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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

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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_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_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_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_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_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.

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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, nitrooxy ($-O-NO_2$) compounds, such as 3-nitrooxypropanol (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 eructated 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.

Nitrooxy 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 nitrooxy 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

Table 1 Ingredients and chemical composition of the experimental diet

Item	%
Ingredients	
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	
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

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 CH_4 emissions were measured simultaneously, and the samples of blood and rumen content were collected on d 4–5 and d 5–6, respectively.

Measuring CH_4 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 CH_4 production was calculated as follows:

$$CH_4 \text{production (L/d)} = \Sigma \left[(C_i - C_{i-1}) \times V_c + V_f \times (C_i - CO_i) \right]/1000$$

Where C_i = the CH_4 concentration (mL/m^3) of the internal chamber at the i 60-min; CO_i = the CH_4 concentration of external environment at the i 60-min; V_c = the chamber volume (83.9 m^3); and V_f = the gas volume pumped from each chamber over each 60-min measurement (0.24 m^3).

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]: 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)).

Apparent total tract digestibility and energy balance
The apparent total tract digestibility and energy balance of each goat were estimated by daily total collection of feces and urine from d 1–4 during experimental weeks 3 and 9. All refusals and feces of individual goats were dried at 55 °C for 72 h in forced air ovens to a constant weight and subsample (about 100 g, wool removed) was ground through a 1-mm screen for further analysis. Urine was collected through a funnel into buckets and acidified by adding 100 mL of 10% (vol/vol) sulfuric acid to prevent microbial degradation and the loss of volatile ammonia-N. These samples were determined the contents of dry matter (DM), ash, and crude protein (CP) [22]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were measured using the filter bag method with sodium sulfite and heat-stable α-amylase (Ankom° A200I fiber analyser, ANKOM Technology, Macedon, NY, USA). The BW was recorded twice daily after milking. The gross energy (GE) content of the samples was analyzed in an automatic adiabatic bomb calorimeter (model 1600 Parr Instrument Co., Moline, IL, USA). Digestible energy (DE) was calculated as the difference between energy intake and fecal energy; the energy lost as CH_4 was calculated as the CH_4 emitted in L/ day × 39.54 kJ/L; metabolizable energy (ME) was the difference between DE and the sum of the energy in urine and CH_4.

Collection 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

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(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 (2×250) 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 $\geq$ 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 CH_4) 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 $0.05 \leq P < 0.10$.

## Results

Methane production and lactation performance

The persistent and combined effects of FUM and NPD supplementation on CH_4 production and milk parameters are shown in Table 2 and Fig. 1. Both FUM and NPD supplementation persistently inhibited ($P < 0.05$) the CH_4 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 CH_4 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 ($P <$

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Table 2 Effects of the dietary treatments on feed intake, milk performance and methane production of the dairy goats

Item	Treatment[1]				SEM	P-value					
	CON	FUM	NPD	FN		Week	FUM	NPD	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											
CH_4, L/d	32.7[a]	26.4[b]	28.3[b]	25.9[b]	0.91	0.031	0.030	0.036	0.081	0.142	0.342
CH_4, MJ/d	1.30[a]	1.05[a]	1.12[a]	1.03[a]	0.036	0.031	0.030	0.036	0.081	0.142	0.342
CH_4/DMI, L/kg	19.2[a]	15.6[b]	15.7[b]	15.3[b]	0.43	0.077	0.004	0.005	0.010	0.197	0.272
CH_4/FCM, L/kg	26.4	23.7	18.0	19.0	2.56	0.296	0.750	0.043	0.512	0.845	0.094

[1]Means by treatment was the pooled data from goats at weeks 3 and 9, $n = 3$ for the measurements related to CH_4 and $n = 6$ for the others

