The compound 3-nitrooxypropanol (3-NOP) is a promising methane inhibitor, which performs well in inhibiting methane emission and does not affect animal feed intake and digestibility. However, it causes a significant increase in hydrogen production while suppressing methane emission, resulting in a waste of feed energy. Vitamin B12 is a key factor in the propionate production pathway and thus plays an important role in regulating the hydrogen utilization pathway. In this study, the effects of 3-NOP combined with vitamin B12 supplementation on rumen fermentation and microbial compositional structure in dairy cattle were investigated by simulating rumen fermentation in vitro. Experiments were performed using a 2 × 2-factorial design: two 3-NOP levels (0 or 2 mg/g dry matter) and 2 vitamin B12 levels (0 or 2 mg/g dry matter). Three experiments were performed, each consisting of 4 treatments, 4 replicates, and 4 blanks containing only inoculum. The combined supplementation of 3-NOP and vitamin B12 reduced methane emission by 12% without affecting dry matter digestibility. The combined addition of 3-NOP and vitamin B12 significantly increased the concentration of propionate and reduced the concentration of acetate and the acetate to propionate ratio. At the bacterial level, 3-NOP increased the relative abundances of Christensenellaceae_R-7_group and Lachnospiraceae_NK3A20_group. Vitamin B12 increased the relative abundances of unclassified_f__Prevotellaceae and Prevotellaceae_UCG-003 and decreased the relative abundance of Lachnospiraceae_NK3A20_group. At the archaeal level, the combination of 3-NOP and vitamin B12 increased the relative abundances of Methanobrevibacter_sp._Abm4, OTU1125, and OTU95 and decreased the relative abundances of uncultured_methanoogenic_archaeon_g__Methanobrevibacter, OTU1147, OTU1056, and OTU55. The results indicated that 3-NOP combined with vitamin B12 could alleviate rumen hydrogen emission and enhance the inhibition of methane emission compared with 3-NOP alone. 

**Key words:** 3-nitrooxypropanol, rumen microbiota, vitamin B12, propionate formation, methanogenesis

### INTRODUCTION

The greenhouse effect is an urgent global environmental problem. Greenhouse gases including carbon dioxide (CO2), methane (CH4), nitrous oxide (N2O), and ozone (O3) can absorb atmospheric infrared radiation to cause climate change and global warming (Lashof and Ahuja, 1990). Methane is a major greenhouse gas produced during agricultural production (Alvarez-Hess et al., 2019a), especially in ruminant livestock production (Hook et al., 2010). Ruminant gastrointestinal fermentation and feces are major sources of CH4, contributing to 80% of total greenhouse gas emission in livestock farming (Wubah and Hagestien, 2002; Ellis et al., 2007; Persson et al., 2015). Although CH4 is the second major greenhouse gas after CO2, the effect of CH4 on global warming is 25 times greater than that of CO2. Because of the short residence time of CH4 in the atmosphere, the concentration of CH4 in the atmosphere can respond to CH4 emission reduction activities relatively quickly (IPCC, 2001; Forster et al., 2007). In addition, the release of CH4 into the atmosphere as a byproduct of rumen microbial fermentation is also a waste of energy for the animal (Mitsumori and Sun, 2008; Alvarez-Hess et al., 2019b). It has been reported that 2 to 12% of the total energy intake is lost in the form of CH4 in ruminants (Johnson and Johnson, 1995). Therefore, researchers and livestock workers in various countries have carried out a lot of research, hoping to formulate effective strategies to mitigate CH4 emission. Among the many measures to reduce CH4 emission in ruminants, the use of CH4 inhibitors seems to be a more effective strategy (Romero-Pérez et al., 2015a).
The compound 3-nitroxypropanol (3-NOP) is a \( \text{CH}_4 \) inhibitor with molecular structure similar to methyl coenzyme M designed and developed by Duval and Kindermann (2012). It inactivates the key enzyme methyl coenzyme M reductase required for \( \text{CH}_4 \) production by oxidizing the active site Ni (I) of methyl coenzyme M, so as to inhibit \( \text{CH}_4 \) production (Duin et al., 2016). In various studies in vivo or in vitro, 3-NOP had a good inhibitory effect on \( \text{CH}_4 \) emission and had no effect on animal DMI (Martínez-Fernandez et al., 2014; Reynolds et al., 2014; Romero-Pérez et al., 2015a; Melgar et al., 2021). For example, in a long-term experiment conducted by Hristov et al. (2015), 3-NOP reduced rumen \( \text{CH}_4 \) emission by 30% without affecting DMI, milk yield, and fiber digestibility, and this significant effect remained throughout the 12-wk experiment. Although 3-NOP can effectively reduce \( \text{CH}_4 \) emission, it also causes a significant increase in hydrogen (\( \text{H}_2 \)) emission (van Gastelen et al., 2020; Melgar et al., 2021).

