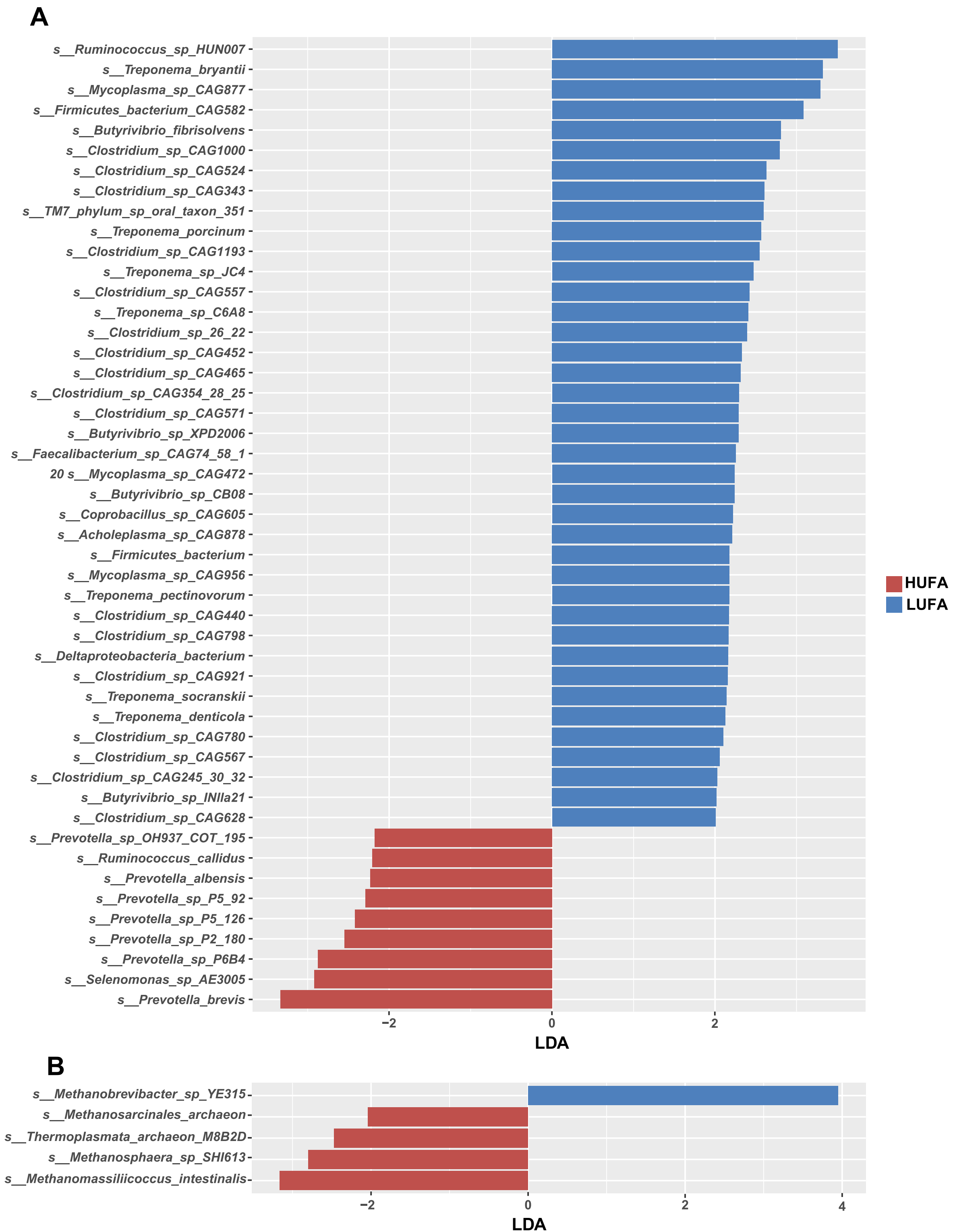

Fig. 2. Differential rumen bacterial and archaeal species between LUFA and HUFA cows. (A) Significantly different bacterial species. (B) Significantly different archaeal species. LUFA = low unsaturated fatty acid; HUFA = high unsaturated fatty acid. Significantly differences were tested by linear discriminant analysis (|LDA| > 2) and $P < 0.05$.