[a,b]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
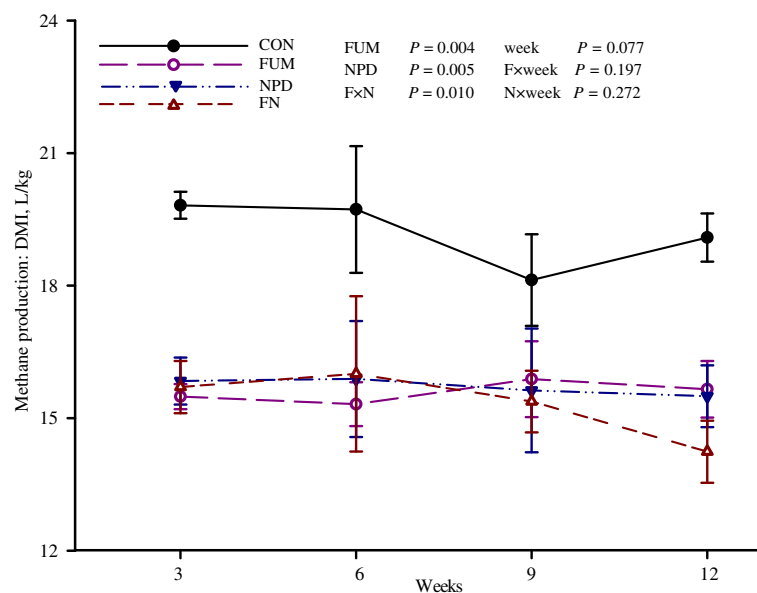
Fig. 1 Dynamic and combined effects of fumaric acid (FUM) and N-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide (NPD) on methane yield (L/kg DMI, mean ± standard error) in the dairy goats. CON, Control; FN, FUM + NPD

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 CH_4 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 $R_{ANOSIM} = 0.145$, $P = 0.001$), particularly changed the structure of the phylum Firmicutes. Within the phylum Firmicutes, the relative abundances of the genera *Ruminococcus*, *Succiniclasticum*, *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	Treatment[1]				SEM	P-value		
	CON	FUM	NPD	FN		FUM	NPD	F × 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
CH_4/GE	4.52[a]	3.73[b]	3.75[b]	3.70[b]	0.072	0.001	0.001	0.002

[1]Means by treatment was the pooled data from goats at weeks 3 and 9, $n = 3$ for the measurements related to CH_4 and $n = 6$ for the others
[a,b]Means in the same row with different superscripts 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, *BW* Body weight, *GE* Gross energy, *DE* Digestible energy, *NEL* Net energy for lactation, *ME* Metabolizable energy, *SEM* Standard error of means

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

Item	Treatment[1]				SEM	P-value					
	CON	FUM	NPD	FN		Week	FUM	NPD	F × N	F × week	N × week
pH	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 × 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, A:P Acetate: propionate ratio, 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 CH₄ 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 ± 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

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

Item	Treatment[1]				SEM	P-value					
	CON	FUM	NPD	FN		Week	FUM	NPD	F × N	F × week	N × week
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	1.42[b]	1.79[a]	1.77[a]	1.79[a]	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
Succiniclasticum	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	0.13[b]	0.14[b]	0.10[b]	0.19[a]	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

