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Altering Methane Emission, Fatty Acid Composition, and Microbial Profile during In Vitro Ruminant Fermentation by Manipulating Dietary Fatty Acid Ratios

Xiaoge Sun [1,2], Qianqian Wang [1], Zhantao Yang [1], Tian Xie [1], Zhonghan Wang [1], Shengli Li [1] and Wei Wang [1,*]

[1] State Key Laboratory of Animal Nutrition, Beijing Engineering Technology Research Center of Raw Milk Quality and Safety Control, College of Animal Science and Technology, China Agricultural University, Beijing 100193, China; xiaogesun@163.com (X.S.); qq_221w@163.com (Q.W.); yzt980929@163.com (Z.Y.); s20203040614@cau.edu.cn (T.X.); wzh15675854231@163.com (Z.W.); lisheng0677@163.com (S.L.)

[2] Institute of Agricultural Sciences, Department of Environmental and Systems Science, ETH Zurich, 8092 Zurich, Switzerland

* Correspondence: wei.wang@cau.edu.cn

Abstract: This study evaluated the effects of different dietary n-6/n-3 polyunsaturated fatty acid (PUFA) ratios on in vitro ruminant fermentation. Methane production, fatty acid composition, and microbial profiles were compared after the in vitro fermentation of rumen fluid collected from cows that had been fed isoenergetic and isoproteic experimental diets at three different n-6/n-3 ratios: 3.04 (HN6, high n-6 source), 2.03 (MN6, medium n-6 source), and 0.8 (LN6, low n-6 source). The fermented rumen fluid pH and total volatile fatty acid (VFA) levels were significantly decreased ($p < 0.05$) in the HN6 group as compared with those in the MN6 and LN6 groups. Additionally, the HN6 group produced a significantly lower ($p < 0.05$) proportion of methane than the MN6 group during in vitro fermentation. The MN6 and LN6 groups had significantly increased ($p < 0.05$) levels of C18:2n6 and C18:3n3 in the fermented rumen fluid, respectively, as compared with the HN6 group. The Chao 1 diversity index value was lower ($p < 0.05$) in the HN6 group than in the MN6 and LN6 groups. The observed species richness was significantly lower ($p < 0.05$) in the HN6 group than in the MN6 group. The reduced relative abundances of *Lachnospiraceae UCG-006* and *Selenomonas* in the HN6 group resulted in lower pH and VFA levels (i.e., acetate, propionate, butyrate, and total VFA) during in vitro fermentation. Furthermore, n-6 and n-3 PUFAs were toxic to *Butyrivibrio_2* growth, resulting in high levels of incomplete biohydrogenation. Taken together, the study findings suggest that supplementation of high-forage diets with high levels of n-6 PUFAs could reduce methane emissions, whereas both VFA concentration and pH are reduced.

Keywords: fatty acid; methane; rumen simulation technique; microbial composition; fermentation

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1. Introduction

Meat and dairy products from ruminants are sought-after sources of nutrients for human diets. Fatty acids (FAs) are a major component of lipids in animal products, among which polyunsaturated fatty acids (PUFAs) comprise the main components of cell membranes and play a key role in various biological processes such as the immune system [1,2], enzyme activities, cell proliferation and differentiation, and receptor expression [3]. The two major PUFA families include the n-3 (omega-3) and n-6 (omega-6) FAs.

Studies using a variety of animal models have demonstrated that n-6 and n-3 PUFAs regulate the expression of several genes associated with carbohydrate and lipid metabolism as well as hormonal responses [4,5]. For example, dietary supplementation with octadeca carbon FA (including n-3 and n-6 PUFAs) seemed to reduce methane emissions from ruminants due to biohydrogenation and the toxic effects of free FAs on both methanogens and protozoa [6]. In ruminants, biohydrogenation transformed PUFAs into saturated FAs

and thereby decreased the amount of PUFAs for absorption, which in turn affected the microbial community associated with rumen fermentation. However, some studies have reported that despite the biohydrogenation of unsaturated FAs, the remaining n-6 and n-3 PUFAs that escape from the rumen and are absorbed by the intestine can still increase the n-3 or n-6 PUFAs in meat FA and play a key role in cellular function and animal performance [7,8]. Additionally, supplementation with n-3 or n-6 PUFAs has been found to reduce the methane output of dairy cows [9] and to increase the levels of conjugated linoleic acid or vaccenic acid in the rumen [10,11], which are beneficial to human health [12]. The negative effects of dietary fat supplementation depend on the fat type (chain length, degree of unsaturation), process (oil, coated, raw, or processed oilseeds), basal diet composition (forage ratio), and dosage [13–15].

The rumen simulation technique is widely used to precisely control the conditions and factors that affect fermentation in dairy cows and to measure end-product formation [16,17]. Previous studies have evaluated the effects of different fat sources on ruminant fermentation in vitro [18,19]. However, these studies mainly investigated the effects of the addition of oil sources on ruminant fermentation. Thus far, the effects of different ratios of n-3 to n-6 PUFA on in vitro rumen fermentation and microorganism-associated changes in methane production have rarely been explored. Therefore, the effects of specific fat sources on digestibility, rumen fermentation, and the microbial population need to be assessed.

The objectives of the current study were to evaluate the effects of dietary fat sources with different PUFA ratios (high saturated FA source, high n-6 source, low n-6 source) on in vitro ruminant fermentation patterns, including pH, gas production, FA profile, and microbial diversity.

2. Materials and Methods

All experimental procedures were approved by the Ethical Committee (Grant number: AW1110202-2, 25 August 2019) of the College of Animal Science and Technology at China Agriculture University (Beijing, China).

2.1. Fermentation Substrates

Three isoenergetic and isoproteic experimental diets, referenced to the dry dairy cow diet containing high forage, were designed as fermentation substrates (Table 1). Forage has a relatively higher n-3 fatty acid content than grain feed. Therefore, the n-6/n-3 ratio was relatively low compared to other studies using low-forage diets for lactating dairy cows. Normally, the n-6/n-3 ratio in a dry cow diet is approximately 2:1, therefore we chose the ratios of 2.03:1, 3.04:1, and 0.8:1 for our study. The diets with high n-6 PUFA levels (HN6, n-6/n-3 ratio = 3.04), moderate saturated FAs levels (MN6, n-6/n-3 ratio = 2.03), and low n-6 PUFA levels (LN6, n-6/n-3 ratio = 0.8) contained 9.67% extruded soybean, 1.51% hydrogenated FAs, and 4.47% extruded flaxseeds, respectively, on a dry matter (DM) basis. Hydrogenated FAs were obtained from Menergy (Yihai Kerry Group, Wuhan, China). Extruded soybeans enriched with n-6 PUFAs and extruded flaxseeds enriched with n-3 PUFAs were obtained from the Henan Shennong Feed Technology Company (Zhengzhou, China).

All samples were dried in an oven at 65 °C for 48 h until they reached a constant weight; they were subsequently ground in a small hammer mill (Thomas Scientific, Swedesboro, NJ, USA) and passed through a 1 mm sieve (40 mesh). The DM content of the total mixed ration (TMR) was determined by oven drying at 135 °C for 2 h, as previously described [20]. The crude protein (CP) content was determined using a Rapid N Exceed nitrogen and protein analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany) according to the manufacturer's instructions [21]. The crude fat content was determined by ether extraction (EE) according to method 920.39 of the Association of Official Analytical Chemists [21]. Feed samples were also analyzed for acid detergent fiber (ADF) and neutral detergent fiber (NDF) content using the ANKOM 2000i automatic fiber analyzer (Beijing Anke Borui Technology Co., Ltd., Beijing, China), as described by Van Soest et al. [22].

Table 1. Ingredients and chemical composition of the diets.