metabolism” (7.84% ± 0.84%), “metabolism of cofactors and vitamins” (6.65% ± 0.55%), “carbohydrate metabolism” (5.92% ± 0.49%), “replication and repair” (5.76% ± 0.39%), “glycan biosynthesis and metabolism” (4.77% ± 0.46%), and “energy metabolism” (4.14% ± 0.43%), while only one third-level pathway (ko00680) was abundant in the LUFA group (Fig. 4A, |LDA| > 2 and $P < 0.05$), whereas 12 third-level pathways (ko00910, ko00790, ko00770, ko00541, ko00525, ko00520, ko00400, ko00330, ko00311, ko00130, ko00052, and ko00040) were enriched in the HUFA group (Fig. 4B, |LDA| > 2 and $P < 0.05$).

Fig. 4C and D shows the correlation between dominant genera (average abundance > 0.5%) and different functions. At the genus

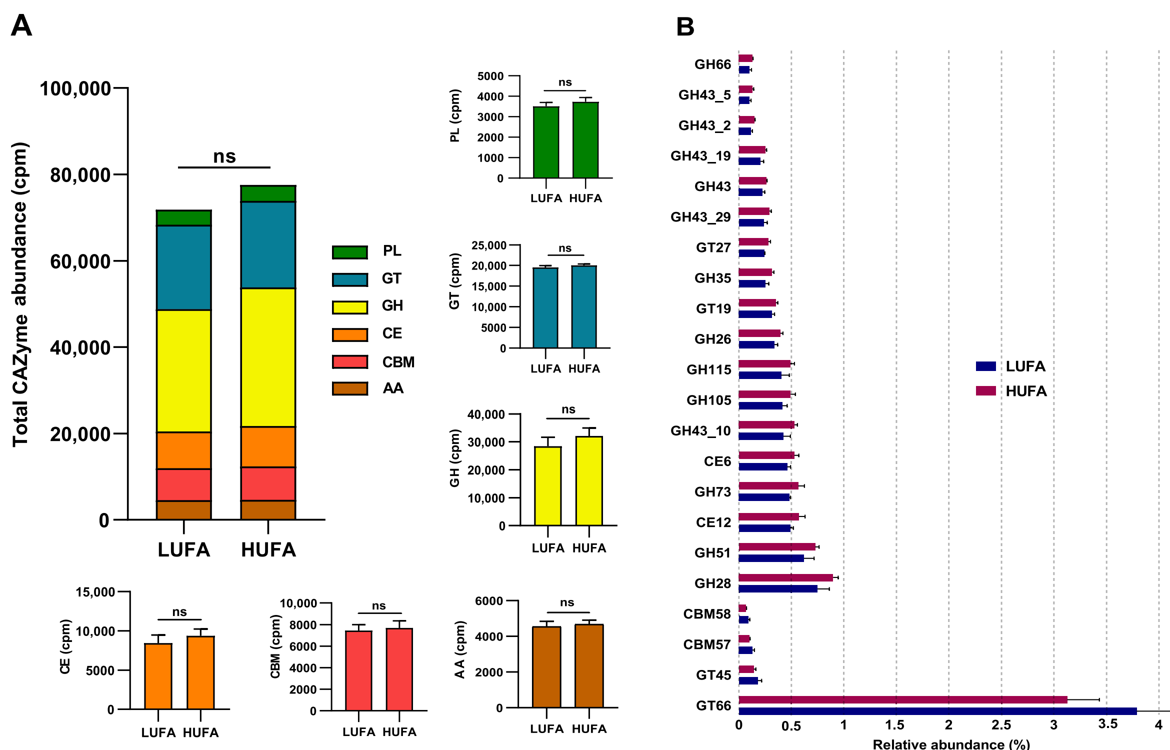

Fig. 3. The composition of CAZymes and differential CAZymes between LUFA and HUFA cows. (A) The abundance and composition of CAZymes. (B) Significantly different CAZymes. cpm = count per million; CAZymes = carbohydrate-active enzymes; PL = polysaccharide lyases; GT = glycosyl transferases; GH = glycoside hydrolases; CE = carbohydrate esterases; CBM = carbohydrate-binding modules; AA = auxiliary activities. LUFA = low unsaturated fatty acid; HUFA = high unsaturated fatty acid. Significances of CAZymes in Fig. 3A were tested by Wilcoxon rank-sum test, ^{ns} $P > 0.05$. Significantly differences in Fig. 3B were tested by linear discriminant analysis (|LDA| > 2) and $P < 0.05$.