[1]Means by treatment was the pooled data from 5 goats at weeks 3, 6, 9 and 12
[a,b]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
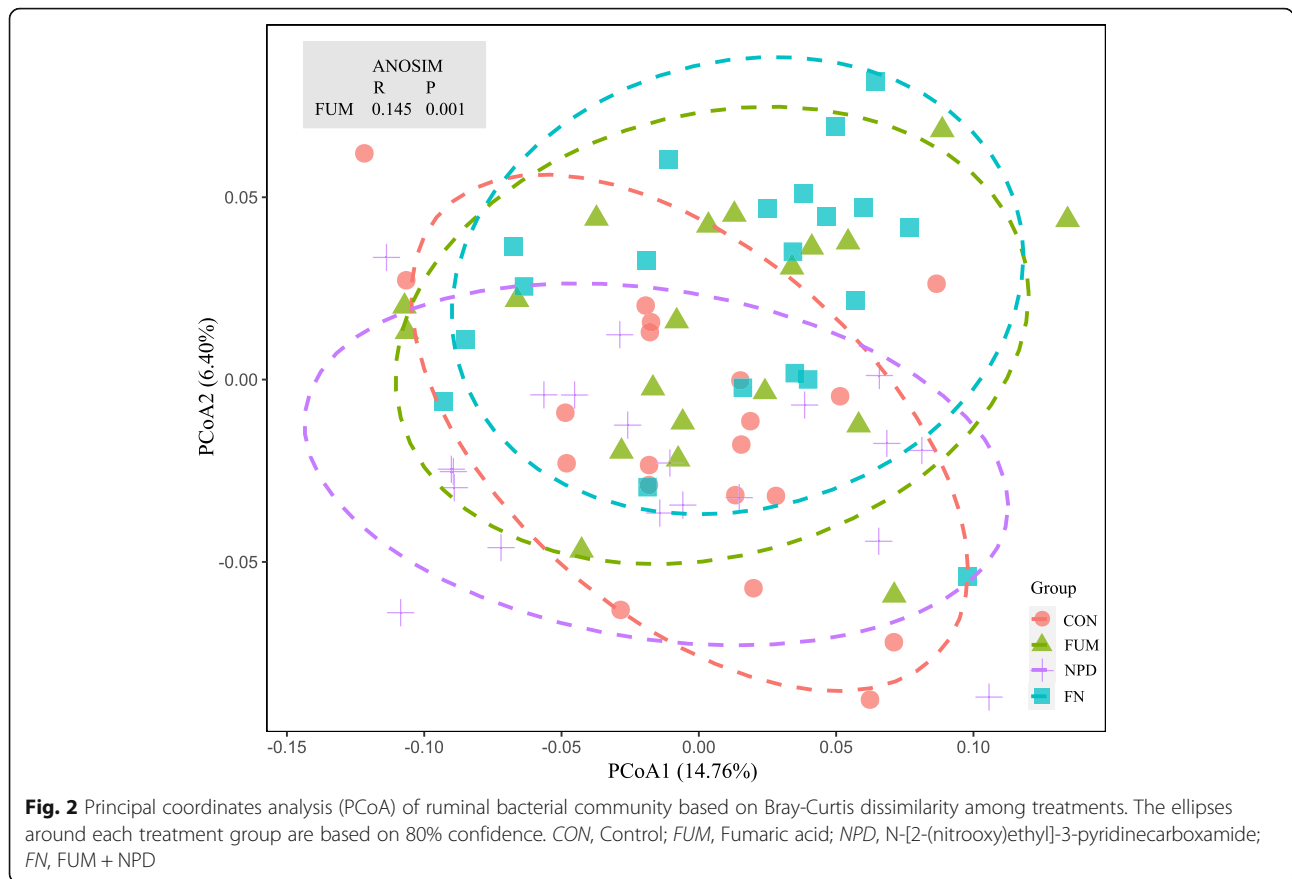*CON* Control, *FUM* Fumaric acid, *NPD* N-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide, *FN* FUM+ NPD

TMR, which could not allow for continual consumption and decreasing CH_4 emissions throughout the day as mixed inclusion of 3-NOP in the TMR [32, 34]. This premise is consistent with the greater reduction in CH_4 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 CH_4 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.

Long-term effects of FUM on CH_4 production and ruminal VFA profiles

The persistence of the CH_4-decreasing effect is an important criterion in evaluating the potential of CH_4 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 CH_4 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 CH_4 yield by 1.80 L [17], which is much lower than the reduction (on

Fig. 2 Principal coordinates analysis (PCoA) of ruminal bacterial community based on Bray-Curtis dissimilarity among treatments. The ellipses around each treatment group are based on 80% confidence. *CON*, Control; *FUM*, Fumaric acid; *NPD*, N-[2-(nitrooxy)ethyl]-3-pyridinecarboxamide; *FN*, FUM + NPD

average of 6.25 L) observed in the current study, supporting the earlier theory that the mechanism of FUM action in CH_4 suppression was not only attributable to its function as an H-acceptor [17, 38]. *Prevotella ruminicola*, *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

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

Item	Treatment[1]				SEM	*P*-value					
	CON	FUM	NPD	F × N		Week	FUM	NPD	F × N	F × week	N × week
T-AOC, U/mL	3.54[b]	4.55[a]	4.88[a]	4.41[a]	0.235	0.001	0.269	0.021	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