Ingredients	HN6 (DM %)	MN6 (DM %)	LN6 (DM %)
Oat hay	41.49	41.80	41.87
Corn silage	28.30	29.58	28.53
Corn fine	10.93	9.12	9.23
Soybean meal	8.61	16.99	14.90
Hydrogenated FA	0.00	1.51	0.00
Whole soybean	9.67	0.00	0.00
Flaxseed	0.00	0.00	4.47
Mineral vitamin premix	1.00	1.00	1.00
Total	100.00	100.00	100.00
Nutrition (DM basis)			
DM (kg/d)	12.51	12.42	12.4
CP (%)	13.77	13.85	13.78
NE_L(Mcal/kg)	1.49	1.48	1.49
NFC (%)	33.46	34.60	33.29
NDF (%)	41.95	42.00	42.41
EE (%)	4.01	3.93	4.01
UFA (%)	2.74	1.37	2.73
C16:0 (g/d)	50.70	175.55	46.00
n-6 (g/d)	178.23	102.03	121.48
n-3 (g/d)	58.63	45.65	150.68
n-6: n-3	3.04:1	2.03:1	0.8:1

DM: Dry matter, CP: Crude protein, NE_L: Net energy of lactation, a calculated value according to NRC (2001), NFC: Non-fibrous carbohydrate, calculated as: 100—[(NDF–NDFCP) + CP + ether extract + ash]. NDF: Neutral detergent fiber, EE: Ether extract, UFA: Unsaturated fat acid, n-6: C18:2 (Linoleic acid, LA), n-3: C18:3 (α- linolenic acid, ALA). HN6: Higher n-6 fatty acid treatment (n6/n3, 3.04:1), MN6: Moderate n-6 fatty acid treatment (n6/n3, 2.03:1), LN6: Lower n-6 fatty acid treatment (n6/n3, 0.8:1). Premix: 1 kg of premix included vitamin A 440,000 IU, vitamin D3 110,000 IU, vitamin E 4000 IU, niacin 400 mg, Ca 152 g, P 41g, Cu 750 mg, Mn 1,140 mg, Zn 2,970 mg, I 30 mg, Se 36 mg.

2.2. Rumen Fluid Preparation

Samples of rumen fluid were collected from three multiparous Holstein cows (daily milk yield: 25.3 ± 2.1 kg) via permanent rumen fistulas during the mid-lactation period. The cows were provided free access to water and TMR twice daily (6:00 am and 6:00 pm), with ad libitum consumption. The TMR diet was formulated using the CPM-Dairy software to meet the nutritional requirements recommended by NRC [23]. The dry matter intake (DMI) of the cows was 19.8 kg/d. The net energy of lactation and the crude protein content of the feed was 1.68 Mcal/kg and 17.3%, respectively. Two hours after the morning feeding, the rumen content was obtained from each cow and squeezed into a plastic thermos through four layers of cheesecloth. The collected rumen fluids were combined in a 5 L beaker, mixed thoroughly, blanketed with CO2, and heated to 39 °C in a preheated water bath.

2.3. Equipment and In Vitro Incubation

Buffer solution (pH 6.87) was prepared according to Menke [24], and CO_2 was continuously injected into the buffer solution for approximately 30 min prior to inoculation. For each incubation bottle (capacity: 120 mL), a total of 0.5 g substrate, 25 mL filtered rumen fluid, and 50 mL pre-heated buffer were added. All bottles (3 treatments × 6 replicates × 5 time points = 90 bottles) were purged with N2 to create anaerobic conditions, rapidly sealed with butyl rubber stoppers and Hungate's screw caps, and then immediately connected to the AGRS-III automated trace gas recording system (China Agricultural University, Beijing, China) via medical transfusion tubes. The AGRS-III system can be used to record cumulative gas production (GP) in real time, according to Yang et al. [25]. Samples were collected after incubation at 39 °C for 3, 6, 12, 24, and 48 h. Meanwhile, bottles (3 treatments × 6 replicates = 18 bottles) prepared according to the same procedures were separately connected to pre-emptied air bags and incubated at 39 °C for 48 h for further gas composition analysis.

2.4. Sampling

After incubation, the bottles were disconnected from the AGRS-III system and air bags. The pH value of the fermented material was measured immediately using a Starter 300 handheld pH meter (Ohaus Instruments Co., Ltd., Shanghai, China). The fermented material was filtered through a pre-dried and weighed nylon bag (300 mesh, 9 cm × 14 cm) to collect the fermented rumen fluid. The nylon bag was rinsed under tap water, squeezed manually, and dried in the oven at 65 °C for 48 h to determine the in vitro DM disappearance (IVDMD). The filtered and fermented rumen fluid was divided among five 2 mL sterile tubes. Two tubes were centrifuged at 4000× *g* for 15 min at 4 °C, the supernatants were mixed with 0.2 mL meta-phosphoric acid solution (250 g/L) at 4 °C for 30 min, and the mixtures were centrifuged at 10,000× *g* for 10 min at 4 °C. Subsequently, the supernatants were collected for ammonium nitrogen (NH3-N) and volatile fatty acid (VFA) analyses. Two tubes were stored at −20 °C for further FA profile analysis and microbial protein (MCP) analysis. The remaining tube was stored at −80 °C for further microbial community analysis. Gas samples (1 mL), obtained from the air bags using a gas-tight Hamilton syringe, were used for gas profile analysis.

2.5. Chemical Analysis and Calculations

NH3-N and MCP levels in the fermented rumen fluid were determined by spectrophotometry, as described by Verdouw et al. [26] and Cui et al. [27], respectively. VFA concentrations in the filtered rumen sample were measured by gas chromatography using the 6890N GC system (Agilent Technologies, Avondale, PA, USA), as previously reported by Zhang and Yang [28]. CH_4, CO_2, and H_2 concentrations in the gas sample were analyzed using the 6890N GC (Agilent Technologies) equipped with a thermal conductivity detector, as described by Cui et al. [27]. The reference gas contained 1% H_2, 8% CH_4, and 40% CO_2 (PanGas, Dagmersellen, Switzerland).

Samples (50–500 mg) of freeze-dried TMR were accurately weighed and transferred into the incubation tubes. Each of the tubes were slowly filled with 1 mL of benzene-containing internal standard, 1 mL of benzene, and 3 mL of freshly made 5% methanolic HC1 (prepared by slowly adding 10 mL of acetyl chloride to 100 mL of anhydrous methanol) so that the solvents would fall over the material without touching the side walls of the tube. After being tightly capped, the culture tubes were vortexed for 1 min at a slow speed in order for the material to remain 2–3 cm from the bottom. The tightly sealed tubes were heated in a water bath at 70 °C for 2 h; if the solvent escaped, 2 mL of benzene was added after cooling, and the tube was returned to the water bath to ensure complete methylation. FA methylation in the fluid was performed as previously described [29]. Either the fatty acid methyl esters obtained from the feed or the rumen fluid was determined by gas chromatography using a fused silica capillary column (DB-23, 60.0 m × 0.25 mm × 0.25 µm, US) and the 6890N GC (Agilent Technologies) equipped with a flame ionization detector. Results for each FA were expressed as mg/g of rumen culture sample.

IVDMD was calculated by determining the weight change of the substrate (DM basis) before and after in vitro incubation.

The cumulative GP data were fitted to a nonlinear model [30] as follows:

$$GP_{48} = A/(1 + (C/48)^B) \quad (1)$$

where GP_{48} represents the total gas production (mL/g dietary DM) at time 48(h); A represents the asymptotic gas production (mL/g dietary DM) at a constant fractional rate (c) per unit time; B represents a parameter reflecting the shape of the curve; C represents the time (h) at which the maximum gas production rate reaches $\frac{1}{2}$, and t represents the time at which the gas is recorded.

The maximum rate of substrate degradation (RmaxS,/h) was calculated as follows [31]:

$$TRmaxS = C \times (B-1)^{(1/B)} \quad (2)$$

$$RmaxS = (B \times TRmaxS^{(B-1)}/(C^B + TRmaxS^B) \quad (3)$$

2.6. DNA Extraction and Determination

Ruminal fluid DNA was extracted using the TIANGEN® TIANamp Stool DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China). Total DNA concentration and purity were determined using the NanoDropND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The V3-V4 region of the 16S rRNA gene was amplified according to the method described by Hao et al. [32]. The forward primer sequence 341F (CCTACGGGNGGCWGCAG) and the reverse sequence 806R (GGACTACHVGGGTATCTAAT) were used, as described by Guo et al. [33]. Amplicons were extracted from 2% agarose gels, purified using the AxyPrepDNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR green chemistry (SuperReal PreMix Plus, Tiangen Biotech Co., Ltd., Beijing, China).