level, abundance of *g_Clostridium* and *g_Butyrivibrio* was positively correlated with the CH_4 metabolic pathway (Fig. 4C, $P < 0.05$). In addition, abundance of *g_Methanobrevibacter* and *g_Methanosphaera* was positively correlated with CH_4 metabolism; however, the correlation was weak (Fig. 4C, $P > 0.05$). *g_Prevotella* and *g_Bacteroides* abundances were positively correlated with the pathways enriched in the HUFA groups, wherein *g_Bacteroides* abundance was correlated (Fig. 4C, $P < 0.05$). In contrast, *g_Clostridium*, *g_Methanobrevibacter*, *g_Methanosphaera*, and *g_Methanobacterium* abundances were negatively correlated with the pathways enriched in the HUFA groups, wherein *g_Methanobrevibacter* and *g_Methanobacterium* abundances were correlated with most pathways (Fig. 4C, $P < 0.05$). At the species level, *s_Clostridiales_bacterium*, *s_bacterium_F083*, *s_Mycoplasma_sp_CAG877*, and *s_Lachnospiraceae_bacterium_G41* abundances were positively correlated with CH_4 metabolism (Fig. 4D, $P < 0.05$). The abundance of 6 *s_Prevotella* sp. was positively correlated with some pathways enriched in the HUFA groups, and *s_Methanobrevibacter_millerae* and *s_Methanobrevibacter_sp_YE315* abundances were negatively correlated with these pathways (Fig. 4D, $P > 0.05$). Furthermore, *s_bacterium_P201* abundance was positively correlated with most of these pathways, whereas *s_Ruminococcus_sp_HUN007* abundance was negatively correlated with most of these pathways (Fig. 4D, $P < 0.05$).

3.6. The gene abundance of related enzymes in methanogenesis

The gene abundance of the enzymes involved in the hydrogenotrophic pathway of reducing CO_2 to 5-methyl-H_4MPT (EC:1.2.7.12, EC:2.3.1.101, EC:3.5.4.27, EC:1.5.98.1, and EC:1.5.98.2) was higher in the LUFA group than that in the HUFA group (Fig. 5A, $P < 0.05$). The level of the enzyme (EC:6.2.1.1) that catalyses the conversion of acetate to 5-methyl-H_4MPT was higher in the LUFA group than in the HUFA group (Fig. 5B, $P < 0.05$). However, the level of the enzyme that catalyses the conversion of methanol to 5-methyl-H4MPT did not change in two groups (Fig. 5C, $P > 0.05$). The levels of enzymes involved in the core steps of methanogenesis (EC:2.1.1.86 and EC:2.8.4.1) were higher in the LUFA group than in the HUFA group (Fig. 5D, $P < 0.05$).

4. Discussion

Increasing the fat content in the diet can have a negative impact on DMI for dairy cows. In particular, the DMI inhibition becomes stronger with increased UFA content in dietary fat (Rego et al., 2005). Although the DMI decreased in the HUFA group in our study, we did not observe milk production decrease, and similar results were found in previous studies (Castro et al., 2019; Ferlay et al., 2013; Neveu et al., 2013). However, lower values of 3.5% FCM and ECM were observed in the HUFA group compared to those in the LUFA group, which was mainly due to the reduction in milk fat in our study. Previous studies also showed that increasing the UFA content in the diet reduced milk fat in dairy cows (Klop et al., 2016). This phenomenon can be attributed to the fact that incomplete hydrogenation of UFA in the rumen produces trans-FAs, which can inhibit the formation of milk fat. Wherein CLA-t10c12 is an important factor causing low milk fat syndrome in dairy cows (Harvatine et al., 2009, 2018). Our study found that the UFA content increased in the HUFA group, which resulted in a decrease in milk fat and fat production in the HUFA group. On the one hand, it was because our FA results showed that CLA-t10c12 was significantly increased in HUFA group; On the other hand, the fat powder supplemented was mainly C16:0 in the LUFA group, which increased milk fat. The C16:0, as a saturated fatty acid, is almost

