Long-term and combined effects of N-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide and fumaric acid on methane production, rumen fermentation, and lactation performance in dairy goats

Zongjun Li†, Xinjian Lei†, Xiaoxu Chen, Qingyan Yin, Jing Shen and Junhu Yao*

Abstract

Background: In recent years, nitrooxy compounds have been identified as promising inhibitors of methanogenesis in ruminants. However, when animals receive a nitrooxy compound, a high portion of the spared hydrogen is eructated as gas, which partly offsets the energy savings of CH\textsubscript{4} mitigation. The objective of the present study was to evaluate the long-term and combined effects of supplementation with N-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide (NPD), a methanogenesis inhibitor, and fumaric acid (FUM), a hydrogen sink, on enteric CH\textsubscript{4} production, rumen fermentation, bacterial populations, apparent nutrient digestibility, and lactation performance of dairy goats.

Results: Twenty-four primiparous dairy goats were used in a randomized complete block design with a 2 × 2 factorial arrangement of treatments: supplementation without or with FUM (32 g/d) or NPD (0.5 g/d). All samples were collected every 3 weeks during a 12-week feeding experiment. Both FUM and NPD supplementation persistently inhibited CH\textsubscript{4} yield (L/kg DMI, by 18.8% and 18.1%, respectively) without negative influence on DMI or apparent nutrient digestibility. When supplemented in combination, no additive CH\textsubscript{4} suppression was observed. FUM showed greater responses in increasing the molar proportion of propionate when supplemented with NPD than supplemented alone (by 10.2% vs. 4.4%). The rumen microbiota structure in the animals receiving FUM was different from that of the other animals, particularly changed the structure of phylum Firmicutes. Daily milk production and serum total antioxidant capacity were improved by NPD, but the contents of milk fat and protein were decreased, probably due to the bioactivity of absorbed NPD on body metabolism.

Conclusions: Supplementing NPD and FUM in combination is a promising way to persistently inhibit CH\textsubscript{4} emissions with a higher rumen propionate proportion. However, the side effects of this nitrooxy compound on animals and its residues in animal products need further evaluation before it can be used as an animal feed additive.

* Correspondence: yaojunhu2004@sohu.com
† Zongjun Li and Xinjian Lei contributed equally to this work.
College of Animal Science and Technology, Northwest A&F University, Yangling 712100, Shaanxi, China

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Keywords: Bacterial populations, Dairy goat, Fumaric acid, Lactation performance, Methane emissions, N-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide, Rumen fermentation

Introduction
Methane (CH$_4$) emissions from ruminants not only contribute to anthropogenic greenhouse gas emissions and enlarge the carbon footprint of dairy or beef production [1] but also drain dietary energy (2% to 12% of gross energy (GE)) [2]. Successful CH$_4$ mitigation strategies should have persistent efficacy and have no adverse effect on feed degradation, animal health, and productivity [1, 3]. In recent years, nitroxy (−O−NO$_2$) compounds, such as 3-nitroxypropanol (3-NOP), have been identified as promising methanogenesis inhibitors [4–8] that specifically dock into the active site of methyl-CoM reductase, a key enzyme in the methanogenesis pathway. As a nitrooxy compound, N-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide (NPD) effectively decreased CH$_4$ production in vitro [8]. When the methanogenesis pathway is inhibited, hydrogen production increased and is excreted as gas (increased by 48- to 100-fold) [6, 8–10], and hydrogen is also a greenhouse gas with high energy [11, 12]. It suggested that the efficiency of hydrogen capture was lower when CH$_4$ production was inhibited. Propionogenesis is the second large hydrogen sink after methanogenesis [13, 14], and it is a more energy-rendering fermentation pathway for animals [14, 15]. Fumaric acid (FUM), a metabolic intermediate of the propionate-forming pathways, has been identified as a promising propionate enhancer and methanogenesis competitor for hydrogen [16, 17]. Therefore, we hypothesized that a combination of NPD and FUM might divert more hydrogen from methanogenesis to propionate synthesis than each inhibitor alone.

Nitroxy compounds have also been used to treat angina [18]. NPD is a nicotinamide derivative and a balanced vasodilator, which is also called Nicorandil, one of the most effective, healthy and widely used angina drugs, because of its functions as a K$^+$/ATP channel opener and NO donor [18]. To our knowledge, the side effects of nitroxy compounds as animal feed additives have been rarely mentioned. The objective of the current study was to evaluate the persistent and combined effects of supplementation with NPD and FUM on CH$_4$ suppression, rumen fermentation, rumen bacterial population, apparent nutrient digestibility, serum total antioxidant capacity, and milk performance in lactating dairy goats.

Methods
All experimental procedures were approved by the Northwest A&F University Animal Care and Use Committee.

Animals, diets, and experimental design
Twenty-four primiparous Guanzhong dairy goats (113 ± 9 days in milk (DIM), 39 ± 3.8 kg of body weight (BW) at the start of the experiment) were chosen from a dairy goat farm (Shaanxi, China) and blocked into six blocks by DIM, BW, and daily milk production (DMP). Animals within each block were randomly assigned to 1 of 4 dietary treatments: control (CON), a basal diet without any additives; basal diet supplemented with FUM (Aladdin®, Shanghai, China) at 34 g/d; basal diet supplemented with NPD (J&K Scientific®, Beijing, China) at 0.5 g/d; and the basal diet supplemented with both FUM (34 g/d) and NPD (0.5 g/d). The supply dose of FUM was based on the data published previously [17], while that of NPD was based on a previous 3-NOP study [6] and a mice study [19]. The ration was fed as total mixed ration (TMR, Table 1) twice daily at 0730 and 1730 h and was provided individually at 105% of the expected feed intake (as-fed basis) based on the amounts of feed offered and

| Ingredient | % |
|------------|---|
| Corn silage | 21.3 |
| Alfalfa hay | 30.8 |
| Ground corn | 22.9 |
| Soybean meal | 6.6 |
| Cottonseed meal | 5.0 |
| Corn germ meal | 3.2 |
| Wheat bran | 8.2 |
| CaHPO$_4$ | 0.5 |
| CaCO$_3$ | 0.5 |
| NaHCO$_3$ | 0.3 |
| Salt | 0.5 |
| Vitamin-mineral premix$^a$ | 0.2 |