Purified amplicons were pooled in equimolar amounts and sequenced using the Miseq PE300 platform (Illumina, Inc., San Diego, CA, USA) to generate paired-end (2 × 300) sequences. The original data were stored in Fastq format. Quality control processing was performed on the measured Fastq data using Trimmomatic v.0.36 and Pear v.0.9.6 software. A sliding window strategy was adopted when using the Trimmomatic software, with the window size set to 50 bp, the average quality value set to 20, and the minimum reserved sequence length set to 120. Pear software was used to remove N′ sequences. The high-quality raw reads were further filtered using fastp criteria (https://github.com/OpenGene/fastp accessed on 10 May 2021). Reads with unknown nucleotides (>10%) were removed. Paired-end clean tags were merged as raw tags using the FLASH v. 1.2.11 software, as described by Magoč and Salzberg [34]. The minimum overlap was set to 10 bp, and the mismatch rate was set to 0.1 to obtain Fasta sequences. Chimeric Fasta sequences were removed using UCHIME against a known database, and a de novo method was used to remove undesirably short sequences at the same time. A total of 1,679,967 raw tags and 1,653,042 clean tags were obtained. On average, 93,332 raw tags and 91,836 clean tags were obtained for each sample. The clean tags were further purified using the QIIME v.1.9.1 pipeline under specific filtering conditions [35]. After removing noise, the high-quality tags were clustered into operational taxonomic units (OTUs) with ≥97% similarity using the Vsearch v.2.7.1 software, according to the UPARSE pipeline [36]. Finally, 467,244 tags and 35,417 OTUs were obtained. On average, 25,958 tags and 1968 OTUs were obtained for each sample. Representative sequences (those with the highest relative abundance in each cluster) were classified using the RDP classifier v. 2.2 software, which is based on a naïve Bayesian model [37], along with the SILVA V.138.1 database (https://www.arb-silva.de/, accessed on 18 May 2021) [38], with confidence threshold values ranging from 0.8 to 1. The abundance statistics of each taxonomy were visualized using the Krona v. 2.6 software [39]. Alpha diversity indices (i.e., Chao 1, observed species, and Shannon) were calculated using QIIME. Sequences were aligned using the MUSCLE v. 3.8.31 software [40] (http://www.drive5.com/muscle/ accessed on 20 May 2021).

2.7. Statistical Methods

The relative abundances of bacterial taxa among the three groups were analyzed by Kruskal–Wallis test using the qvalue package in the R software. Data on bacterial beta-diversity were analyzed based on the unweighted UNIFRAC distance metric in the R software. The data from the bacterial alpha diversity indexes (i.e., Chao 1, observed species, and Shannon), total gas production, CH_4 production, CO2, and O2 were analyzed by one-way analysis of variance (ANOVA) using the SAS version 9.4 software (SAS Institute Inc., Cary, NC, USA). Other data (i.e., IVDMD, pH, VFA, MCP, NH3, gas kinetics) were analyzed by two-way ANOVA using the SAS version 9.4 software. The data were presented as the mean ± standard error of the mean (SEM). Multiple comparisons were further analyzed using Tukey's test for ANOVA ($0.05 \leq p \leq 0.10$) and Dunk's test for Kruskal–Wallis.

Statistical significance was considered when p-value ≤ 0.05, whereas tendencies were assumed for $0.05 \leq p \leq 0.10$.

3. Results

3.1. Effects of Dietary Fat Source on In Vitro Ruminant Fermentation and N-Metabolism

The fermented rumen fluid of the HN6 tended to decrease the pH ($p < 0.10$) when compared with the other two groups (Table 2). In addition, the pH significantly increased ($p < 0.01$) over the incubation period. The IVDMD values did not differ significantly among the three dietary groups and tended to increase linearly ($p < 0.01$) with increasing incubation time (Table 2). No interaction effect was detected between the pH and the IVDMD.

Table 2. Effects of dietary n-6/n-3 PUFA ratio on in vitro ruminant fermentation and N metabolism.

Item [1]	Treatment [2]			Time [3]					SEM [4]	*p*-Value [5]		
	HN6	MN6	LN6	3	6	12	24	48		TR	Time	INT
PH	6.89 ^{B}	6.94 ^{A}	6.94 ^{A}	6.85 ^{c}	6.94 ^{ab}	6.92 ^{b}	6.94 ^{ab}	6.98 ^{a}	0.03	0.09	<0.01	0.12
IVDMD	0.60	0.62	0.60	0.48 ^{d}	0.50 ^{d}	0.56 ^{c}	0.72 ^{b}	0.79 ^{a}	0.02	0.49	<0.01	0.16
NH3-N(mg/dL)	10.46 ^{b}	11.69 ^{a}	10.23 ^{b}	5.33 ^{d}	6.58 ^{c}	7.29 ^{c}	13.47 ^{b}	22.49 ^{a}	0.82	0.04	<0.01	0.14
MCP(μg/L)	640.45 ^{A}	616.17 ^{B}	656.62 ^{A}	640.00 ^{b}	753.82 ^{a}	630.03 ^{b}	614.62 ^{b}	552.75 ^{c}	11.77	0.08	<0.01	0.44
Acetate (mmol/L)	25.60 ^{B}	27.99 ^{A}	27.65 ^{A}	20.09 ^{d}	22.93 ^{c}	24.08 ^{c}	30.02 ^{b}	38.83 ^{a}	0.83	0.09	<0.01	0.07
Butyrate (mmol/L)	3.99 ^{b}	4.40 ^{a}	4.43 ^{a}	2.73 ^{e}	3.67 ^{d}	4.05 ^{c}	4.77 ^{b}	6.21 ^{a}	0.15	<0.01	<0.01	0.14
Propionate (mmol/L)	10.12 ^{b}	11.16 ^{a}	11.12 ^{a}	7.64 ^{d}	8.46 ^{c}	9.11 ^{c}	12.64 ^{b}	16.19 ^{a}	0.40	<0.01	<0.01	0.18
TVFA (mmol/L)	41.33 ^{b}	44.27 ^{a}	45.02 ^{a}	31.27 ^{d}	36.12 ^{c}	38.76 ^{c}	49.22 ^{b}	63.25^{a}	1.42	0.02	<0.01	0.04 *
A:P	2.57	2.52	2.56	2.63 ^{b}	2.71 ^{a}	2.64 ^{b}	2.38 ^{c}	2.40 ^{c}	0.02	0.18	<0.01	0.56

[1] IVDMD: In vitro dry matter digestibility, A:P: Acetic acid: Propionic acid, TVFA: Total volatile fat acid, NH_3-N: Ammonia–N, MCP: Microbial protein. [2] HN6: High n-6 fatty acid treatment (n6/n3, 3.04:1), MN6: Moderate n-6 fatty acid treatment (n6/n3, 2.03:1), LN6: Low n-6 fatty acid treatment (n6/n3, 0.8:1), ^{a,b,c}: Superscript letters show the multiple comparisons using Tukey's test, with different lowercase letters indicating significant differences in the indicators among groups ($p < 0.05$), ^{A,B,C}:a row with different uppercase letters indicating that there was a trend difference among groups ($0.05 \leq p < 0.1$), whereas those denoted by the same letters or no letters are not significantly different. [4] SEM: standard error of the mean. [3] Time: Incubation time, ^{a,b,c,d,e}: Superscript letters show the multiple comparisons using Tukey's test; a row with different lowercase letters indicates a significant difference under time effect factors ($p < 0.05$), whereas those denoted by the same letters or no letters are not significantly different. [4] SEM: standard error of the mean. [5] TR: The overall diet treatment effect between groups. Time: The overall effect of incubation time. INT: The interaction effects between diet treatment and incubation time. *: If the interaction effect factor indicates a significant difference, the detailed information of the data will be expressed in the picture (Figure 1).

The total VFA production was the lowest in the HN6 group. The total VFA production in the LN6 group was greater than that in the MN6 group after incubation for 48 h and that in the HN6 group after 12 h (Figure 1). The HN6 group had lower levels of acetate ($p < 0.10$), propionate ($p < 0.05$), and butyrate ($p < 0.05$) than the LN6 and MN6 groups (Table 2). However, the acetate to propionate ratio did not differ significantly ($p > 0.05$) among the three dietary groups. The total VFA concentration in fermented rumen fluid was affected ($p = 0.04$) by the interaction effect of treatment and time, but no interaction effects were detected for individual VFA concentrations.