The results of a study by Hristov et al. (2015) showed that addition of 3-NOP at 40–80 mg/kg DM of feed decreased \( \text{CH}_4 \) emission by 30% in high-yielding cows, but increased \( \text{H}_2 \) emission by 64-fold, although the intensity of \( \text{H}_2 \) emission declined after a period of time. Hydrogen, which exists in the rumen as a gas (\( \text{H}_2 \)) or in liquid ([\( \text{H} \)]) state (Janssen, 2010), is a key intermediate in rumen microbial fermentation (Hungate, 1967) and an energy substrate for \( \text{CH}_4 \) production by rumen archaea (Ellis et al., 2008). In cows fed 3-NOP, the production of \( \text{CH}_4 \) was inhibited, and the increased hydrogen was excreted in the form of \( \text{H}_2 \) or accumulated in the rumen in the form of [\( \text{H} \)] (Melgar et al., 2020). However, either form is a waste of feed energy. Furthermore, the daily increased \( \text{H}_2 \) emission of dairy cows fed with 3-NOP is much smaller than that produced by [\( \text{H} \)] but not used for \( \text{CH}_4 \) production, so a large amount of [\( \text{H} \)] may be accumulated in rumen (Hristov et al., 2015; Melgar et al., 2020). Therefore, when using 3-NOP to inhibit \( \text{CH}_4 \) production, how to reasonably allocate the fate of [\( \text{H} \)] in rumen is still an important scientific problem to be solved.

Transferring [\( \text{H} \)] to metabolic processes that have nutritional value for animals is an ideal strategy to suppress the loss of digestible energy in the process of \( \text{CH}_4 \) production, while avoiding fermentation inhibition (Lan and Yang, 2019). For example, it has been suggested to shift [\( \text{H} \)] from \( \text{CH}_4 \) to propionate, which may be a way to increase the available metabolizable energy of animals (Martin and Macy, 1985). Propionate is a short chain fatty acid formed by the fermentation of carbohydrates in the diet, and is absorbed and used by animals and participates in the process of gluconeogenesis in the body (Reichardt et al., 2014; Morrison and Preston, 2016). As the main precursor of glucose in cows, propionate accounts for 60% of the amount of glucose released by the liver through gluconeogenesis (Reynolds, 2006; Larsen and Kristensen, 2013; Duplessis et al., 2017). Furthermore, in lactating cows, approximately 80% of the glucose supply comes from gluconeogenesis (Galindo et al., 2011). Three pathways of propionate production are known: the succinate pathway, the propylene glycol pathway, and the acrylate pathway. Of which the succinate pathway is the most predominant one (Louis and Flint, 2017). Propionate production in the succinate pathway involves propionyl-CoA transferase reaction. Vitamin \( \text{B}_{12} \) is a necessary factor for propionyl-CoA transferase, a key enzyme in the formation of propionate in the succinate pathway (Louis and Flint, 2017), and accumulation of succinate was observed in vitamin \( \text{B}_{12} \) deficient cultures of rumen \textit{Prevotella ruminicola} (Strobel, 1992).

In view of the role of vitamin \( \text{B}_{12} \) in propionic acid metabolism, it should play an important role in regulating [\( \text{H} \)] utilization pathway. Therefore, the objectives of this study were to determine the effects of vitamin \( \text{B}_{12} \) on rumen propionate and \( \text{CH}_4 \) production in dairy cows by using in vitro fermentation methods, and to explore whether its combined application with 3-NOP in vitro would have synergistic effects on increasing rumen propionate and reducing \( \text{CH}_4 \) production in dairy cows.

**MATERIALS AND METHODS**

The experiments were conducted at the Institute of Animal Science, Chinese Academy of Agricultural Sciences (Beijing, China), and the animal procedures were approved by the Animal Care and Use Committee of the Chinese Academy of Agricultural Sciences (IAS2021–100; Beijing, China).

**Experimental Design**

Performed in vitro culture experiment to assess the combined effect of 3-NOP and vitamin \( \text{B}_{12} \), the treatments were laid out as a \( 2 \times 3 \)-factorial arrangement in randomized complete block design: two 3-NOP levels (0 or 2 mg/g DM) and 2 vitamin \( \text{B}_{12} \) levels (0 or 2 mg/g DM). The compound 3-NOP levels were selected based on the highest 3-NOP dose used in a previous in vitro experiment (Romero-Pérez et al., 2016). The selection of vitamin \( \text{B}_{12} \) level is based on the addition dose selected by our in vitro experiment of adding vitamin \( \text{B}_{12} \) alone. The experiment consisted of three 24-h in vitro cultures. Each run consisted of 20 samples: 4 treatments × 4 replicates as well as 4 blanks containing only inoculum. The 4 treatments were: control (CON), vitamin \( \text{B}_{12} \), 3-NOP, 3-NOP + vitamin \( \text{B}_{12} \). Reagents...
were added accurately to the fermentation flasks and the volume of liquid in all fermentation flasks was equalized with distilled water. The compound 3-NOP powder (>98%) was purchased from Shanghai Aladdin Biochemical Technology Co. Ltd.; vitamin B₁₂ powder (>98%) was purchased from Solarbio Life Science.