Chemical composition, % of DM

| Item | % |
|------|---|
| DM   | 47.0 |
| EE   | 4.1 |
| Ash  | 6.7 |
| CP   | 18.6 |
| NDF  | 36.1 |
| ADF  | 20.4 |

$^a$Vitamin-mineral premix (per kg): 600 mg of Mn, 950 mg of Zn, 430 mg of Fe, 650 mg of Cu, 30 mg of Se, 45 mg of I, 20 mg of Co, 450 mg of nicotinic acid, 800 mg of vitamin E, 45 kIU of vitamin D, and 120 kIU of vitamin A

Table 1 Ingredients and chemical composition of the experimental diet
refused from the previous day. The FUM and/or NPD was top-dressed on one-quarter of the offered TMR that was fed first to ensure complete intake. All goats were individually housed in 24 tie-stalls in a barn and had free access to water. The goats were milked twice daily at feeding. The milk produced by the goats receiving NPD was discarded.

The feeding experiment lasted 12 weeks, and all samples were collected or measured at weeks 3, 6, 9, and 12. The six blocks of goats were divided into 3 groups by DIM, and the feeding experiment started in a staggered manner for the 3 groups with a 7-d interval so that gas emissions from each group could be measured in turn using the four indoor environmental chambers (each 7.4 m × 4.2 m × 2.7 m) available. Two goats within the same treatment were placed in one chamber and were separated by placing each in a metabolic cage (1.5 m × 1.0 m × 1.5 m). The goats were moved from barn to chambers one day before sample collection and measurements, and no stress responses were observed because they had already adapted to the chambers before the feeding experiment. On d 1–4 during each sample collecting week, total-tract digestibility of dietary nutrients, milk composition, and CH4 emissions were measured simultaneously, and the samples of blood and rumen content were collected on d 4–5 and d 5–6, respectively.

Measuring CH4 emissions and milk performance
Gas emissions in the environmental chambers were measured as previously described [17, 20] with minor changes. Briefly, the daily (22 h; 08:30 to 17:30 and 18:30 to 07:30) gas emissions from each chamber were measured in 3 consecutive days. During the gas measurement, the internal temperature of the chambers was maintained to be the same as the ambient temperature outside the building. The air inside each chamber was mixed for 30 s every 10 min by 4 draft fans. The gases from the four chambers and external environment were continuously and constantly pumped at a rate of 4 L/min by 5 exhaust fans. The pumped gases were analyzed sequentially by an FID sensor (Thermo Scientific 55i, USA), 12 min for each in every 60 min.

The daily CH4 production was calculated as follows:

\[
\text{CH}_4 \text{production (L/d) = } \Sigma (C_i \cdot C_{i,i}) \times V_e + V_f \times (C_{i,i} \cdot C_{CO}) / 1000
\]

Where \( C_i \) = the CH4 concentration (mL/m3) of the internal chamber at the i 60-min; \( CO_i \) = the CH4 concentration of external environment at the i 60-min; \( V_e \) = the chamber volume (83.9 m3); and \( V_f \) = the gas volume pumped from each chamber over each 60-min measurement (0.24 m3).

During each of the two one-hour no-measurement periods, the chamber doors were opened, and the fresh-air exchange fans were running to exchange fresh air. Meanwhile, the goats were milked and fed, and the samples of milk and orts of individual goats were collected. During these 3 consecutive days, the morning and evening milk production of each goat were recorded and mixed, and 50 mL was subsampled and stored at 4°C until analysis for milk composition. Milk samples were analyzed for fat, protein, lactose, and milk urea nitrogen (MUN) using an infrared milk analyzer (MilkoScan FT 120, FOSS, Hillerød, Denmark) within 24 h. Fat corrected milk (FCM) was calculated according to NRC (2001) [21]:

\[
\text{milk fat yield (kg/d) × 16.216 + milk yield (kg/d) × 0.4324,}
\]

and net energy for lactation (NEL, Mcal/d) = milk yield (kg/d) × ((0.0929 × percent fat) + (0.0563 × percent true protein) + (0.0395 × percent lactose)).

Collections and analysis of blood samples
Blood samples were collected from an external jugular vein into two 10-mL blood tubes before the morning feeding on two consecutive days in each sample collection week. The sample in the tube was allowed to clot at room temperature for 30 min and centrifuged (3000 × g, 15 min) thereafter to obtain serum, which was stored at −80°C for later analysis. Serum malondialdehyde (MDA) concentration, total antioxidant capacity (T-AOC), and the activities of serum glutathione peroxidase
(GSH-Px) and superoxide dismutase (SOD) were analyzed using respective commercial kits (Jiancheng Bioengineering Institute, Nanjing, China).

**Collection and analysis of ruminal samples**

Ruminal content samples were collected using an oral tube and a hand vacuum pump at 6 h after the morning feeding in 2 consecutive days in each sample collection week. To minimize saliva contamination, approximately 50 mL of ruminal fluid was discarded before sample collection. Ruminal pH was measured immediately after sampling. Rumen fluid was subsampled for analysis of volatile fatty acids (VFA, 5 mL with 1 mL of 25% metaphosphoric acid added), organic acids (5 mL), and microbiota (45 mL), and then stored at −80 °C until analysis.