NH3-N levels were lower ($p < 0.10$) in the HN6 and LN6 groups than in the MN6 group, and NH3-N accumulated over the incubation period (Table 2). MCP synthesis was significantly increased ($p < 0.05$) in the HN6 and LN6 groups as compared to that in the MN6 group. MCP levels increased ($p < 0.05$) over the incubation period, reaching a maximum level at 6 h before significantly decreasing ($p < 0.05$) to the lowest level at 48 h (Table 2). NH3-N and MCP levels did not display interaction effects with treatment and time.

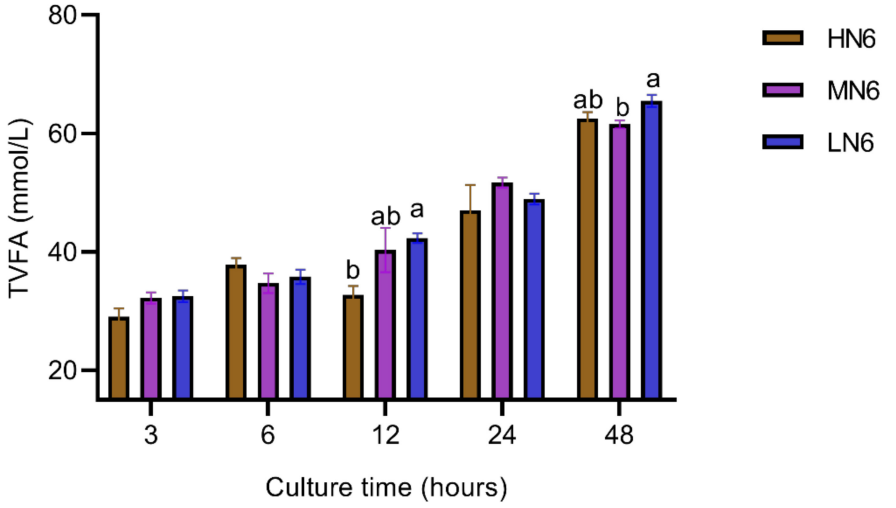


Figure 1. Effects of dietary n-6/n-3 ratio on in vitro total volatile fatty acid (VFA) production at each incubation time. HN6, high n-6 fatty acid treatment (n-6/n-3 ratio = 3.04:1); MN6, moderate n-6 fatty acid treatment (n-6/n-3 ratio = 2.03:1); LN6, low n-6 fatty acid treatment (n-6/n-3 ratio = 0.8:1); TVFA, total volatile fatty acids. Different superscript letters at the same time point indicate a significant difference ($p < 0.05$), whereas the same or no superscript letters indicate no significant difference. Data are represented by the mean ± SEM.

3.2. Effects of Dietary Fat Source on In Vitro Gas Kinetics

As shown in Figure 2, the total cumulative GP after incubation for 48 h and the asymptotic GP were unchanged by the three dietary treatments, while the maximum rate of substrate degradation (RmaxS) was significantly higher ($p < 0.05$) in the MN6 group than in the HN6 and LN6 groups.

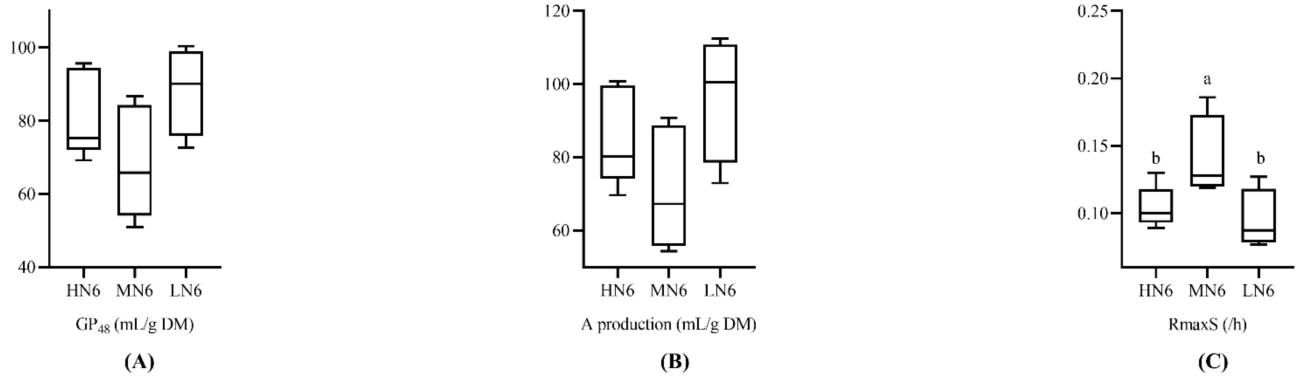


Figure 2. Effects of dietary n-6/n-3 ratio on in vitro gas production and kinetic parameters. (**A**) Cumulative gas production (mL/g DM) after incubation for 48 h. (**B**) Asymptotic gas production (mL/g DM). (**C**) Maximum rate of substrate digestion (/h). Different superscript letters indicate a significant difference ($p < 0.05$), whereas the same or no superscript letters indicate no significant. Data are represented by the mean ± SEM. HN6, High n-6 fatty acid treatment (n-6/n-3 ratio = 3.04:1); MN6, Moderate n-6 fatty acid treatment (n-6/n-3 ratio = 2.03:1); LN6, Low n-6 fatty acid treatment (n-6/n-3 ratio = 0.8:1).

3.3. Effects of Dietary Fat Source on In Vitro Gas Emission Production

A significantly lower ($p < 0.05$) CH_4 production was observed in the HN6 group than in the MN6 group, while the production of CH_4 did not differ between the MN6 and LN6 groups (Figure 3A). The production of O2 was significantly decreased ($p < 0.05$) in the HN6 and LN6 groups as compared to that in the MN6 group (Figure 3C).

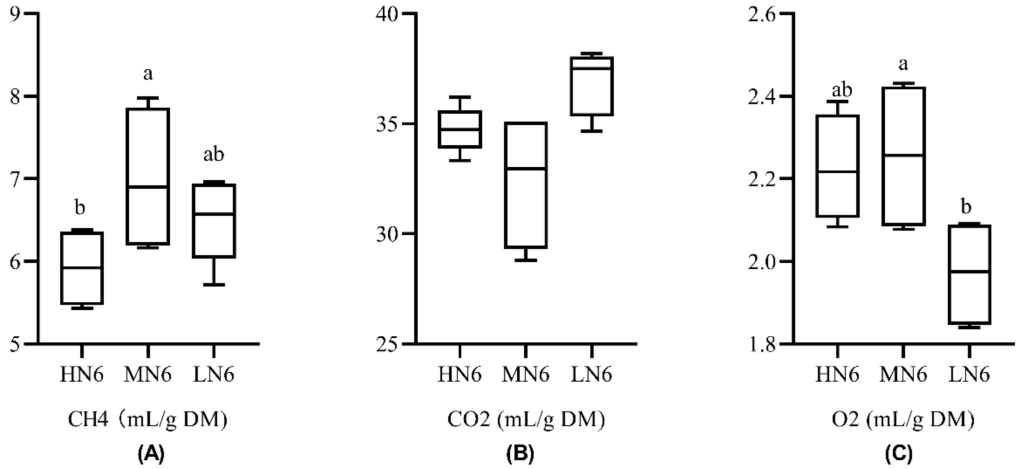


Figure 3. Effects of dietary n-6/n-3 ratio on in vitro CH_4, CO2, and O2 production. (**A**) CH_4 production (mL/g DM). (**B**) CO_2 production (mL/g DM). (**C**) O_2 production (mL/g DM). Different superscript letters indicate a significant difference ($p < 0.05$), whereas the same or no superscript letters indicate no significant difference. Data are represented by the mean ± SEM. HN6, High n-6 fatty acid treatment (n-6/n-3 ratio = 3.04:1); MN6, Moderate n-6 fatty acid treatment (n-6/n-3 ratio = 2.03:1); LN6, Low n-6 fatty acid treatment (n-6/n-3 ratio = 0.8:1).