In Vitro Incubation

Three lactating multiparous Holstein cows with permanent ruminal fistulas served as donors of ruminal fluid (3 ± 1 of parity; 618 ± 100 kg of BW; 23 ± 2.8 kg of milk yield/d). Donor cows received a TMR diet, which consisted (DM basis) of corn silage (25.7%), alfalfa hay (18.6%), steam flaked corn (26.0%), soybean meal (7.4%), cottonseed meal (7.4%), beet meal (5.6%), distillers dried grains with solubles (7.4%), and minerals and vitamins (1.9%). The rumen fluid was collected about 2 h after morning feeding. And the rumen fluid from the 3 donor cows was mixed together in equal volumes and placed in a pre-heated thermost, and then brought back to the laboratory within 30 min. The rumen fluid was then filtered through 2 layers of gauze to remove impurities and then diluted with warm (39°C) buffer (each liter of solution containing 8.75 g of NaHCO₃, 1.00 g of NH₄HCO₃, 1.43 g of Na₂HPO₄, 1.55 g of KH₂PO₄, 0.15 g of MgSO₄·7H₂O, 0.52 g of Na₂S, 0.017 g of CaCl·2H₂O, 0.015 g of MnCl₂·4H₂O, 0.002 g of CoCl·6H₂O, 0.012 g of FeCl₃·6H₂O, and 1.25 mg of resazurin) as inoculum. The inoculum was placed in a water bath at 39°C with constant agitation to ensure adequate mixing of the rumen fluid and buffer (1:2, vol/vol). The whole process was performed under continuous flushing with CO₂. The fermentation substrate was the same TMR offered to the cows, which was dried at 55°C for 48 h and passed through a 1-mm screen with a Wiley mill (Arthur H Thomas Co). Under anaerobic conditions of continuous washing with CO₂, the fermentation substrate and inoculum were mixed thoroughly. When the incubation time reached 24 h, all the fermentation flasks were taken out and put into ice water to terminate the fermentation. The samples were then collected and preserved for subsequent analysis.

Sample Collection and Analysis

Total gas production from each air bag was measured using a calibrated glass syringe (100 mL, Häberle Labortechnik). A portable pH meter (Seven Go portable pH meter, Mettler-Toledo) was used to measure the pH values of the in vitro incubations. The liquid sample was collected after it had been completely cooled. Liquid samples (2.5 mL × 3) were collected from each fermentation flask, respectively for the analysis of VFA and microorganisms. The liquid collected from each bottle was centrifuged at 15,000 × g for 10 min at 4°C. Then the supernatant was acidified with 0.15 mL of 25% metaphosphate and stored at −20°C for VFA analysis. Samples for microbial DNA extraction and 16S rRNA gene sequencing and analysis were immediately frozen in liquid nitrogen and then transferred to a −80°C freezer. All the biomass materials in each bottle were separately filtered through a pre-weighed nylon bag (8 cm × 12 cm, 42 µm). The nylon bags were repeatedly rinsed with tap water until the effluent became clear and then oven-dried at 55°C for 48 h for measuring the dry matter degradation (DMD). Methane production was calculated by CH₄ concentration and total gas volume. The concentrations of CH₄ and VFA were determined using an Agilent 7890B gas chromatograph (7890B, Agilent Technologies).

DNA Extraction and 16S rRNA Genes Sequencing and Analysis

Rumen microbial DNA was extracted using cetyltrimethylammonium bromide (CTAB) plus bead beating (Jin et al., 2017). The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific). The hypervariable region V3–V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by an ABI Gene Amp 9700 PCR thermocycler (ABI). The V4–V5 region of the archaeal 16S rRNA gene was amplified by primers 524F10extF (5′-GTGYCAGCCGCCGCGGTAA-3′) and Arch958R-modR (5′-YCCGCGGTGTA VTCCAA TT-3′). The PCR amplification of 16S rRNA gene was performed as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles (bacteria) or 33 cycles (archaea) of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and
extension at 72°C for 45 s, and single extension at 72°C for 10 min, and end at 4°C. The PCR mixtures contained 5 × TransStart FastPfu buffer (4 µL), 2.5 mM dNTP (2 µL), forward primer [(5 µM) 0.8 µL], reverse primer [(5 µM) 0.8 µL], TransStart FastPfu DNA Polymerase (0.4 µL), template DNA (10 ng), and finally double-distilled H2O up to 20 µL. The PCR reactions were performed in triplicate. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences) according to the manufacturer’s instructions and quantified using a Quantus Fluorometer (Promega). Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina) according to standard protocols by Majorbio Bio-Pharm Technology Co. Ltd.

The data were analyzed on the free online platform of Majorbio Cloud Platform (www.majorbio.com). Different samples were identified based on unique barcodes. The 16S rRNA sequences were perfectly matched to different samples, quality filtered by fastp (version 0.20.0), and merged by FLASH (version 1.2.7; Magoč and Salzberg, 2011); (1) filtered the bases with a tail quality value below 20 of the reads, and set a 50 bp window. If the average quality value in the window was lower than 20, the back-end bases would be truncated from the window, filtered the reads with the quality control value below 50 bp, and removed the reads containing N bases; (2) according to the overlap relationship between PE reads, paired reads were spliced into a sequence with a minimum overlap length of 10 bp. Then the primers and barcodes were removed, and chimera was filtered to obtain valid reads. After filtration, the valid reads of bacterial and archaeal communities were 2,491,440 and 2,990,098, respectively, and the average lengths of valid reads were 418 and 428 bp, respectively. Valid reads were clustered with operational taxonomic units (OTU) at a similarity cutoff of 97% using UPARSE (version 7.1; Edgar et al., 2011). Classification analysis with a confidence threshold of 0.8 and the SILVA database (version 138) was performed using the RDP classifier (version 2.11; Quast et al., 2013). Alpha diversity was analyzed using the QIIME 2 package, and the indices Simpson, Shannon, Chao1, coverage, and ACE were calculated. Principal coordinate analysis was performed based on weighted UniFrac distances (Lozupone et al., 2007). Differences in bacterial and archaeal relative abundances were visualized by extended error bar plots with bioinformatics software (STAMP). Welch’s 2-sided test was used, with a Welch’s inversion test of 0.95 (Parks et al., 2014).