Ruminal VFA concentration was determined using gas chromatography (Agilent Technologies 7820A GC system, Palo Alto, CA, USA) as described by Li et al. [23]. Ruminal organic acid (fumarate, succinate, and lactate) concentration was determined using an Agilent 1260 high-performance liquid chromatography system as done in previous studies [24, 25].

**Bacterial community analysis**

Rumen content samples of each goat from each week were freeze-dried and mixed. Microbial genomic DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction. The concentration and purity of the DNA samples were analyzed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc., Madison, WI, USA). The V4-V5 hypervariable region (515F-926R) of the 16S rRNA gene was amplified using the primers: 5′-GTGYCAGCMGCCGCGGTAA-3′ and 5′-CCGYCAATTYMTTTRAGTTT-3′ [26] and paired-end sequenced (2 x 250) on the Illumina MiSeq platform.

The paired-end reads were quality-filtered, assembled, and trimmed as described previously [27]. The trimmed sequences were clustered into operational taxonomic units (OTUs) at ≥ 97% sequence similarity using Uclust in QIIME [28]. Subsequently, the OTUs were taxonomically assigned using the Silva 16S rRNA databases (SSU132; https://www.arbsilva.de/) at a confidence threshold of 80%.

**Statistical analysis**

The duplicate measurements (i.e. VFA and CH₄) of individual goats within each sampling week were averaged as one replicate for the statistical analysis. All data were analyzed as a repeated measures ANOVA using the PROC MIXED program in SAS 9.2 (SAS Institute Inc., Cary, NC, USA). The statistical model included NPD, FUM, week, and NPD × FUM, NPD × week, FUM × week, and NPD × FUM × week. interactions as fixed effects, and goat and block as random errors. Sampling week was treated as a repeated measure and goat as a subject. The most desirable covariance structure (unstructured, compound symmetric, and first-order autoregressive) for analysis was determined according to the smallest Bayesian information criterion [23, 29]. When there was a treatment × week interaction, differences among treatments at each sampling week were reanalyzed using the MIXED procedure with NPD, FUM and NPD × FUM interaction as fixed factors, and block as a random error. When there was an NPD × FUM interaction, Tukey’s multiple comparison test was used to assess differences among treatment means.

The alpha diversity of the samples was estimated using the abundance-based coverage (ACE) estimators, Shannon diversity index, and observed OTUs. Beta diversity of the samples was computed using principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity [30] in R v.3.6.3 (http://www.R-project.org). Permutational multivariate analysis of variance was performed using the ANOSIM function in the R package vegan to compare the statistical difference in microbial composition across the experimental periods and between treatments.

Statistical significance was declared at P < 0.05, while tendency was declared at 0.05 ≤ P < 0.10.

**Results**

**Methane production and lactation performance**

The persistent and combined effects of FUM and NPD supplementation on CH₄ production and milk parameters are shown in Table 2 and Fig. 1. Both FUM and NPD supplementation persistently inhibited (P < 0.05) the CH₄ emissions in goats either expressed as L/d (by 19.1% and 13.4%, respectively) or as L/kg DMI (by 18.8% and 18.1%, respectively) without influencing DMI. A negative interaction (P = 0.01) was observed between FUM and NPD in CH₄ yield (L/kg DMI). The NPD supplementation increased (P < 0.05) the DMP, improved feed conversion efficiency expressed as DMP/DMI, and tended to increase the daily FCM production and FCM/DMI, but it decreased (P < 0.05) the fat and protein content of the milk without changing milk fat and protein yields. NPD by time interaction was detected for milk protein content (P = 0.029), decreasing milk protein content to a greater extent over time (−9.1% at weeks 3 vs. −20.8% at weeks 9). FUM supplementation had no effects on DMP but decreased (P = 0.008) milk fat content and tended to decrease (P = 0.065) daily fat yield. In addition, most of the milk parameters changed over time, with DMP (P = 0.01) and lactose content (P = 0.06) decreasing, whereas fat and protein contents increasing (P <
Table 2 Effects of the dietary treatments on feed intake, milk performance and methane production of the dairy goats