3.4. Effect of Dietary Fat Source on Fatty Acid Profile

The C16:1 level was greater in the MN6 group than in the LN6 and HN6 groups after incubation for 3 h, but it did not differ at other incubation times (Figure 4A). Moreover, the LN6 group had higher C18:3n3 levels after incubation for 3 and 6 h as compared with the HN6 and MN6 groups, but the levels did not differ at 12 and 24 h (Figure 4B). The C20:1 level was significantly greater ($p < 0.05$) in the LN6 group than in the MN6 group, especially after incubation for 6 h, whereas the C20:1 level did not change over the incubation period in the HN6 group as compared with that in the LN6 and MN6 groups (Table 3, Figure 4C). The HN6 and LN6 groups had significantly higher ($p < 0.05$) levels of C18:2n6 and C18:3n3, respectively, as well as higher C18:1n9c levels as compared to the MN6 group (Table 3). A significantly higher ($p < 0.05$) level of the C16:0 FAs was observed in the MN6 group as compared to that in the HN6 and LN6 groups. The C24:0 FAs were higher in LN6 than in HN6 at an incubation time of 3 h; inversely, HN6 FAs were higher at 6 h as compared to those in HN3 (Figure 4E). Other saturated FAs (i.e., C12:0, C14:0, C15:0, C17:0, C18:0, C20:0, C22:0) were not affected by the dietary n-6/n-3 ratio (Table 3, Figure 4D). Both C16:0 and C16:1 levels were significantly greater ($p < 0.05$) in the MN6 group than in the LN6 and HN6 groups (Table 3). The n-6/n-3 ratio was significantly lower ($p < 0.05$) in the LN6 group than in the MN6 and HN6 groups.

Incubation time also had an influence on FA fermentation in vitro. Overall, unsaturated FA levels decreased with increasing incubation time such that C18:1n9, C18:2n6, and C18:3n3 levels decreased and achieved relative stability after incubation for 12 h, whereas C16:1, C20:1, and C22:1 levels were reduced to zero after incubation for 48 h. The levels of medium-chain (<C18) saturated FAs (C12:0, C14:0, C:15, C17:0) increased over the incubation period. C16:0, C18:0, C20:0, and C22:0 levels reached a maximum after incubation for 6 h and then decreased to their lowest levels at 48 h. In contrast, the C24:0 level steadily decreased during incubation, from 3 to 48 h.

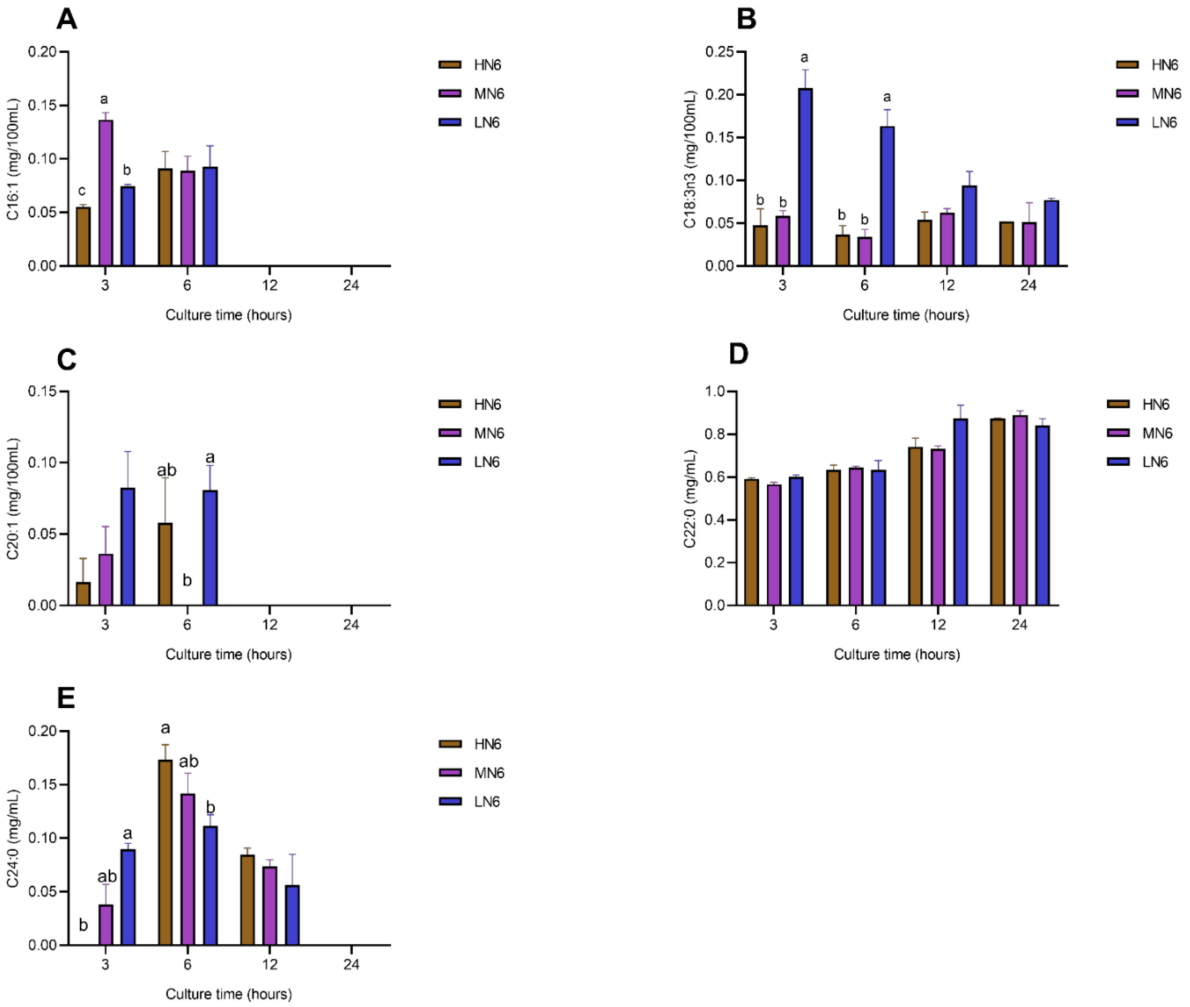


Figure 4. Effects of dietary n-6/n-3 ratio on in vitro fatty acid profiles. (**A**–**E**): The concentration of C16:1, C18:3, C20:1, C22:0, and C24:0 in the fermentation fluid. Different superscript letters at the same time point indicate a significant difference ($p < 0.05$), whereas the same or no superscript letters indicate no significant difference. Data are represented by the mean ± SEM. HN6, High n-6 fatty acid treatment (n-6/n-3 ratio = 3.04:1); MN6, Moderate n-6 fatty acid treatment (n-6/n-3 ratio = 2.03:1); LN6, Low n-6 fatty acid treatment (n-6/n-3 ratio = 0.8:1).

3.5. Effect of Dietary Fat Source on Microbial Profile

The Chao 1 diversity index value of the HN6 group was significantly lower ($p < 0.05$) than that of the MN6 and LN6 groups, which did not differ (Figure 5). The observed species richness was significantly lower ($p < 0.05$) for the HN6 group than for the MN6 group (Figure 5). Shannon diversity index values did not differ among the three dietary groups (Figure 5). The overall picture of the microbial composition of the samples in the three groups was obtained by PCoA (Figure 6), and the *p*-value of PERMANOVA was 0.210, showing that microbial communities were similar in the tested conditions.

Table 3. Effects of dietary n-6/n-3 PUFA ratio on fatty acid profiles in fermented rumen fluid in vitro (mg/100 mL).