The raw reads were deposited into the National Center for Biotechnology Information Sequence Read Archive database (Accession Number: SRP327717).

### Statistical Analysis

All data presented were the average of the 3 experiments. The analysis of in vitro fermentation parameters (pH, DMD, total gas, CH4, and VFA), and abundance and diversity of bacteria and archaea were performed using the MIXED procedure of SAS 9.4 version (SAS Institute Inc.). The data were tested for normality using the UNIVARIATE procedure of SAS. The model used for data analysis was as follows:

\[ Y_{ij} = \mu + P_i + S_j + PS_{ij} + e_{ij} \]

where \( Y_{ij} \) is the observed value, \( \mu \) is the overall mean, \( P_i \) is the fixed effect of treatment with 3-NOP, \( S_j \) is the fixed effect of treatment with vitamin B12, \( PS_{ij} \) is the interaction effect of treatment with 3-NOP + vitamin B12, and \( e_{ij} \) is the random error. The difference was significant when \( P \leq 0.05 \), and showed a trend when 0.05 < \( P \leq 0.10 \).

### RESULTS

#### Methane Production and Fermentation Characteristics

The effects of 3-NOP, vitamin B12, and their interactions on fermentation characteristics and CH4 production are shown in Table 1. The addition of 3-NOP reduced the total gas production (\( P = 0.02 \)), whereas vitamin B12 had no effect on the total gas production (\( P = 0.30 \)). The addition of 3-NOP and vitamin B12 both respectively decreased the CH4 production by 9% (\( P = 0.30 \)). The compound 3-NOP in combination with vitamin B12 decreased CH4 production by 12% (\( P = 0.26 \)). Both 3-NOP (\( P < 0.01 \)) and vitamin B12 (\( P = 0.08 \)) increased the pH of the fermentation liquid. All treatments had no effect on DMD. Corresponding to the increased pHi, both 3-NOP (\( P < 0.01 \)) and vitamin B12 (\( P = 0.03 \)) significantly decreased the concentration of total VFA. The compound 3-NOP significantly decreased the concentrations of acetate (\( P < 0.01 \)) and valerate (\( P < 0.01 \)), significantly increased the concentration of propionate (\( P = 0.02 \)), and tended to decrease the concentration of isobutyrate (\( P = 0.06 \)). Vitamin B12 significantly decreased the concentration of propionate (\( P < 0.01 \)) and increased the concentration of propionate (\( P = 0.04 \)), whereas it had no effect on other VFA (\( P > 0.1 \)). Both 3-NOP (\( P < 0.01 \)) and vitamin B12 (\( P < 0.01 \)) significantly reduced the ratio of acetate to propionate. The combination of 3-NOP and vitamin B12 significantly increased the concentration of propionate and decreased the concentration of acetate and the ratio of acetate to propionate.
Changes of Microbial Community

Effects on Bacterial Community. The α diversity of bacterial community is shown in Table 2. The coverage of sample OTU was more than 99% and there was no significant difference among treatments, indicating that the accuracy and repeatability of sequencing results were ideal for analysis. Alpha diversity indices such as Shannon, Simpson, ACE, and Chao1 did not differ significantly among treatments \((P > 0.05)\), indicating that the addition of 3-NOP and vitamin \(B_{12}\) did not affect the richness and diversity of the bacterial community. Propionyl-CoA analysis based on the weighted UniFrac distance algorithm showed no obvious separation of each treatment group from CON (Figure 1). Similarity analysis (ANOSIM) revealed no significant difference in bacterial community structure between each treatment group and CON \((r = 0.01, P = 0.58)\).

A total of 18 phyla of bacteria were identified in rumen samples. Among them, Bacteroidota \((48.7\%)\) and Firmicutes \((47.5\%)\) had higher relative abundances...
and were the 2 dominant phyla (Figure 2A). A total of 18 dominant genera were identified which accounted for more than 1% of the total sequence. These genera were: Rikenellaceae_RC9_gut_group (18.0%), Prevotella (10.9%), Succinivibrio (9.2%), NK4A214_group (7.9%), Christensenellaceae_R-7_group (6.4%), norank_f__F082 (6.3%), norank_f__UCG-011 (5.4%), norank_f__Bacteroidales_RF16_group (3.8%), Prevotellaceae_UCG-003 (3.3%), norank_f__Muribaculaceae (2.5%), Selenomonas (1.6%), Lachnospiraceae_NK3A20_group (1.6%), unclassified_f__Prevotellaceae (1.5%), Ruminococcus (1.4%), Acetitomaculum (1.3%), Ruminococcus_gauvreauii_group (1.3%), Butyribrio (1.0%), and Veillonellaceae_UCG-001 (0.9%; Figure 2B). The experimental treatments had no significant effect on the relative abundance of bacterial phyla and most genera. The changes in the relative abundance of bacteria at the phylum and genus level are shown in Table 3. At the phylum level, we observed no significant difference in the relative abundance of Bacteroidota and Firmicutes among the treatment groups. The compound 3-NOP significantly increased the relative abundances of Christensenellaceae_R-7_group (P < 0.05) and Lachnospiraceae_NK3A20_group (P < 0.05). Vitamin B_{12} tended to decrease the relative abundances of Rikenellaceae_RC9_gut_group (P = 0.07) and Lachnospiraceae_NK3A20_group (P = 0.06).