| Item          | Treatment† | SEM | P-value |
|---------------|------------|-----|---------|
|               | CON FUM   | NPD | FN      | Week | F × N | F × week | N × week |
| DMI, kg       | 1.70       | 1.70 | 1.81    | 1.70 | 0.080 | 0.005    | 0.465    | 0.511   | 0.495   | 0.190   | 0.958   |
| DMP, kg       | 1.24       | 1.29 | 1.61    | 1.52 | 0.127 | 0.010    | 0.887    | 0.035    | 0.607   | 0.736   | 0.235   |
| FCM, kg       | 1.30       | 1.19 | 1.59    | 1.37 | 0.125 | 0.207    | 0.213    | 0.085    | 0.662   | 0.569   | 0.259   |
| DMP/DMI       | 0.73       | 0.74 | 0.89    | 0.90 | 0.063 | 0.001    | 0.862    | 0.021    | 0.974   | 0.915   | 0.128   |
| FCM/DMI       | 0.76       | 0.69 | 0.87    | 0.81 | 0.065 | 0.046    | 0.310    | 0.091    | 0.912   | 0.713   | 0.160   |
| NEL, MJ/d     | 3.77       | 3.41 | 4.45    | 3.84 | 0.349 | 0.171    | 0.183    | 0.131    | 0.724   | 0.628   | 0.213   |
| Milk composition, % |           |     |         |       |       |          |         |          |         |          |         |
| Fat           | 3.90       | 3.24 | 3.41    | 2.91 | 0.189 | 0.001    | 0.008    | 0.045    | 0.683   | 0.505   | 0.208   |
| Protein       | 3.87       | 3.65 | 3.26    | 3.20 | 0.168 | 0.001    | 0.412    | 0.007    | 0.633   | 0.963   | 0.029   |
| Lactose       | 4.15       | 3.97 | 4.10    | 3.97 | 0.128 | 0.062    | 0.248    | 0.825    | 0.842   | 0.736   | 0.239   |
| MUN, mg/L     | 3.92       | 3.89 | 4.03    | 3.50 | 0.172 | 0.157    | 0.125    | 0.435    | 0.161   | 0.238   | 0.997   |
| Milk composition yield, g/d |           |     |         |       |       |          |         |          |         |          |         |
| Fat           | 46.9       | 39.1 | 55.0    | 43.8 | 4.78  | 0.420    | 0.065    | 0.202    | 0.726   | 0.423   | 0.307   |
| Protein       | 46.0       | 43.7 | 52.2    | 48.4 | 3.57  | 0.005    | 0.409    | 0.152    | 0.839   | 0.758   | 0.258   |
| Lactose       | 52.0       | 52.0 | 65.7    | 60.3 | 0.91  | 0.006    | 0.642    | 0.076    | 0.651   | 0.950   | 0.115   |
| Methane emissions |           |     |         |       |       |          |         |          |         |          |         |
| CH₄, L/d     | 32.7a     | 26.4b | 28.3b   | 25.9b | 0.91  | 0.031    | 0.030    | 0.036    | 0.081   | 0.142   | 0.342   |
| CH₄, MJ/d    | 1.30a     | 1.05a | 1.12a   | 1.03a | 0.036 | 0.031    | 0.030    | 0.036    | 0.081   | 0.142   | 0.342   |
| CH₄/DMI, L/kg | 19.2a    | 15.6b | 15.7b   | 15.3b | 0.43  | 0.077    | 0.004    | 0.005    | 0.010   | 0.197   | 0.272   |
| CH₄/FCM, L/kg| 26.4      | 23.7 | 18.0    | 16.8 | 2.56  | 0.296    | 0.750    | 0.043    | 0.512   | 0.845   | 0.094   |

†Means by treatment was the pooled data from goats at weeks 3 and 9, n = 3 for the measurements related to CH₄ and n = 6 for the others

*Means with different superscripts within a row differ (P < 0.05). The P-values for all the F × N × week. interactions were higher than 0.05, and they were not listed in the table.

CON Control, FUM Fumaric acid, NPD N-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide, FN FUM+ NPD, DMI Dry matter intake, DMP Daily milk production, FCM Fat corrected milk, MUN Milk urea nitrogen, NEL Net energy for lactation, SEM Standard error of means

Fig. 1 Dynamic and combined effects of fumaric acid (FUM) and N-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide (NPD) on methane yield (L/kg DMI, mean ± standard error) in the dairy goats. CON, Control; FN, FUM+ NPD
0.01) over time. The time-dependent observations are in line with data reported by Waite et al. [31].

Apparent total tract digestibility and energy balance
The apparent total tract digestibility of nutrients (DM, NDF, ADF, and CP) was not affected by FUM or NPD (Table 3). No change in GE, DE, ME, daily BW change, or energy loss in feces and urine were observed, either. The loss of energy as CH₄ relative to total GE intake was decreased ($P < 0.01$) by both FUM and NPD, and a negative interaction occurred between these two inhibitors.

Rumen fermentation parameters and bacterial community
The NPD supplementation did not affect any of the measured parameters of rumen fermentation (Table 4). FUM supplementation increased the molar proportion of rumen propionate ($P = 0.006$) but decreased the rumen butyrate proportion ($P = 0.002$), A:P ratio ($P = 0.018$), VFA hydrogen ratio ($P = 0.005$) and the concentrations of fumarate ($P = 0.003$) and succinate ($P = 0.025$). FUM supplementation did not affect rumen total concentration of VFA, pH or the concentration of lactate.

After concatenation and quality filtering, a total of 3.32 M sequences (41,462 per sample) were obtained from the 80 rumen samples. The OTUs were assigned to 22 phyla, 37 classes, 59 orders, 66 families, and 72 genera. At the phylum level, Bacteroidetes (64.7%), Firmicutes (19.4%) and Proteobacteria (6.5%) were predominant. The NPD supplementation did not affect bacterial community composition or diversity (Table 5 and Fig. 2). The bacterial community structure in the animals fed FUM differed from that of the other (Bray-Curtis RANOSIM = 0.145, $P = 0.001$), particularly changed the structure of the phylum Firmicutes. Within the phylum Firmicutes, the relative abundances of the genera Ruminococcus, Succinivibacterium, Clostridium and Shuttleworthia were increased ($P < 0.05$) by FUM, and the genera Coprococcus and Selenomonas tended to gain higher relative abundance. On the other hand, the

### Table 3 Effects of the dietary treatments on the dietary apparent nutrient digestibility and energy balance of the dairy goats