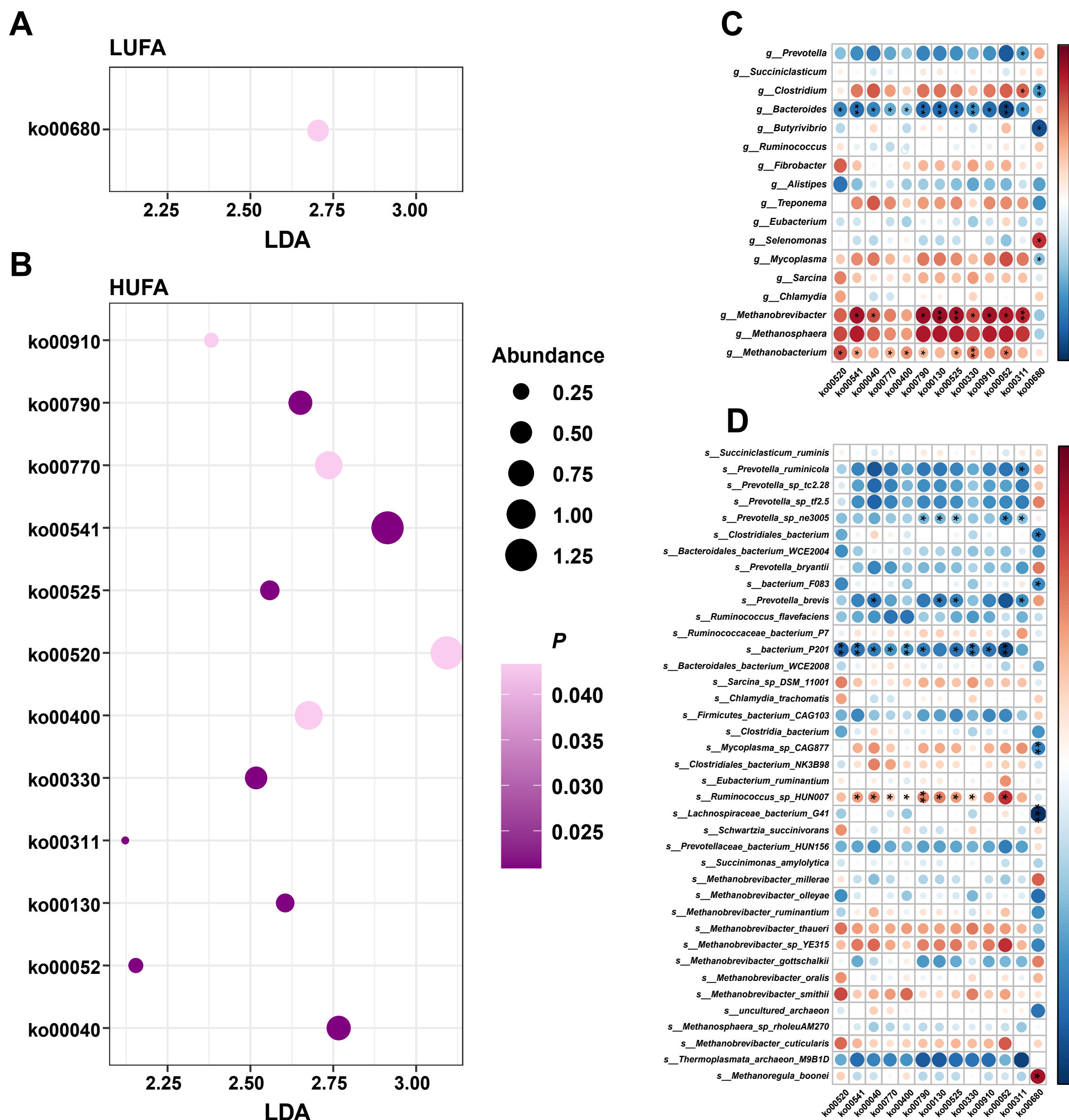

Fig. 4. Differential KEGG function pathways between LUFA and HUFA groups and its relationship with different species. (A) LUFA group significantly enriched metabolic pathway. (B) HUFA group significantly enriched metabolic pathways. (C) Spearman's rank correlations between rumen dominant genus (average abundance > 0.5%) and different KEGG function pathways. (D) Spearman's rank correlations between rumen dominant species (average abundance > 0.5%) and different KEGG function pathways. KEGG = Kyoto Encyclopaedia of Genes and Genomes; ko = KEGG Orthology; LUFA = low unsaturated fatty acid; HUFA = high unsaturated fatty acid. Significance of KEGG function pathways were tested by linear discriminant analysis (|LDA| > 2) and $P < 0.05$. In Spearman's rank correlations heatmap, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