Vitamin B_{12} significantly increased the relative abundance of unclassified_f__Prevotellaceae (P < 0.05) and tended to increase the relative abundance of Prevotellaceae_UCG-003 (P = 0.06).

**Effects on Archaeal Community.** The α diversity of archaeal community is shown in Table 2. A total of 1,041 OTU were identified in the 4 treatments. The coverage of sample OTU over 99% was close to 100%, and we observed no significant difference among treatments, indicating that the accuracy and repeatability of sequencing results were ideal for analysis. Alpha diversity indices such as Shannon, Simpson, ACE and Chao1 did not differ significantly among treatments (P > 0.05), indicating that the addition of 3-NOP and vitamin B_{12} did not affect the richness and diversity of the archaeal community. Propionyl-CoA analysis based on the weighted UniFrac distance algorithm showed no obvious separation of each treatment group from CON (Figure 3). ANOSIM revealed no significant difference in archaeal community structure between each treatment group and CON (r = 0.03, P = 0.20).

At the phylum level, Euryarchaeota and unclassified_k__norank_d__Archea represented the 2 main archaeal communities, of which Euryarchaeota accounted for an average of 98.5% (Figure 4A). At the genus level, genera that accounted for ≥1% of the total sequences were selected for analysis, and 3 dominant genera were
identified: *Methanobrevibacter* (95.3%), *Methanosphaera* (3.2%), and *unclassified_k__norank_d__Archaea* (1.2%; Figure 4B). No effect of 3-NOP or vitamin B$_{12}$ on archaeal microbial community was observed at the phylum and genus levels. The species and OTU levels of archaeal community were further analyzed to investigate changes in the archaeal microbial community (Table 4). At the species level, vitamin B$_{12}$ tended to decrease the relative abundance of *uncultured_methanogenic_archaeon_g_ _Methanobrevibacter* ($P = 0.07$). The compound 3-NOP tended to decrease the relative abundances of *unclassified_g__Methanobrevibacter* ($P = 0.07$) and *uncultured_methanogenic_archaeon_g_ _Methanobrevibacter* ($P = 0.06$), whereas tended to increase the relative abundance of *Methanobrevibacter_sp._AbM4* ($P = 0.09$). Combined supplementation of 3-NOP and vitamin B$_{12}$ had a significant interaction effect on increasing the relative abundance of *Methanobrevibacter_sp._AbM4* ($P < 0.05$) and decreasing the relative abundance of *uncultured_methanogenic_
archaeon_g__Methanobrevibacter (P < 0.05), and we observed a trend of interaction on decreasing the relative abundance of unclassified_g__Methanobrevibacter (P = 0.07). At the OTU level, vitamin B_{12} significantly reduced the relative abundance of OTU55 (P < 0.05). The compound 3-NOP significantly decreased the relative abundances of OTU55 (P < 0.05) and OTU1147 (P < 0.05), and had a trend of decreasing the relative abundance of OTU1056 (P = 0.08). The compound 3-NOP significantly increased the relative abundances of OTU1125 (P < 0.05) and OTU95 (P < 0.05). Combined supplementation of 3-NOP and vitamin B_{12} had a significant interaction effect on increasing the relative abundances of OTU1125 and OTU95 and decreasing the relative abundances of OTU1056, OTU55, and OTU1147 (P < 0.05).

**DISCUSSION**

**Effects on Methanogenesis**

In the current study, the addition of 3-NOP alone or vitamin B_{12} alone reduced the CH_{4} emission by 9%. Similar to other previous studies on cows, 3-NOP significantly reduced CH_{4} emission (Haisan et al., 2014; Reynolds et al., 2014; Hristov et al., 2015; Lopes et al., 2016). The observed effectiveness of inhibiting CH_{4} emission in studies of 3-NOP ranges from 7 to 60% (Haisan et al., 2014; Reynolds et al., 2014), but in most studies, 3-NOP reduces CH_{4} emission by an average of 30% in cases where it is provided to cows by way of mixing with TMR for ad libitum feeding (Hristov et al., 2015; Lopes et al., 2016). In addition, in 2 in vitro experiments, the use of 3-NOP at a dose similar to that in our study reduced CH_{4} emission by 70–80% (Romero-Pérez et al., 2015a, 2017). However, different with our study, these 2 in vitro studies used high-grain diets as the fermentation substrates, whereas the forage-based diet was used in our study. The use of different dietary types as fermentation substrates may induce potential differences in the effects on inhibiting CH_{4} emission. The compound 3-NOP was more effective in CH_{4} alleviation when it was added to the high-grain diet. For example, the same supplementation of 200 mg of 3-NOP/kg of DM reduced CH_{4} emission by 38% in beef cattle consuming a forage-based diet and by 84% in beef cattle consuming a high-grain diet (Vyas et al., 2016). Another reason for the lower efficacy of 3-NOP in inhibiting CH_{4} emission observed in the current study may be caused by the difference methods of administration of 3-NOP between in vitro addition and directly mixing into cow feed. Ungerfeld
Table 4. Difference in the relative abundances of archaeal community among treatments in vitro