Fatty Acid [1]	Treatment [2]			Fermentation Time (h) [3]				SEM [4]	*p*-Value [5]		
	HN6	MN6	LN6	3	6	12	24		TR	Time	INT
C12:0	0.153	0.148	0.160	0.135 ^{b}	0.129 ^{b}	0.173 ^{a}	0.175 ^{a}	0.003	0.133	<0.001	0.448
C14:0	0.363	0.367	0.377	0.264 ^{b}	0.311 ^{b}	0.440 ^{a}	0.456 ^{a}	0.012	0.763	<0.001	0.487
C15:0	0.353	0.353	0.368	0.246	0.331	0.469	0.386	0.012	0.746	<0.001	0.659
C16:0	5.820 ^{b}	7.983 ^{a}	6.092 ^{b}	10.588 ^{a}	4.379 ^{c}	5.875 ^{b}	5.691 ^{bc}	0.347	0.002	<0.001	0.165
C16:1	0.040 ^{b}	0.056 ^{a}	0.042 ^{b}	0.089 ^{a}	0.091 ^{a}	0.000 ^{b}	0.000 ^{b}	0.006	0.012	<0.001	0.001 *
C17:0	0.177	0.177	0.199	0.116 ^{d}	0.161 ^{c}	0.203 ^{b}	0.247 ^{a}	0.007	0.490	<0.001	0.442
C18:0	11.247	11.414	13.669	6.176 ^{c}	11.813 ^{a}	10.401 ^{b}	10.11 ^{b}	0.738	0.209	<0.001	0.612
C18:1n9c	1.569 ^{a}	1.523 ^{b}	1.872 ^{a}	2.415 ^{a}	1.844 ^{b}	1.159 ^{c}	1.156 ^{c}	0.085	0.019	<0.001	0.793
C18:2n6	0.716 ^{a}	0.697 ^{ab}	0.606 ^{b}	0.855 ^{a}	0.843 ^{a}	0.459 ^{b}	0.507 ^{b}	0.026	0.120	<0.001	0.543
C18:3n3	0.047 ^{b}	0.051 ^{b}	0.136 ^{a}	0.104 ^{a}	0.078 ^{b}	0.072 ^{b}	0.061 ^{b}	0.007	<0.001	0.004	<0.001 *
C20:0	0.156	0.156	0.166	0.082 ^{c}	0.270 ^{a}	0.155 ^{b}	0.130 ^{bc}	0.010	0.859	<0.001	0.093
C20:1	0.020 ^{ab}	0.009 ^{b}	0.041 ^{a}	0.045 ^{a}	0.046 ^{a}	0.000	0.000	0.005	0.019	<0.001	0.038 *
C22:0	0.707	0.708	0.737	0.586 ^{d}	0.868 ^{a}	0.788 ^{b}	0.637 ^{c}	0.015	0.278	<0.001	0.039 *
C22:1n9	0.085	0.075	0.084	0.107 ^{ab}	0.121 ^{a}	0.099 ^{b}	0.000 ^{c}	0.053	0.384	<0.001	0.193
C24:0	0.063	0.063	0.064	0.142 ^{a}	0.042 ^{c}	0.070 ^{b}	0.000 ^{d}	0.006	0.992	<0.001	0.001 *
n6/n3	12.669 ^{a}	11.062 ^{b}	4.851^{c}	9.427	10.860	6.983	9.215	0.551	<0.001	0.008	0.093

[1] n6/n3: The ratio of C18:2n6 to C18:3n3. [2] HN6: High n-6 fatty acid treatment (n6/n3, 3.04:1), MN6: Moderate n-6 fatty acid treatment (n6/n3, 2.03:1), LN6: Low n-6 fatty acid treatment (n6/n3, 0.8:1). ^{a,b,c}: Superscript letters show the multiple comparisons using Tukey's test; different superscripts mean significant differences in the indicators among groups ($p < 0.05$). [3] Time: Incubation time, ^{a,b,c}: Superscript letters show the multiple comparisons using Tukey's test; a row with different superscripts means significant differences under time effect factors exist ($p < 0.05$), whereas those denoted by the same letters or no letters are not significantly different. [4] SEM: standard error of the mean. [5] TR: The overall diet treatment effect between groups. Time: The overall effect of incubation time. INT: The effects of interaction between diet treatment and incubation time. *: If the interaction effect factor is significantly different, the detailed information of the data will be expressed in the picture (Figure 4).

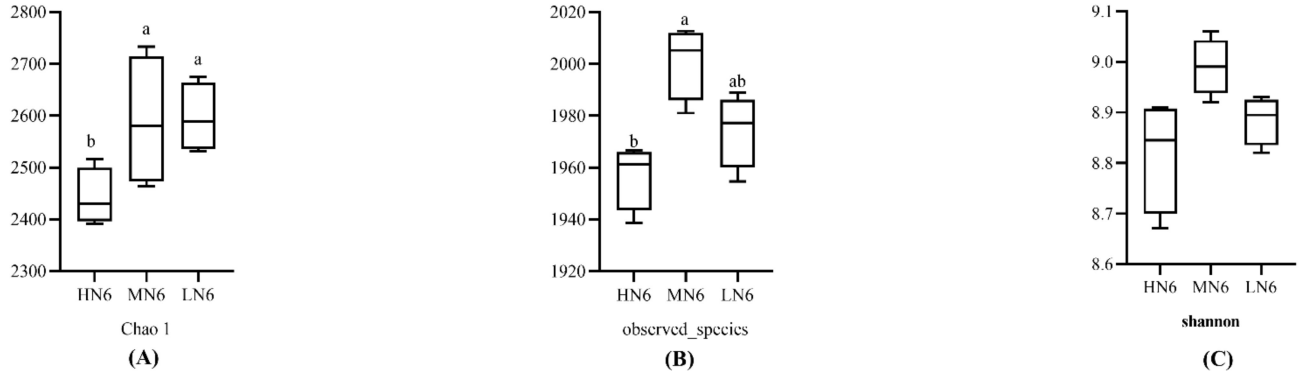


Figure 5. Alpha diversity indices for bacterial communities in fermented ruminal fluid. (**A**) Chao 1 diversity index. (**B**) Number of observed species. (**C**) Shannon diversity index. Different superscript letters indicate significant differences among dietary groups ($p < 0.05$), whereas the same or no superscript letters indicate no significant difference. Data are represented by the mean ± SEM. HN6, High n-6 fatty acid treatment (n-6/n-3 ratio = 3.04:1); MN6, Moderate n-6 fatty acid treatment (n-6/n-3 ratio = 2.03:1); LN6, Low n-6 fatty acid treatment (n-6/n-3 ratio = 0.8:1).

The genera influenced by the three dietary treatments are listed in Table 4. The relative abundances of *Ruminococcaceae_UCG-002*, *Lachnospiraceae_UCG-006*, *Selenomonas*, *Ruminiclostridium_6*, and *Ruminococcaceae_UCG-010* were significantly lower ($p < 0.05$) in the microbial community of the HN6 group than in the microbial community of the LN6 group. Meanwhile, the relative abundances of *Prevotellaceae_UCG-003* and *Methanobrevibacter* were significantly higher ($p < 0.05$) in the microbial community of the HN6 group than in the microbial community of the LN6 group (Table 4). The relative abundances of *Methanobrevibacter* and *Butyrivibrio_2* were lower ($p < 0.05$) in the MN6 group than in the LN6 group. Meanwhile, the relative abundances of *Lachnospiraceae_UCG-006*, *Prevotellaceae_UCG-003*, and *Butyrivibrio_2* were increased ($p < 0.05$) in the MN6 group as compared to those in the HN6 group.