unchanged in the rumen, so it was usually used to improve dairy milk fat (Loften et al., 2014). The milk FA profile is directly related to the dietary fatty acid profile. C4:0-C14:0 and some C16:0 are synthesised de novo in the mammary gland (Walter et al., 2020), whereas C16:0, and other FAs with a carbon chain greater than 16, are usually directly obtained from the diet or during body fat mobilisation (Moate et al., 2007). In previous studies, an increase in UFA in the diet of dairy cows resulted in a decrease in the saturated fatty acid (SFA) content in milk, and the content of some intermediates (e.g. conjugated linoleic acid) increased due to incomplete biohydrogenation of UFA in the rumen (Kliem et al., 2019; Vargas-Bello-Perez et al., 2018; Vargas-Bello-Pérez et al., 2019). Similar results were observed in this study. The ratio of ω-6 to ω-3 FA in dietary fat might alter feed intake, body fat metabolism, nutrient distribution, and production performance of dairy cows (Greco et al., 2015; Liu et al., 2020; Newman et al., 2002; Papadopoulos et al., 2009; Silvestre et al., 2011). Our study showed that the concentration of Σω-3 and Σω-6 PUFA was higher in the HUFA group than in the LUFA group, and the ratio of ω-6 to ω-3 PUFA was lower. Although the percentage of milk fat decreased, the profile of milk FAs was more in line with modern human nutritional requirements in the HUFA group.

Volatile fatty acids are the main fermentation product of macromolecular nutrients in the rumen, where acetate, propionate, and butyrate are the primary VFA. The VFA yield and composition are related to the feed intake and the dietary composition of dairy cows (Dijkstra et al., 1993). The TVFA and acetate contents in the rumen of dairy cows fed rubber seed oil and flaxseed oil were lower than those of the control group (Pi et al., 2019). Furthermore, the contents of butyrate and NH_3—N decreased with an increase in