| Item | 0          | VB | NOP | P-value |
|------|------------|----|-----|---------|
| species level abundance (%) |            |    |     |         |
| Uncultured_archaeon_g_Methanobrevibacter | 62.62      | 65.06 | 64.18 | 65.04  | 0.22 | 0.10 | 0.24 |
| Unclassified_g_Methanobrevibacter | 13.39      | 12.46 | 12.12 | 11.62  | 0.07 | 0.19 | 0.07 |
| Methanobrevibacter_sp._AbM4 | 7.03       | 6.73 | 6.81 | 6.73   | 0.31 | 0.26 | 0.24 |
| Uncultured_rumen_methanogen_g_Methanobrevibacter | 6.99      | 6.30 | 6.27 | 6.02   | 0.06 | 0.07 | 0.04 |
| Unclassified_g_Methanosphaera | 2.78       | 2.59 | 2.72 | 2.61   | 0.75 | 0.18 | 0.40 |
| OTU level abundance (%) |            |    |     |         |
| OTU893 | 55.64      | 58.03 | 56.40 | 57.07  | 0.44 | 0.10 | 0.36 |
| OTU1056 | 9.05       | 8.23 | 8.02 | 7.64   | 0.08 | 0.24 | 0.02 |
| OTU1125 | 6.97      | 7.03 | 7.78 | 7.97   | 0.01 | 0.93 | 0.01 |
| OTU195 | 6.75       | 6.50 | 7.54 | 7.63   | 0.09 | 0.42 | 0.02 |
| OTU55 | 4.68       | 4.23 | 3.98 | 3.84   | 0.01 | 0.02 | 0.01 |
| OTU1147 | 3.72      | 3.48 | 3.29 | 3.17   | 0.04 | 0.26 | 0.04 |

n = 15.

Data were analyzed using two 3-nitrooxypropanol levels (0 or 2.0 mg/g DM) and 2 vitamin B12 levels (0 or 2.0 mg/g DM).

NOP = 3-nitrooxypropanol; VB = vitamin B12; NPV = the interaction between 3-nitrooxypropanol and vitamin B12.

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(2018) reported that the methods of administration were an important factor affecting the efficacy of 3-NOP in inhibiting CH4 emission. The compound 3-NOP is volatile and metabolized rapidly in rumen fluid due to the increase of temperature after entering the rumen (Romero-Pérez et al., 2015b; Van Wesemael et al., 2019). Mixing 3-NOP into TMR can create synchronization between the gradual entry of 3-NOP into the rumen along with the fermentation of the feed, and this synchrony may elevate the inhibitory potential of 3-NOP on CH4 emission (Romero-Pérez et al., 2015b). Thus, 3-NOP addition after mixing with feed is a key measure for the intake of 3-NOP to be evenly distributed throughout the day. Similar to the current findings, a study by Reynolds et al. (2014) adding 3-NOP directly to the rumen via a rumen fistula reduced CH4 discharge by 9.8%. To our knowledge, the current study was the first to report the effect of vitamin B12 in suppressing CH4 emission, and the combination of 3-NOP with vitamin B12 elevated the effect of suppression compared with either alone (9 to 12%).

**Effects on Rumen Fluid Incubation**

In the current study, neither 3-NOP nor vitamin B12 affected DMD. This result was similar to the previous studies that supplementation of 3-NOP to cows did not affect DMI (Haisan et al., 2014; Reynolds et al., 2014; Hristov et al., 2015; Lopes et al., 2016; Haisan et al., 2017). Consistent with previous studies, supplementation with 3-NOP was generally accompanied by an increase in pH and a decrease in total VFA.