| Item                      | Treatment¹ | SEM | $P$-value |
|---------------------------|------------|-----|-----------|
|                          | CON | FUM | NPD | FN | FUM | NPD | $F \times N$ |
| Apparent nutrient digestibility, % |     |     |     |     |     |     |     |
| DM                        | 63.8 | 64.3 | 63.8 | 64.0 | 0.76 | 0.648 | 0.792 | 0.865 |
| NDF                       | 42.0 | 42.1 | 41.4 | 41.9 | 1.33 | 0.802 | 0.777 | 0.901 |
| ADF                       | 37.7 | 40.1 | 38.6 | 39.5 | 1.22 | 0.191 | 0.900 | 0.566 |
| CP                        | 82.0 | 81.8 | 82.1 | 81.1 | 0.56 | 0.291 | 0.611 | 0.497 |
| Energy intake, MJ/d       |     |     |     |     |     |     |     |     |
| GE                        | 28.8 | 28.5 | 30.6 | 28.2 | 1.37 | 0.355 | 0.578 | 0.469 |
| DE                        | 18.7 | 18.5 | 19.8 | 18.3 | 0.86 | 0.373 | 0.591 | 0.472 |
| ME                        | 16.5 | 16.3 | 17.8 | 16.2 | 0.76 | 0.310 | 0.457 | 0.400 |
| Energy loss, MJ/d         |     |     |     |     |     |     |     |     |
| Faeces                    | 10.1 | 10.0 | 10.8 | 9.9  | 0.57 | 0.374 | 0.594 | 0.508 |
| Urine                     | 0.90 | 1.14 | 0.87 | 1.08 | 0.182 | 0.239 | 0.818 | 0.947 |
| Methane                   | 1.30 | 1.06 | 1.14 | 1.04 | 0.041 | 0.007 | 0.066 | 0.134 |
| Energy retention          |     |     |     |     |     |     |     |     |
| NEL, MJ/d                 | 3.87 | 3.46 | 4.47 | 3.79 | 0.328 | 0.116 | 0.179 | 0.694 |
| BW change, g/d            | 47.5 | 43.0 | 62.5 | 52.1 | 8.32 | 0.383 | 0.167 | 0.731 |
| Utilisation of gross energy, % |     |     |     |     |     |     |     |     |
| DE/GE                     | 64.9 | 65.3 | 64.7 | 65.0 | 0.74 | 0.651 | 0.756 | 0.920 |
| ME/GE                     | 57.1 | 57.5 | 58.1 | 57.4 | 1.04 | 0.902 | 0.675 | 0.627 |
| Milk/GE                   | 13.5 | 12.0 | 14.6 | 13.5 | 1.00 | 0.208 | 0.223 | 0.853 |
| CH₄/GE                    | 4.52ab | 3.73ab | 3.75ab | 3.70ab | 0.072 | 0.001 | 0.001 | 0.002 |

¹Means by treatment was the pooled data from goats at weeks 3 and 9, $n = 3$ for the measurements related to CH₄ and $n = 6$ for the others

²Means in the same row with different superscripts differ significantly ($P < 0.05$). The $P$-values for all the $F \times N \times$ week. interactions were higher than 0.05, and they were not listed in the table

CON Control, FUM Fumaric acid, NPD N-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide, FN FUM+ NPD, BW Body weight, GE Gross energy, DE Digestible energy, NEL Net energy for lactation, ME Metabolizable energy, SEM Standard error of means
genera *Oscillospira* and *RFN20* decreased their relative abundance ($P < 0.05$).

**Serum total antioxidant capacity**

Supplementation with NPD increased the activity of serum T-AOC and tended to increase the activity of SOD ($P = 0.053$) (Table 6). The FUM supplementation decreased the concentration of MDA ($P = 0.031$) and increased the activity of T-AOC in the serum. An interaction ($P = 0.007$) between FUM and NPD was detected for the activity of T-AOC.

**Discussion**

**Long-term effects of NPD on daily CH$_4$ production**

To our knowledge, no *in vivo* studies have been published on the effects of NPD in CH$_4$ production since its first evaluation via *in vitro* fermentation [8]. Consistent with the effects of 3-NOP [6, 10], a most researched nitrooxy compound, supplementation with NPD resulted in a reduction (by 18.1%) in CH$_4$ emissions in dairy goats, and the inhibitory effect persisted throughout the 12-week treatment. A recent meta-analysis based on dairy and beef cattle trials showed that an average dose of 123 mg 3-NOP per kilogram of feed dry matter (FDM) reduced CH$_4$ emissions by $29.3 \pm 5.63\%$ [32], which is higher than our observation (by 18.1%). The extent of methane inhibition by 3-NOP is dependent on the dose and administration technique [32, 33]. The dose of NPD in the current study was 276 mg per kilogram of FDM, equivalent to 158 mg of 3-NOP/FDM based on the molecular weight and mole of the nitrooxy group. At a similar supply dose (150 mg/kg FDM), the extent of CH$_4$ emission reductions ($-18.1\%$) by NPD in the present study was also much lower than that ($-36\%$) by 3-NOP [34]. Considering the high oxidability of nitrooxy groups and the low redox potential of the rumen environment, nitrooxy groups can be reduced in the rumen [7], and it has been shown that the antimethanogenic effects of 3-NOP are the highest within 6 h after feeding [10]. When NOP was dosed into the rumen of dairy cattle, Reynolds et al. [35] found that CH$_4$ production dropped substantially immediately after dosing, but the effect was only sustained for 1 to 2 h. Therefore, one possible explanation for the lower CH$_4$ emission reduction by NPD observed in this study could be the administration technique of top-dressing on the

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**Table 4 Effects of the dietary treatments on ruminal fermentation parameters and alpha diversity of microbial community**