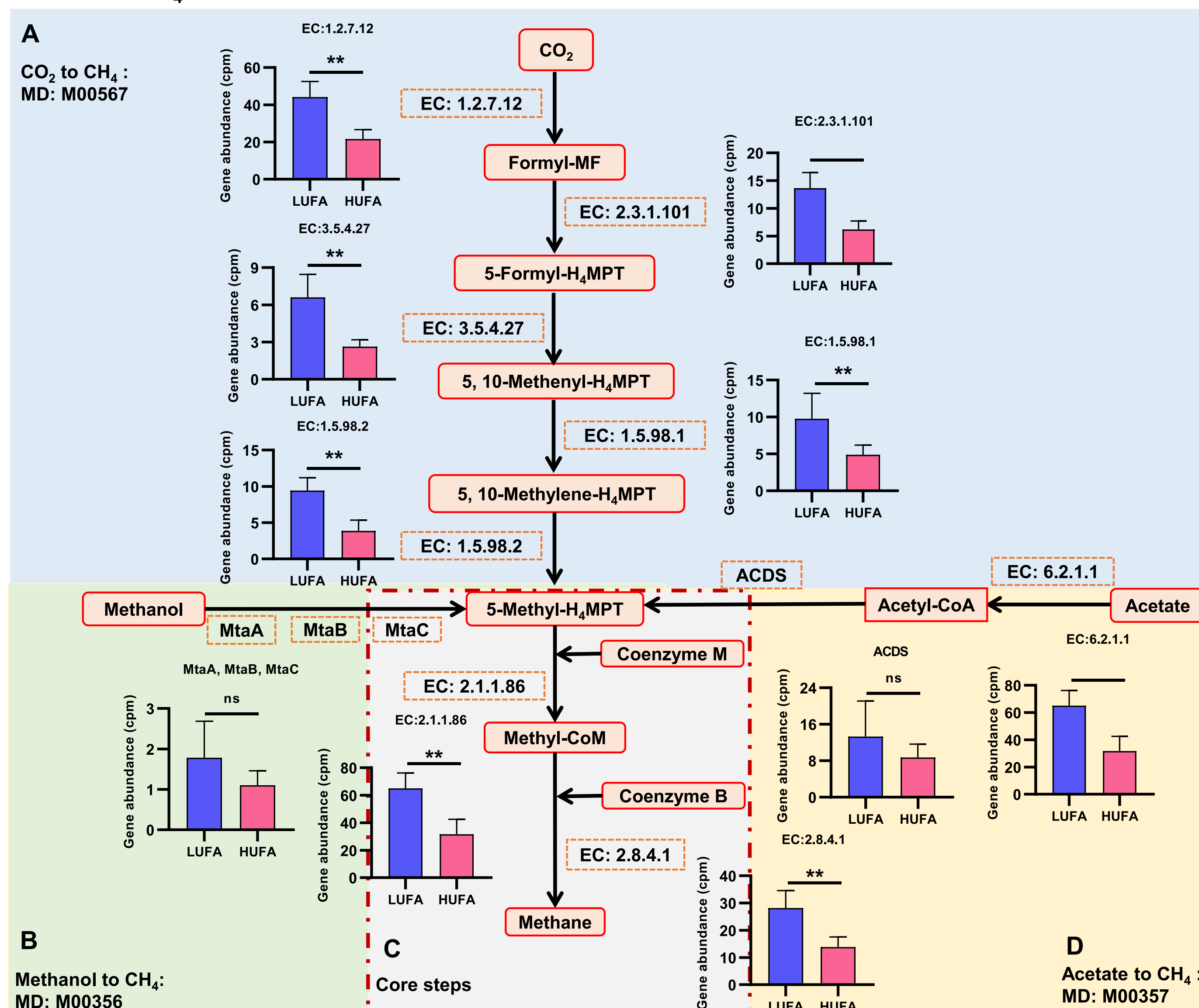

Fig. 5. The methanogenesis pathways and gene abundance of related enzymes between the LUFA and HUFA groups. (A) Carbon dioxide (CO_2) methanogenesis pathway (KEGG pathway entry MD: M00567). (B) Methanol methanogenesis pathway (KEGG pathway entry MD: M00356). (C) Core steps in methanogenesis pathway. (D) Acetate methanogenesis pathway (KEGG pathway entry MD: M00357). KEGG = Kyoto Encyclopaedia of Genes and Genomes; cpm = count per million; ko = KEGG Orthology; H_4MPT = tetrahydromethanopterin; EC: 1.2.7.12 = formylmethanofuran dehydrogenase subunit A, B, C, E; EC: 2.3.1.101 = formylmethanofuran-tetrahydromethanopterin N-formyltransferase; EC:3.5.4.27 = methenyltetrahydromethanopterin cyclohydrolase; EC:1.5.98.1 = methylenetetrahydromethanopterin dehydrogenase; EC:1.5.98.2 = 5,10-methylenetetrahydromethanopterin reductase; EC:2.1.1.86 = tetrahydromethanopterin S-methyltransferase subunit A, B, C, D, E, F, G, H; EC:2.8.4.1 = methyl-coenzyme M reductase alpha, beta and gamma subunit; EC:6.2.1.1 = acetyl-CoA synthetase; ACDS = acetyl-CoA decarbonylase/synthase, CODH/ACS complex subunit beta, gamma and delta; MtaA = coenzyme M methyltransferase; MtaB = methanol-5-hydroxybenzimidazolylcobamide Co-methyltransferase; MtaC = methanol corrinoid protein. LUFA = low unsaturated fatty acid; HUFA = high unsaturated fatty acid. Significance of the gene abundance of enzymes were tested by Wilcoxon rank-sum test, ^{ns} $P > 0.05$, $^{**}P < 0.01$.