![Figure 4](image-url)
while inhibiting methanogenesis (Guyader et al., 2017; Ungerfeld, 2018). Meanwhile, as we hypothesized, vitamin B12 had a significant effect on fermentation patterns and methanogenesis, resulting in a decreased acetate to propionate ratio and CH4 production. Vitamin B12 plays an important role in the production of propionate, and the pathway to propionate formation provides an alternative sink for the use of metabolic hydrogen compared with acetate, and an inverse correlation between methanogenesis and propionate formation has been reported (Janssen, 2010). This may explain that the addition of vitamin B12 resulted in a decreased acetate to propionate ratio and lower CH4 production in the current study. Supplementation of 3-NOP also decreased the concentration of acetate, increased that of propionate, and decreased the acetate to propionate ratio. It is worth mentioning that these changes in VFA concentrations were most significant when 3-NOP was combined with vitamin B12. In addition, the decrease of isobutyrate and valerate concentrations was also observed in the 3 treatment groups. Therefore, the decrease of total VFA concentration in the current study may be due to the reduced concentrations of acetate, isobutyrate and valerate. Cows supplemented with 3-NOP typically observed reduced concentration of acetate. This might be related to the accumulation of [H] due to the inhibition of CH4 production, because the increase of [H] concentration in ruminen will reduce the production of acetate. (van Lingen et al., 2016; Melgar et al., 2020). Although the emission of H2 was not measured in this study, multiple previous studies have observed that 3-NOP suppresses CH4 emission while resulting in a dramatic increase in H2 emission. The H2 that is excreted as gas is only a fraction of the excess H2 that is produced as a result of inhibited CH4 synthesis. So, there is still a significant amount of [H] diverted to other metabolic pathways (Hristov et al., 2015; van Gastelen et al., 2020; Zhang et al., 2020; Melgar et al., 2021). Propionate generation is the thermodynamically most favorable pathway in the case of [H] accumulation (Ellis et al., 2008, Janssen, 2010). When CH4 generation is inhibited, propionate generation becomes an alternative pathway that uses [H] (Mcallister and Newbold, 2008), which might explain the increased propionate concentration in the current study. However, the increased production of propionate by 3-NOP does not fully explain the metabolic fate of [H]. Vitamin B12 is an important coenzyme of the propionate production pathway and can become deficient when propionate production increases (Frobish and Davis, 1977). This may explain the increase in propionate with the addition of vitamin B12 in the current study. Moreover, when 3-NOP was combined with vitamin B12, the magnitude of the increase in the concentration of propionate was greater compared with the addition of 3-NOP alone, which may to some extent alleviate the problem of increased H2 emission caused by 3-NOP addition.

Effects on Microbial Community

Bacteroidota and Firmicutes were the dominant phyla in the current study, which was consistent with several previous studies of in vitro fermentation in cows (Wang et al., 2018, 2021; Liu et al., 2022). Bacteroidota and Firmicutes are widely recognized as the most abundant and common phyla in rumen (Henderson et al., 2015; Sun et al., 2019). Firmicutes play an important role in fiber and cellulose breakdown and can produce H2. The main function of Bacteroidota is to degrade proteins and carbohydrates, and it is a utilizer of H2 (Comtet-Marre et al., 2017; Lan and Yang, 2019). Prevotella is generally the predominant bacterial genus commonly found in the rumen and comprises multiple species (Kim et al., 2011). Prevotella has a variety of extracellular degrading enzymes that degrade carbohydrates into short chain fatty acid, and different species produce final fermentation products formed differently by extracellular degrading enzymes, with acetate or propionate as the final fermentation product (Stevenson and Weimer, 2007; Emerson and Weimer, 2017). Although we observed functional differences among species of Prevotella, propionate and succinate appear to be the major fermentation products of most Prevotella species (de Menezes et al., 2011; De Vadder et al., 2016). Furthermore, the abundance of some Prevotella species was generally positively associated with the low CH4-producing phenotype and high concentration of vitamin B12 in the rumen (Kittelmann et al., 2014; Franco-Lopez et al., 2020). In the current study, the addition of vitamin B12 increased the relative abundances of Prevotellaceae_UCG-003 and unclassified_f__Prevotellaceae. Martinez-Fernandez et al. (2016) showed that Prevotella could increase propionate production when CH4 production was inhibited. Therefore, the increase of propionate concentration and the decrease of CH4 production in the current study were partly related to the change of the relative abundance of Prevotella. Lachnospiraceae_NK3A20_group is a fiber degrading bacterium, whose members can ferment various sugars to produce acetate, H2 and CO2 (Xing et al., 2006). In the current study, vitamin B12 decreased the relative abundance of Lachnospiraceae_NK3A20_group, whereas 3-NOP increased the relative abundance of Lachnospiraceae_NK3A20_group. As mentioned above, many studies have shown that the addition of 3-NOP increased H2 emission. Therefore, the significant increase in the abundance of Lachnospiraceae_NK3A20_group
after the addition of 3-NOP may lead to increased \( \mathrm{H}_2 \) production. In contrast, vitamin \( \mathrm{B}_{12} \) supplementation may inhibit fiber fermentation and reduce acetate production by reducing the relative abundance of \( \text{Lachnospiraceae}_\text{NK3A20}\_\text{group} \). \( \text{Christensenellaceae}_R-7\_\text{group} \) is a member of Firmicutes, which is usually associated with good health and better digestive system function (Morotomi et al., 2012; Goodrich et al., 2014; Chen et al., 2020). The multiple metabolites secreted by the \( \text{Christensenellaceae}_R-7\_\text{group} \) are related to feed efficiency and may play an important role in promoting digestive tract health (Xie et al., 2020). Several previous studies have shown that the abundance of the \( \text{Christensenellaceae}_R-7\_\text{group} \) appears reduced in individuals with intestinal inflammation (Goodrich et al., 2014), and higher abundance of the \( \text{Christensenellaceae}_R-7\_\text{group} \) in healthy individuals compared with individuals with intestinal disease was observed (Pittayanon et al., 2020). In the current study, 3-NOP addition significantly increased the relative abundance of \( \text{Christensenellaceae}_R-7\_\text{group} \), indicating that 3-NOP addition may also play a beneficial role in the health of the digestive tract of dairy cows.