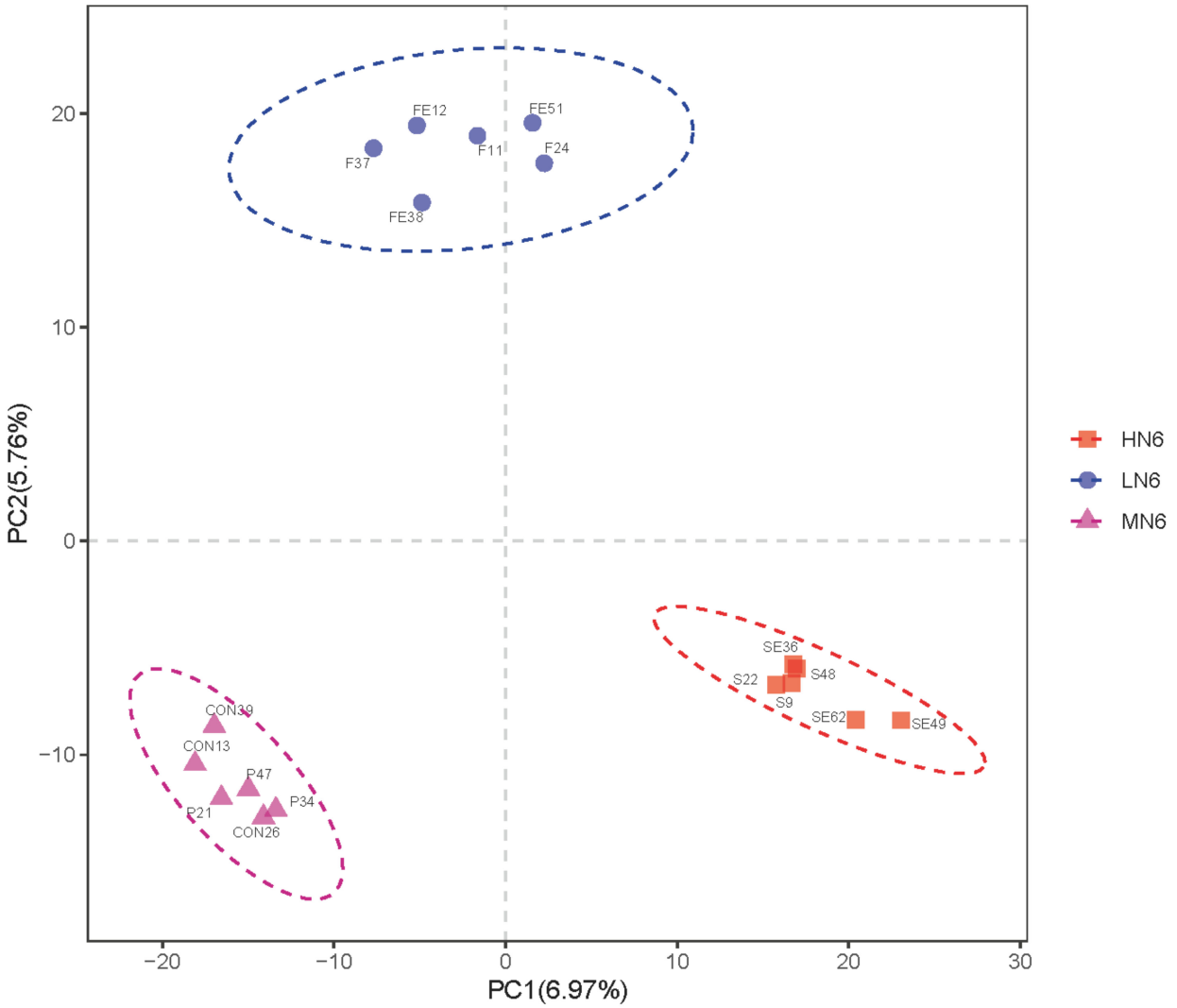


Figure 6. Beta diversity indices for bacterial communities in fermented ruminal fluid. Data are represented by the mean ± SEM. HN6, High n-6 fatty acid treatment (n-6/n-3 ratio = 3.04:1); MN6, Moderate n-6 fatty acid treatment (n-6/n-3 ratio = 2.03:1); LN6, Low n-6 fatty acid treatment (n-6/n-3 ratio = 0.8:1).

Table 4. Effects of dietary n-6/n-3 PUFA ratio on the relative abundance of bacterial genes (%).

Microorganism	Treatment			SEM	*p*-Value
	HN6	MN6	LN6		
Anaerorhabdus_furcosa	0.136	0.131	0.074	0.009	0.060
Butyrivibrio_2	0.573 ^{b}	0.664 ^{a}	0.526 ^{b}	0.017	0.024
Lachnospiraceae_NK4A136	0.091	0.099	0.132	0.006	0.060
Lachnospiraceae_UCG-006	0.130 ^{a}	0.091 ^{b}	0.131 ^{a}	0.005	0.020
Methanobrevibacter	0.035 ^{a}	0.042 ^{a}	0.013 ^{b}	0.004	0.001
Prevotellaceae_UCG-003	1.264 ^{a}	0.960 ^{b}	0.928 ^{b}	0.191	0.025
Prevotellaceae_UCG-004	0.433	0.275	0.280	0.045	0.053
Ruminococcaceae_UCG-002	0.555 ^{b}	0.666 ^{ab}	0.714 ^{a}	0.021	0.038
Ruminococcaceae_UCG-010	1.366 ^{c}	1.522 ^{b}	1.666 ^{a}	0.038	0.001
Ruminiclostridium_6	0.158 ^{a}	0.132 ^{ab}	0.113 ^{b}	0.005	0.033
Selenomonas	0.059 ^{b}	0.100 ^{a}	0.091 ^{a}	0.007	0.017

HN6: High n-6 fatty acid treatment (n6/n3, 3.04:1), MN6: Moderate n-6 fatty acid treatment (n6/n3, 2.03:1), LN6: Low n-6 fatty acid treatment (n6/n3, 0.8:1). SEM: standard error of the mean. ^{a,b,c}: Different superscripts mean significant differences in the indicators among groups ($p < 0.05$), whereas those denoted by the same letters or no letters are not significantly different.

4. Discussion

Previous researchers have reported that the addition of dietary lipids trigger various changes in rumen fermentation depending on the amount and type of FA supplemented and the diet composition [41–43]. Moreover, unsaturated FAs are reportedly toxic to many species of rumen bacteria, particularly those that are involved in fiber digestion [44]. Furthermore, relatively higher dietary levels of vegetable oil (5.8 or 7% DM) induced the decrease of DMI and organic matter digestibility [9,45]. However, several studies did not report the negative effect of dietary C18 PUFAs on fiber degradation, especially at relatively lower levels of oil addition (≤5% dietary DM) [46–48]. Thus, the similar IVDMD values obtained among dietary groups in the current study could be due to the relatively low level of fat supplementation (4% DM). In addition, previous studies have suggested that C18 PUFA supplementation has little effect on rumen pH [49–51] and total VFA concentration [51–53], which contradicts the results of the current study, in which the HN6 diet induced lower pH and VFA concentrations (i.e., acetate, propionate, butyrate, and total VFA) as compared to the other diets; however, no difference was observed between the LN6 and MN6 diets. These inconsistencies may be due to differences in the fat type and the proportion of dietary forage used among the studies since a TMR containing 70% forage and 30% concentrate was used in the current study. Nevertheless, the study results concur with previous reports, in which high-forage diets supplemented with oil enriched with n-6 PUFAs (soybean oil) interfered with ruminal fermentation in dairy cows [54]; on the other hand, oil enriched with n-3 PUFAs (flaxseed oil) [53] and saturated FAs (palm oil) [52] had no effects on ruminal pH and total VFAs, respectively. Notably, the acetate to propionate ratio was not affected by any of the dietary treatments, which concurred with another study demonstrating that the FA type did not affect the acetate to propionate ratio in the rumen [55].

The n-6 PUFAs were hypothesized to decrease some microorganism activities associated with high-forage diets. *Ruminococcaceae_UCG-002* was previously reported to be negatively correlated with glucose production [56], while *Ruminococcaceae_ UCG-010* was associated with digestive fibers [57]. *Prevotellaceae UCG-003* belongs to the genus *Prevotella*, members of which play a key role in the metabolism of carbohydrates such as sugar, starch, and xylan, and protein [58]. The relative abundances of *Ruminococcaceae_UCG-002* and *Ruminococcaceae_ UCG-010* were decreased in the HN6 group, but those of *Prevotellaceae UCG-003* were increased. These results imply that a high n-6 PUFA diet could enhance the metabolism of carbohydrates and reduce fiber digestibility. *Lachnospiraceae UCG-006* was reportedly negatively correlated with acetic acid and butyric acid levels, but positively correlated with the mRNA expression of adenosine monophosphate-activated protein kinase-α (AMPK-α) [59]. These results might explain the lower VFA concentrations observed in the HN6 group, which may have been caused by the inhibited growth of *Lachnospiraceae UCG-006*. Selenomonas spp. are obligate *saccharolytic* bacteria that participate in the fermentation of soluble sugars and lactate in the rumen [60]. Lactic acid is a major metabolic product of the genus *A. furcosa* [61]. These results could partially explain the lower pH in the rumen fluid of the HN6 group, in which the high n-6 PUFA diet decreased the relative abundance of *Selenomonas*. Taken together, these results indicate that oil with a high ratio of n-6/n-3 added to the high-forage diet had detrimental effects on the pH and VFAs during in vitro ruminant fermentation as compared with oil enriched with a relatively high n-6/n-3 ratio.