dietary UFA (Boland et al., 2020). The fat levels of the two treatment groups in our experiment were the same; therefore, no significant difference was found in primary VFA. Furthermore, the decreased iso-butyrate and iso-valerate content of the HUFA group in our experiment was consistent with the experimental results mentioned above. The ratio of rumen acetate to propionate decreased in the HUFA group; however, the acetate and propionate content was not significantly different between the two groups. This could be due to the lower abundance of *Methanobrevibacter* in the HUFA group, resulting in more hydrogen being used to produce propionate instead of CH_4 (Newbold et al., 2005); therefore, causing a greater change in propionate content than in acetate, resulting in a lower ratio of acetate to propionate. Our results also revealed that the level of NH_3—N was lower in the HUFA group than in the LUFA group. The functional analysis of the current study suggested that the HUFA group significantly enriched the pathways of nitrogen metabolism and some amino acid metabolism in this study. This may accelerate the use of nitrogen in the rumen, thus reducing the concentration of NH_3—N.

Analysis of the rumen metagenome revealed that UFA affected rumen microorganisms, which altered rumen function. Our results of alpha diversity showed that only the bacterial Shannon index differed between the two groups, and the significant decrease in the rumen Shannon index in the HUFA group might be related to

the toxic effects of UFA on microorganisms (Lourenço et al., 2010). *Bacteroidetes* are known to degrade various carbohydrates (McKee et al., 2021). They were significantly enriched in the HUFA group and were significantly and positively correlated with all pathways that were enriched in the HUFA group. *Prevotella* is the most abundant bacterial genus in *Bacteroides* and is one of the most abundant core bacteria in the rumen of dairy cows (Stevenson and Weimer, 2007). *Prevotella* encodes a variety of GHs, especially xylanase GH-10 and β-xylanase GH-43; therefore, it effectively degrades xylan and pectin (Dao et al., 2021). The result of our study showed that abundances of some *Prevotella* spp. were significantly enriched in the HUFA group, and those with GH-43 were significantly enriched in the HUFA group, consistent with the findings of previous research. Furthermore, *Prevotella* can effectively degrade starch and protein (Dao et al., 2021; Potempa et al., 2009). Our correlation results showed that abundance of *Prevotella* was positively correlated with all significantly enriched pathways in the HUFA group, including some metabolic pathways associated with carbohydrates and nitrogen. This indicated that the addition of UFA might promote the metabolism of carbohydrates and nitrogen in the rumen.

The LEfSe results illustrated that the relative abundances of two dominant bacterial genera (*Clostridium* and *Butyrivibrio*) decreased in the HUFA group. *Butyrivibrio* group bacteria occupy a dominant position in the process of C18:3n-3 and C18:2n-6 biohydrogenation, and cis-9, trans-11 CLA, and other intermediate products would be produced in the process of biohydrogenation (Boeckaert et al., 2008; Lourenço et al., 2010). With accumulation of intermediates (such as cis-9 and trans-11 CLA) or changes in percentage content in the rumen of sheep and dairy cow rumen (Boeckaert et al., 2008; Toral et al., 2012), the relative abundance of bacteria in the *Butyrivibrio* group did not change, however the structure of bacteria in the *Butyrivibrio* group did change. However, it has been shown the relative abundance of *Butyrivibrio* decreases to some extent when C18:3n-3 and C18:2n-6 accumulates in the rumen of goats (Lv et al., 2016). Similar results were observed in our experiment. A study found that *Clostridiales* also played an important role in rumen biohydrogenation (Huws et al., 2011). Similar to the *Butyrivibrio* changes, our results also showed that abundance of *Clostridium* decreased in the HUFA group. The relative abundances of these bacteria decreased, which may be due to the toxic effects of UFA on rumen microorganisms.