Archaea uses \( \mathrm{H}_2 \) to produce \( \mathrm{CH}_4 \) in the rumen to maintain a lower \( \mathrm{H}_2 \) partial pressure. This low \( \mathrm{H}_2 \) partial pressure rumen environment is conducive to rumen microorganisms fermenting digestible fiber in the feed (Ferry, 1993; Zhou et al., 2010). \( \text{Euryarchaeota} \) is the dominant archaea phylum and the only methanogenic microorganisms currently known in the rumen, which contains many different species (Hook et al., 2010). In the current study, 98.5% of the total sequences of the archaeal community belonged to this dominant phylum. The species of methanogens are mainly divided into hydrogen-trophic, acetate-trophic and methyl-trophic types based on the difference of methanogenic substrates (\( \mathrm{H}_2 \), acetate, formate, and methylamine, respectively; Lan and Yang, 2019). At the genus level, similar to previous studies, \( \text{Methanobacter} \) was the dominant genus and was the most common methanogen (Zhou et al., 2009; Weimar et al., 2017). At the phylum and genus level, we observed no effect of treatment on the archaeal community. Methane production may be related to the abundance of specific methanogens species rather than the total number of methanogens. Shi et al. (2014) reported that if the total number of methanogens in the rumen did not differ, the composition of the methanogenic community may be an important factor in determining \( \mathrm{CH}_4 \) production. A similar conclusion was made in a study of the influence of nonforage fiber on rumen microbes in cows, where changes in archaeal community structure contributed to the reduction in \( \mathrm{CH}_4 \) production (Wang et al., 2018). At the species and OTU levels, 3-NOP + vitamin \( \mathrm{B}_{12} \) increased the relative abundances of \( \text{Methanobrevibacter}_\text{sp.}_\text{Abm}4 \), OTU1125, and OTU95 and decreased the relative abundances of uncultured\_methanogenic\_archaeon\_g\_Methanobrevibacter, OTU1147, OTU1056, and OTU55. \( \text{Methanobrevibacter}_\text{sp.}_\text{Abm}4 \) is both a hydrogen-trophic and methyl-trophic methanogen (Leahy et al., 2013). \( \text{Methanobrevibacter}_\text{sp.}_\text{Abm}4 \) encodes only a few sticky proteins and can synthesize coenzyme M on its own. So, the external environment has little influence on its growth (Kumar et al., 2014). The final step of all \( \mathrm{CH}_4 \) production reactions is catalyzed by coenzyme M (Ermõl et al., 1997). The molecular structure of 3-NOP is similar to coenzyme M, which can inactivate methyl coenzyme M reductase, block the combination of coenzyme M and coenzyme B, and inhibit the production of \( \mathrm{CH}_4 \) (Duin et al., 2016). Thus, the abundance of \( \text{Methanobrevibacter}_\text{sp.}_\text{Abm}4 \) increased upon inhibition of \( \mathrm{CH}_4 \) production by 3-NOP. In addition, the methanogenic efficiency of different methanogens species is considered to be a more important factor than methanogens abundance in affecting \( \mathrm{CH}_4 \) production (Shi et al., 2014). For example, \( \text{Methanobrevibacter} \) can use 1 mol of \( \mathrm{CO}_2 \) to produce 1 mol of \( \mathrm{CH}_4 \) (Hook et al., 2010), whereas \( \text{Methanosphaera} \) consumes 4 moles of methanol to produce 3 moles of \( \mathrm{CH}_4 \) (Fricke et al., 2006). Therefore, it is possible that the addition of 3-NOP and vitamin \( \mathrm{B}_{12} \) may have altered the archaeal community structure by altering the abundance of methanogens, thereby inhibiting \( \mathrm{CH}_4 \) production.

CONCLUSIONS

In the current study, we studied the effects of 3-NOP and vitamin \( \mathrm{B}_{12} \) on VFA, \( \mathrm{CH}_4 \) production, and microbial community structure of dairy cows by in vitro rumen fermentation. The combined addition of 3-NOP and vitamin \( \mathrm{B}_{12} \) significantly decreased \( \mathrm{CH}_4 \) emission, increased propionate concentration, decreased acetate to propionate ratio, and changed rumen fermentation pattern. Moreover, compared with the addition of 3-NOP alone, the addition of 3-NOP in combination with vitamin \( \mathrm{B}_{12} \) further enhanced the suppression effect on \( \mathrm{CH}_4 \) emission, with more [H] being transferred to the production of propionate. By analyzing the changes of bacterial and archaeal communities in the rumen, we demonstrated the effects of supplementation with 3-NOP + vitamin \( \mathrm{B}_{12} \) on rumen fermentation and \( \mathrm{CH}_4 \) production, providing a basis for the application of 3-NOP + vitamin \( \mathrm{B}_{12} \). In conclusion, 3-NOP added in combination with vitamin \( \mathrm{B}_{12} \) may be a meaningful strategy to suppress \( \mathrm{CH}_4 \) emission in rumen. However, further studies are still needed to evaluate whether the same effect could be achieved in vivo in a longer period.
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