| Item                        | Treatment | SEM | $P$-value | Week | FUM | NPD | F × N | F × week | N × week |
|-----------------------------|-----------|-----|-----------|------|-----|-----|-------|---------|----------|
| pH                          | CON 6.39  | 6.50| 6.46 6.39 | 0.037| 0.001| 0.661| 0.561| 0.033   | 0.943    | 0.659    |
| Total VFA production, mmol/L| 79.2      | 75.8| 77.5 77.2 | 3.07 | 0.001| 0.555| 0.978| 0.624   | 0.624    | 0.534    |
| Individual VFA molar proportion, % |          |     |          |      |     |      |       |         |          |          |
| Acetate                     | 65.7      | 66.4| 65.4 65.3 | 0.46 | 0.001| 0.541| 0.142| 0.424   | 0.620    | 0.088    |
| Propionate                  | 18.1      | 18.9| 17.8 19.6 | 0.40 | 0.164| 0.006| 0.525| 0.222   | 0.394    | 0.627    |
| Butyrate                    | 12.7      | 11.5| 13.1 11.5 | 0.37 | 0.001| 0.002| 0.603| 0.629   | 0.940    | 0.196    |
| Valerate                    | 1.13      | 1.12| 1.19 1.21 | 0.795| 0.771| 0.795| 0.077| 0.641   | 0.923    | 0.534    |
| A/P                         | 3.67      | 3.54| 3.69 3.34 | 0.089| 0.098| 0.018| 0.307| 0.243   | 0.367    | 0.479    |
| VFA hydrogen ratio          | 8.25      | 7.84| 8.29 7.41 | 0.196| 0.299| 0.005| 0.327| 0.238   | 0.415    | 0.619    |
| Fumarate, mmol/L            | 0.10      | 0.07| 0.10 0.08 | 0.007| 0.001| 0.003| 0.98  | 0.451   | 0.431    | 0.582    |
| Succinate, mmol/L           | 2.54      | 2.02| 2.39 1.80 | 0.225| 0.008| 0.025| 0.425| 0.880   | 0.458    | 0.445    |
| Lactate, mmol/L             | 1.14      | 0.82| 0.79 0.78 | 0.132| 0.001| 0.226| 0.165| 0.241   | 0.752    | 0.222    |

Relative abundances of fumarate-utilizing bacteria, %

| Prevotella ruminicola       | 9.50      | 9.52| 8.83 9.70 | 0.772| 0.089| 0.574| 0.760| 0.591   | 0.724    | 0.831    |
| Fibrobacter succinogenes    | 1.51      | 1.67| 1.71 1.32 | 0.184| 0.054| 0.530| 0.687| 0.162   | 0.510    | 0.845    |
| Selenomonas ruminantium     | 0.18      | 0.23| 0.20 0.30 | 0.035| 0.001| 0.059| 0.192| 0.461   | 0.187    | 0.759    |

Alpha diversity of microbial community

| Observed OTUs               | 2786      | 2956| 2756 2776 | 96.8 | 0.442| 0.345| 0.297| 0.454   | 0.392    | 0.883    |
| ACE                         | 5239      | 5528| 5212 5160 | 171.3| 0.027| 0.503| 0.273| 0.340   | 0.725    | 0.987    |
| Shannon                     | 8.08      | 8.07| 7.99 8.17 | 0.089| 0.226| 0.352| 0.954| 0.323   | 0.354    | 0.693    |

1 Means by treatment was the pooled data from goats at weeks 3, 6, 9 and 12, $n = 5$ for the microbial measurements and $n = 6$ for the others.

The $P$-values for all the $F \times N \times$ week. interactions were higher than 0.05, and they were not listed in the table.

CON Control, FUM Fumaric acid, NPD N-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide, FN FUM+ NPD, A/P Acetate: propionate ratio, SEM Standard error of means.

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TMR, which could not allow for continual consumption and decreasing CH₄ emissions throughout the day as mixed inclusion of 3-NOP in the TMR [32, 34]. This premise is consistent with the greater reduction in CH₄ emissions (59%) observed when 280 mg of 3-NOP/kg FDM was mixed with beef cattle TMR [36] than that (33%) when up to 345 mg of 3-NOP/kg FDM was top-dressed on the same background diet [37]. Differences in molecular structure may also be responsible for the different CH₄ mitigation potentials between 3-NOP and NPD [8], and future studies are needed to compare the two nitrooxy compared both in vivo and in vitro to determine their relative efficacy.