This study found that abundance of *Euryarchaeota* and *Methanobrevibacter* in the rumen of the HUFA group was significantly decreased by an increase in the proportion of UFA in the diet. Previous studies showed that the relative abundance of total methanogens decreased when flaxseed oil was added to dairy cow diets, and the relative abundance of *Methanosphaera stadtmanae, Methanobrevibacter smithii,* and *Methanobrevibacter ruminantium* decreased by over 50% on average (Boland et al., 2020), which was consistent with the above results. In addition, we found significant enrichment of other methanogens (*Methanomassillicoccus*, *Methanobacterium,* etc.) in HUFA group, which may be due to the dominance of *Methanobrevibacter* in the archaea genus, when the relative abundance of *Methanobrevibacter* decreased, reducing the competition between methanogens for substrate. We analysed the rumen KEGG pathway and found that the CH_4 metabolism pathway was significantly enriched in the LUFA group. The reason was that the abundance of *Methanobrevibacter* was significantly enriched in the LUFA group. The correlation results showed that abundances of *Methanobrevibacter* and *Methanosphaera* were positively correlated with the CH_4 metabolism pathway, which further explains the relationship between rumen methanogens and the CH_4 metabolism pathway. Furthermore, abundances of *Butyrivibrio* and *Clostridium* were significantly positively correlated with CH_4 metabolism in the rumen. This finding indicated that bacterial abundances in the rumen were also closely related to CH_4 metabolism, as hydrogen produced by rumen bacteria fermenting nutrients is an important reducing substance in the pathway of methanogenesis (Du et al., 2020; Ungerfeld, 2020).

Previous research found that each form of UFA (crude flaxseed, extruded flaxseed or flaxseed oil) added to the diet significantly reduced CH_4 production, especially when flaxseed oil was added, where CH_4 production decreased by approximately 64% (Martin et al., 2008). In another study, CH_4 production in dairy cows linearly decreased with an increase in UFA supplementation levels, whether the roughage was the hay or corn silage, respectively (Martin et al., 2016). In our study, increasing the dietary UFA content reduced the daily CH_4 yield and CH_4 yield per kg DMI of dairy cows by 21.60% and 15.17%, respectively. Further analysis of rumen microorganisms and rumen function revealed that the addition of UFA significantly reduced the relative abundance of *Methanobrevibacter* and the CH_4 metabolism pathway. This was consistent with the reduction in CH_4 production described above. Furthermore, we analysed the abundance of genes encoding enzymes involved in the three methanogenesis pathways. We found that in the pathway of hydrogenotrophic methanogenesis, the gene abundances of all enzymes were lower in the HUFA group; in the pathway of acetoclastic methanogenesis, the gene abundance of enzymes required for acetate incorporation to acetyl-CoA was lower in HUFA group. Furthermore, the gene abundance of all enzymes in the pathway shared by the three methanogenic pathways was lower in the HUFA group. This means that the rumen methanogenesis pathways in the LUFA group cows were more active. In summary, increasing the proportion of UFA in the diet significantly reduced the relative abundance of *Methanobrevibacter* in the rumen. Therefore, the abundance of multiple enzymes in rumen methanogenesis decreased with increasing UFA, reducing the activity of the CH_4 metabolism pathway in the rumen and ultimately reducing CH_4 emissions from dairy cows.

5. Conclusion

This study demonstrates that increasing the dietary content of UFA plays an important role in the decrease in rumen CH_4 production in dairy cows. High-throughput sequencing analysis reveals that UFA inhibited *Methanobrevibacter* in the rumen, thus decreasing the relative abundance of multiple enzymes during methanogenesis. Therefore, the CH_4 metabolism pathway was reduced in the rumen, which ultimately decreases CH_4 production in the rumen. Furthermore, an increase in the UFA content did not affect milk production. Although milk fat decreased, the ΣUFA, ΣCLA, Σω-3PUFA, and Σω-6PUFA levels were higher in the HUFA group, and the ratio of ω-6PUFA to ω-3PUFA was lower. Therefore, the profile of milk FAs was more in line with modern human nutritional needs in the HUFA group. This finding can be attributed to a decrease in CH_4, which improved the efficiency of energy use of dairy cows, promoted the utilisation of carbohydrates and nitrogen by *Bacteroides*, and increased the metabolism of carbohydrates and nitrogen in the rumen. This study provides new evidence to explore CH_4 emission reduction measures for dairy cows.

Author contributions

Zhantao Yang: Formal analysis, Methodology, Investigation; Visualization, Writing - Original draft preparation; **Yuhui Zheng:** Investigation; **Siyuan Liu**, **Tian Xie**, **Qianqian Wang** and **Zhonghan Wang**: Methodology; **Wei Wang** and **Shengli Li**: Conceptualization, Project administration, Supervision, and Writing - Review & Editing.

Availability of data and material

The rumen metagenome sequences were deposited into the national center for biotechnology information (NCBI) Sequence Read Archive (SRA) under the accession number PRJNA932960.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.06.003.

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