VFA concentrations increased with increasing incubation time. Increased pH after incubation for 3 h could be explained by the accumulation of NH_3-N in fermented rumen fluid over the incubation period. Additionally, lower NH_3-N concentrations and higher MCP concentrations were observed in the HN_6 and LN_6 groups. These results could indicate that the addition of unsaturated fat sources decreased the NH_3-N concentration in the rumen [62] and enhanced the effectiveness of microbial nitrogen synthesis [63]. This may be due to the fact that the addition of high n-3 or n-6 PUFA can reduce the protozoa numbers [64]. This is in line with a previous study, which reported that plant oil (enriched with n-3 or n-6 C18 PUFA) supplementation in ruminant diets could depress

the rumen ammonia concentration [65]. Ammonia is usually assumed to be produced by the decomposition of bacterial protein [66], which is often associated with the presence of protozoa [67]. According to Hristov et al. [68], the number of protozoa has a positive correlation with ammonia concentrations; reducing the number of protozoa could inhibit bacterial protein breakdown in the rumen. MCP levels were the highest after incubation for 6 h and decreased to their lowest levels at 48 h, which could be due to decreased substrate levels and the decomposition of MCP over the incubation period. Moreover, IVDMD values were not affected in the current study, which is likely due to the low FA concentration (<5%) used to supplement the diet. A previous study reported that lipid blends in the diet reduced IVDMD values when FA concentrations were more than 5%, but not at lower concentrations [69].

Generally, the addition of oil enriched with n-6 or n-3 PUFAs to a high-forage diet has been shown to reduce the production of fermentation gases [70]. Despite the cumulative GP and the asymptotic GP not being affected in the current study, the maximum rates of substrate digestion in the HN_6 and LN_6 groups were lower than that in the MN_6 group. Additionally, the HN_6 and LN_6 groups produced less CH_4 than the MN_6 group, particularly the HN_6 group. These results were consistent with the majority of studies reporting that dietary unsaturated fat supplementation can depress enteric methane emissions, although the extent of inhibition varied [71,72]. In particular, studies have reported that supplementation with C18 PUFA-rich oils inhibited methane production in vitro [73] or in vivo [74], and the extent differed according to the degree of unsaturation and the inclusion level [6]. This indicates that the n-6/n-3 ratio can also depress methane production as the different ratios will modify the degree of unsaturation of the dietary fat. In our study, it seems that the high n-6/n-3 ratio of additional PUFA is more efficient in decreasing CH_4. Moreover, some studies have reported that high-forage diets might increase the inhibitory effect of PUFAs on CH_4 production. For example, Martin et al. [9] reported that dairy cows fed high-forage diets supplemented with various forms of oilseed enriched with n-3 PUFAs exhibited a 10% reduction in CH_4 production (g/kg DMI) for unprocessed seeds and a 49% reduction for crude oil from the seeds. Other studies demonstrated that the supplementation of a forage-based diet with oil enriched with n-6 PUFAs reduced the CH_4 production of beef cattle (g/kg of DMI) by 17% [75] or 15% [76]. Taken together, these results indicate that supplemental PUFAs could reduce CH_4 emissions, and the extent of CH_4 inhibition may be influenced by the proportion of forage in the diet.

The biohydrogenation of unsaturated FAs in the rumen and propionate production are considered to be a competitive pathway for hydrogen use that inhibits CH_4 production. However, the results of the current study indicate that CH_4 and propionate levels were both decreased in the HN_6 group. This inconsistency may be due to the small amount of total metabolizable hydrogen used in the biohydrogenation of endogenous PUFAs [77], but it may also reflect the direct effect on methanogenesis performed by rumen microorganisms [78]. However, a decreased relative abundance in methanogenic bacteria was not detected in the HN_6 group, possibly because only bacterial relative abundance was measured and the ciliate protozoal population, considered to be one of the main methanogenesis microorganisms, was not detected [79]. Interestingly, the relative abundance of *Methanobrevibacter*, an anaerobic archaean that produces methane [80], was decreased in the HN6 group. These results suggest that higher n-3 PUFA levels might reduce CH_4 emission.

The unexpected high O_2 production may be due to the rumen fluid collected from cannula cows. Wang et al. [81] reported that the laparotomy incision is usually bigger than the diameter of the cannula, and that the muscle and skin support around the cannula can be compromised, leading to leakage of fermentation gases and rumen content. The leakage of fermentation gases from the rumen could then result in the increases in concentrations of N_2 and O_2. The influx of O_2 into the headspace of the rumen has the potential to alter its strictly anaerobic microbial ecosystem, consequently increasing O_2 concentration. This probably has little effect on the fermentation process. However, the specific reason is still unclear, and we intend to focus on a study regarding O_2 in the future.

The highest levels of C16:0, C18:2n6, and C18:3n3 were detected in the MN6, HN6, and LN6 groups, respectively, which reflected the higher amounts of these FAs in the diets. This finding indicates that the n-6/n-3 ratio in the diet affects the FA composition in the ruminal fluid. Additionally, relatively high levels of C18:1n9c (and cis-C18:1 isomers), intermediaries of the biohydrogenation of unsaturated FAs [82], were detected among the three dietary groups, although the C18:1n9c concentrations in the HN_6 and LN_6 groups were higher than that in the MN6 group. However, the amount of stearic acid (C18:0), the end product of the biohydrogenation of unsaturated FAs [82], was not influenced by the diet. These results could reflect the incomplete biohydrogenation of unsaturated C18 FAs, which concurs with unsaturated C18 FA (C18:1n9c, C18:2n6, and C18:3n3) concentrations decreasing after incubation for 12 h but remaining stable at 24 and 48 h. However, unsaturated C16:1 and C22:1 were not detected at 48 h, suggesting that the complete biohydrogenation of these unsaturated FAs occurred. Notably, Kalscheur et al. [83] reported that incomplete biohydrogenation occurred to a greater extent in cows supplemented with C18 FA-enriched fat. Furthermore, the HN_6 and LN_6 groups displayed decreased relative abundances of *Butyrivibrio_2* as compared with the MN6 group. *Butyrivibrio_2* is reportedly involved in biohydrogenation [84]. The results suggest that n-6 and n-3 PUFAs were toxic to *Butyrivibrio_2* growth, resulting in the incomplete biohydrogenation of unsaturated FAs.

For the rumen fermentation, the first 12 h of fermentation activities were very fast, and the biohydrogenation process mainly occurred during this period. The fatty acid composition is highly related to biohydrogenation. Therefore, the effect of fat addition on the rumen fluid fatty acid will be different between the beginning and end stages. This is the reason for the interaction effect in some FAs. This can also explain why some saturated fatty acids such as C22:0 increased (C22:1 biohydrogenation) in the first couple of hours but decreased in the last few hours after a breakdown in the rumen. In addition, fatty acids < 16 carbon mainly come from de novo synthesis using VFA [85]. As a result, C12:0 and C14:0 both increased with time. As for the fatty acids $\geq$ C16, which mainly come from feed, these would partially catabolize to VFA or CO2 [86]. Thus, the C16:0 decreased with time.

Beyond the aforementioned microorganisms, the Chao 1 diversity index value and the observed species richness were the lowest in the HN6 group, indicating that an increase in the n-6/n-3 ratio decreased bacterial community diversity and abundance. In addition, the HN6 group displayed the lowest relative abundance of *Lachnospiraceae NK4A136* and the highest relative abundance of *Ruminiclostridium_6*. *Lachnospiraceae NK4A136* is considered to be a potential probiotic and exerts anti-inflammation effects [87]. Higher *Ruminiclostridium_6* abundance was positively correlated with cytokine levels [88]. Therefore, n-6 PUFA supplementation might have pro-inflammatory effects on ruminants, whereas n-3 PUFAs might have anti-inflammatory effects.

5. Conclusions

In conclusion, the study findings suggest that manipulating the n-6/n-3 ratio of dairy cow feed by adding extruded flaxseeds or soybeans could affect rumen fermentation, fatty acid composition, and the microbial community. Furthermore, the results indicate that supplementation with a high n-6 PUFA source decreased bacterial community richness and diversity, specifically reducing the relative abundance of *Lachnospiraceae UCG-006* and *Selenomonas*, which resulted in lower ruminant pH and VFA (i.e., acetate, propionate, butyrate, and total VFA) levels in vitro. The results also suggest that n-6 and n-3 PUFAs are toxic to *Butyrivibrio_2*, resulting in a higher level of incomplete biohydrogenation in the rumen. Taken together, the study findings suggest that supplementing high-forage diets with high levels of n-6 PUFAs could reduce CH_4 emissions.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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