**Table 5** Effects of the dietary treatments on relative abundances of ruminal dominant bacterial genus (> 0.1%), %

| Item | Treatment | SEM | P-value |
|------|-----------|-----|---------|
| Bacteroidetes, mean = 64.7% | | | |
| Prevotella | 37.4 | 36.5 | 38.3 | 36.3 | 0.95 | 0.166 | 0.147 | 0.719 | 0.571 | 0.857 | 0.791 |
| YRC22 | 2.25 | 1.96 | 2.19 | 1.95 | 0.207 | 0.332 | 0.221 | 0.871 | 0.905 | 0.837 | 0.791 |
| CF231 | 1.42³ | 1.79⁴ | 1.77³ | 1.79³ | 0.098 | 0.953 | 0.069 | 0.100 | 0.098 | 0.339 | 0.704 |
| BF311 | 0.12 | 0.15 | 0.17 | 0.17 | 0.016 | 0.669 | 0.452 | 0.076 | 0.313 | 0.213 | 0.635 |
| Paludibacter | 0.08 | 0.28 | 0.15 | 0.08 | 0.089 | 0.161 | 0.521 | 0.482 | 0.161 | 0.135 | 0.361 |
| (Prevotella) | 0.11 | 0.09 | 0.11 | 0.14 | 0.017 | 0.435 | 0.656 | 0.223 | 0.236 | 0.506 | 0.471 |
| Firmicutes, mean = 19.4% | | | |
| Ruminococcus | 1.83 | 1.94 | 1.67 | 2.05 | 0.102 | 0.001 | 0.034 | 0.811 | 0.184 | 0.891 | 0.749 |
| Succiniclasicum | 1.32 | 1.52 | 1.16 | 1.48 | 0.099 | 0.043 | 0.022 | 0.320 | 0.579 | 0.890 | 0.481 |
| Oscillospira | 1.38 | 0.97 | 1.72 | 0.77 | 0.198 | 0.199 | 0.005 | 0.726 | 0.209 | 0.932 | 0.252 |
| Coprococcus | 1.12 | 1.22 | 1.05 | 1.43 | 0.120 | 0.524 | 0.071 | 0.580 | 0.243 | 0.061 | 0.683 |
| 02d06 | 0.75 | 0.80 | 0.73 | 0.89 | 0.073 | <0.001 | 0.185 | 0.673 | 0.496 | 0.808 | 0.578 |
| Butyrivibrio | 0.79 | 0.86 | 0.66 | 0.83 | 0.079 | 0.005 | 0.146 | 0.310 | 0.575 | 0.158 | 0.480 |
| RFN20 | 0.84 | 0.56 | 0.79 | 0.53 | 0.100 | 0.266 | 0.020 | 0.696 | 0.906 | 0.984 | 0.231 |
| Clostridium | 0.46 | 0.52 | 0.42 | 0.50 | 0.029 | 0.005 | 0.038 | 0.231 | 0.782 | 0.227 | 0.869 |
| Selenomonas | 0.23 | 0.29 | 0.24 | 0.38 | 0.048 | 0.001 | 0.070 | 0.354 | 0.380 | 0.137 | 0.815 |
| Moryella | 0.24 | 0.23 | 0.23 | 0.27 | 0.023 | <0.001 | 0.672 | 0.477 | 0.342 | 0.331 | 0.662 |
| Shuttleworthia | 0.13³ | 0.14² | 0.10³ | 0.19³ | 0.015 | 0.062 | 0.004 | 0.470 | 0.011 | 0.065 | 0.713 |
| Proteobacteria, mean = 6.5% | | | |
| Ruminobacter | 1.67 | 1.76 | 1.50 | 1.14 | 0.223 | 0.828 | 0.548 | 0.102 | 0.342 | 0.097 | 0.875 |
| Succinivibrio | 0.43 | 0.55 | 0.63 | 0.35 | 0.144 | 0.006 | 0.578 | 0.987 | 0.189 | 0.340 | 0.849 |
| Tenericutes, mean = 2.6% | | | |
| Anaeroplasma | 0.53 | 0.39 | 0.41 | 0.43 | 0.049 | <0.001 | 0.216 | 0.397 | 0.150 | 0.142 | 0.007 |
| Fibrobacteres, mean = 1.6% | | | |
| Fibrobacteres | 1.55 | 1.70 | 1.71 | 1.32 | 0.180 | 0.024 | 0.520 | 0.553 | 0.160 | 0.508 | 0.888 |
| Synergistetes, mean = 0.6% | | | |
| TG5 | 0.80 | 0.35 | 0.63 | 0.30 | 0.163 | 0.007 | 0.035 | 0.501 | 0.743 | 0.037 | 0.089 |

¹Means by treatment was the pooled data from 5 goats at weeks 3, 6, 9 and 12
²³Means with different superscripts within a row differ (P < 0.05). The P-values for all the F×N×week. interactions were higher than 0.05, and they were not listed in the table

CON Control, FUM Fumaric acid, NPD N-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide, FN FUM+ NPD

Long-term effects of FUM on CH₄ production and ruminal VFA profiles

The persistence of the CH₄-decreasing effect is an important criterion in evaluating the potential of CH₄ emission reduction strategies [1], and to our knowledge, no studies have been reported to evaluate this criterion for FUM. Supplementation of the diet with FUM enhanced rumen propionate fermentation accompanied by a decrease in CH₄ emissions, consistent with the results of previous studies [16, 17, 38], and these responses persisted over the whole 12-week treatment period. Theoretically, conversion of all 34 g FUM (0.29 mol) to propionate could potentially reduce daily CH₄ yield by 1.80 L [17], which is much lower than the reduction (on
average of 6.25 L) observed in the current study, supporting the earlier theory that the mechanism of FUM action in CH4 suppression was not only attributable to its function as an H-acceptor [17, 38]. Prevotella ruminicolae, Fibrobacter succinogenes and Selenomonas ruminantium have been recognized as rumen fumarate-utilizing bacteria [39, 40], but only the relative abundances of Selenomonas ruminantium tended to be more abundant (P = 0.059) in the animals fed with FUM, which is in agreement with the previous findings in sheep [16]. Instead of increase, the concentrations of rumen fumarate and succinate decreased in the goats fed FUM compared with the goats fed CON, probably due to the substrate stimulatory effects of FUM on fumarate-utilizing bacteria [41], and thus increasing the ruminal activity of the succinate-propionate metabolic pathway.

**Combined effects of FUM and NPD on ruminal hydrogen flow potential**

Hydrogen is an important fermentation intermediate in the rumen [42], mainly originating from the acetate- and butyrate-forming pathways. The produced hydrogen is primarily removed via methanogenesis and

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**Table 6** Effect of dietary treatments on serum antioxidant capacity of the dairy goats

| Item           | Treatment | SEM       | P-value       |
|----------------|-----------|-----------|---------------|
|                | CON       | FUM       | NPD           | F × N     |
| T-AOC, U/mL    | 3.54b     | 4.55a     | 4.88a         | 4.41a     | 0.235 | 0.001 | 0.269 | 0.007 | 0.644 | 0.565 |
| GSH-Px, U/mL   | 286       | 269       | 252           | 276       | 11.8  | 0.001 | 0.769 | 0.273 | 0.105 | 0.898 | 0.428 |
| SOD, U/mL      | 65.8      | 67.5      | 68.5          | 71.4      | 1.59  | 0.001 | 0.167 | 0.053 | 0.691 | 0.227 | 0.570 |
| MDA, μmol/L    | 1.05      | 0.79      | 0.85          | 0.81      | 0.069 | 0.005 | 0.031 | 0.248 | 0.181 | 0.479 | 0.874 |

1Means by treatment was the pooled data from 6 goats at 3, 6, 9 and 12 weeks.

2Means with different superscripts within a row differ significantly (P < 0.05). The P-values for all the F × N × week interactions were higher than 0.05, and they were not listed in the table.

CON Control, FUM Fumaric acid, NPD N-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide, FN FUM + NPD, MDA Malondialdehyde, GSH-Px Glutathione peroxidase, SOD Superoxide dismutase, T-AOC Total antioxidant capacity, SEM Standard error of means.
propionogenesis [13]. The inhibition of methanogenesis is expected to redirect excess hydrogen to propionate synthesis [14, 43]. However, the ruminal proportion of propionate does not always increase when methanogenesis is inhibited by 3-NOP [10, 44], consistent with our results. An increase (by 48- to 100-fold) in eructated gaseous hydrogen is commonly observed *in vitro* or *in vivo* with 3-NOP supplementation [6, 8–10], suggesting that the efficiency of hydrogen capture was lower when CH$_4$ production was inhibited by 3-NOP [12, 45]. Only 54.3% of the hydrogen spared from methanogenesis was diverted to alternate hydrogen-sinks *in vitro* [15], and 31% of the spared hydrogen was released as gas in beef cattle [45]. Consequently, the negative interaction between NPD and FUM was expected as the hydrogen was enough for both methanogenesis and propionogenesis, resulting in their competitive relationship disappearing when both NPD and FUM were supplemented in goats.

If 4 mol of H$_2$ and 1 mol of CO$_2$ are required to yield 1 mol of CH$_4$, the energy loss associated with eructated gaseous H$_2$ is 27% higher than that of CH$_4$. Moreover, the global warming potential of eructated gaseous H$_2$ is close to that of converted CH$_4$ (4 × 5.8: 25, on a CO$_2$-equivalent basis) [11, 12]. In addition, the volume of eructated gaseous H$_2$ and CO$_2$ is 4-fold higher than that of converted CH$_4$, resulting in a risk of rumen flatulence. Taken together, an increase in eructated gaseous H$_2$ partly offsets the advantages of energy savings and reduced environmental concerns by CH$_4$ mitigation. Therefore, it is desirable for the spared H$_2$ to be efficiently diverted to nutritionally beneficial sinks, such as propionate [45]. In this study, FUM showed greater responses in propionate increase when supplemented in combination with NPD than alone (by 10.2% vs. 4.4%), suggesting FUM diverted more hydrogen towards propionate synthesis when supplemented in combination. Because NPD, FUM, and their combination resulted in similar CH$_4$ emissions, it indicates that the release of gaseous H$_2$ was lower from the animals fed both NPD and FUM than those fed NPD alone. Similar results have also been observed in beef cattle when supplementation of 3-NOP was combined with monensin [45], with the combination increasing more propionate proportion than supplementing monensin alone (by 29.8% vs. 11.6%), and the combination decreasing H$_2$ emissions by 79.7% compared with supplementing 3-NOP alone.

**Effects of FUM on lactation performance**
Supplementation with FUM did not affect DMP, although it was accompanied by a series of positive effects, such as inhibiting CH$_4$ production, increasing propionate proportion and the relative abundances of rumen cellulolytic bacterial genera (e.g., *Ruminococcus* and *Clostridium*). The null effect of FUM on DMP is consistent with that observed in dairy cows receiving FUM in previous studies [38, 51]. On the other hand, the inclusion of FUM decreased milk fat content and tended to decrease milk fat yield, without changing other milk components, which are close to the classical characteristics of diet-induced milk fat depression [52]. Similar results were also observed in dairy cows receiving 600 g FUM supplementation per day [51]. The decreased rumen butyrate proportion and acetate-to-propionate ratio in response to FUM might be partially responsible for the lower milk fat because acetate and butyrate are important precursors for the de novo synthesis of milk fatty acids [53, 54]. However, more recent experiments revealed that shifts in the rumen VFA profile do not seem to be a major cause of milk fat depression [52]. Diets known to induce milk fat depression were associated with rumen unsaturated fatty acid biohydrogenation [52], which will be explored in our further research.

**Conclusions**
Using lactating dairy goats as a model, we evaluated the effects of NPD as a direct methanogenesis inhibitor, fumarate as an alternative hydrogen sink, and their combination on CH$_4$ production, rumen fermentation, and...
lactation performance over 12 weeks. Both NPD and FUM persistently inhibited CH₄ emissions without negative influences on DMI or nutrient digestibility. The hydrogen spared from the inhibited methanogenesis by NPD was more likely used for propionate synthesis rather than being eructated as gas when FUM was also supplemented. However, NPD and other nitrooxy compounds need to be further evaluated for their side effects on animal health and their residues in animal products before they can be used as animal feed additives.

Abbreviations

AP: Acetate to propionate ratio; ADF: Acid detergent fiber; BW: Body weight; DE: Digestible energy; DMI: Days in milk; DMP: Daily milk production; DMI: Dry matter intake; FCM: Fat corrected milk; FDM: Feed dry matter; FUM: Fumaric acid; GE: Gross energy; GSH-Px: Glutathione peroxidase; MDA: Malondialdehyde; ME: Metabolizable energy; MUN: Milk urea nitrogen; NEL: Net energy for lactation; NDF: Neutral detergent fiber; NPD: N-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide; VFA: Volatile fatty acids; NEL: Net energy for lactation; NDF: Neutral detergent fiber; NPD: N-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide; VFA: Volatile fatty acids; SMD: Standardized mean difference; SOD: Superoxide dismutase; T-AOC: Total antioxidant capacity

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Authors’ contributions

Conceived and designed the experiments: ZJL and JHY. Performed the experiments: ZJL, XJC, QYY and JS. Analyzed the data: ZJL and XJC. Contributed to the writing of the manuscript: ZJL, XJC and JHY. All authors reviewed and approved the manuscript.

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Availability of data and materials

The original sequence data had been deposited to NCBI with Bioproject accession no. PRJNA703427.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests

There is no conflict of interest among all authors.

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