[1]Means by treatment was the pooled data from 6 goats at 3, 6, 9 and 12 weeks.
[a,b]Means 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 NPD on lactation performance
Beyond expectation, NPD increased DMP by 29.8%, while decreasing milk fat content by − 12.6% and milk protein content by − 15.8%, without changes in nutrient digestibility, rumen VFA profiles, or daily BW gain. Although CH_4 suppression in the rumen has the potential

to improve energy efficiency [45], the mean decrease in CH_4 energy of 0.18 MJ/d per goat by NPD would only convert to 0.04 MJ/d of milk NEL [10, 21], which is much lower than the actual improvement of milk NEL (0.68 MJ) observed in the goats. The NPD dose used in the current study was about 13 mg/kg/d, much higher than the level (1 mg/kg/d) that increased blood flow in mice [19]. A possible explanation for the unexpected improvement of milk NEL by DMP might be that some of the NPD escaped from rumen fermentation and was absorbed by the gut, ultimately exerting its bioactive function, such as increasing blood flow. Indeed, several studies have shown that blood flow was positively associated with milk production and uptake of milk precursors [46–48]. However, the blood flow and blood NPD content were not measured in this study. To support this explanation, we measured another bioactive function of NPD, enhancing antioxidant capacity [49, 50], which is consistent with our observations. Therefore, the side effects of this nitrooxy compound on animal health and its residues in animal products need further evaluation before it can be used as an animal feed additive.

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 explore 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 CH_4 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
A:P: Acetate to propionate ratio; ADF: Acid detergent fiber; BW: Body weight; DE: Digestible energy; DIM: 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; SMD: Standardized mean difference; SOD: Superoxide dismutase; T-AOC: Total antioxidant capacity

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

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.

References
1. Hristov AN, Oh J, Lee C, Meinen R. Mitigation of greenhouse gas emissions in livestock production: a review of technical options for non-CO_2 emissions. Rome: Food and Agriculture Organization of the United Nations; 2013.
2. Johnson KA, Johnson DE. Methane emissions from cattle. J Anim Sci. 1995; 73(8):2483–92. https://doi.org/10.2527/1995.7382483x.
3. Grainger C, Williams R, Eckard RJ, Hannah MC. A high dose of monensin does not reduce methane emissions of dairy cows offered pasture supplemented with grain. J Dairy Sci. 2010;93(11):5300–8. https://doi.org/10.3168/jds.2010-3154.
4. Duval S, Kindermann M. Nitrooxy alkanoic acids and derivatives thereof in feed for reducing methane emission in ruminants, and/or to improve ruminant performance. 2014. http://www.freepatentsonline.com/y2014/0134282.html.
5. Martínez-Fernández G, Abecia L, Arco A, Cantalapiedra-Hijar G, Martín-García AI, Molina-Alcaide E, et al. Effects of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol on ruminal fermentation, microbial abundance, and methane emissions in sheep. J Dairy Sci. 2014;97(6):3790–9. https://doi.org/10.3168/jds.2013-7398.
6. Hristov AN, Oh J, Giallongo F, Frederick TW, Harper MT, Weeks HL, et al. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. Proc Natl Acad Sci. 2015; 112(34):10663–8. https://doi.org/10.1073/pnas.1504124112.
7. Duin EC, Wagner T, Shima S, Prakash D, Cronin B, Yáñez-Ruiz DR, et al. Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol. Proc Natl Acad Sci. 2016;113(22):6172–7. https://doi.org/10.1073/pnas.1600298113.
8. Jin W, Meng Z, Wang J, Cheng Y, Zhu W. Effect of Nitrooxy compounds with different molecular structures on the rumen Methanogenesis, metabolic profile, and methanogenic community. Curr Microbiol. 2017;74(8): 891–8. https://doi.org/10.1007/s00284-017-1261-7.
9. Lopes JC, de Matos LF, Harper MT, Giallongo F, Oh J, Gruen D, et al. Effect of 3-nitrooxypropanol on methane and hydrogen emissions, methane isotopic signature, and ruminal fermentation in dairy cows. J Dairy Sci. 2016; 99(7):5335–44. https://doi.org/10.3168/jds.2015-10832.
10. Melgar A, Harper MT, Oh J, Giallongo F, Young ME, Ott TL, et al. Effects of 3-nitrooxypropanol on rumen fermentation, lactational performance, and resumption of ovarian cyclicity in dairy cows. J Dairy Sci. 2020;103(1):410–32. https://doi.org/10.3168/jds.2019-17085.
11. Rooke JA, Wallace RJ, Duthie C-A, McKain N, de Souza SM, Hyslop JJ, et al. Hydrogen and methane emissions from beef cattle and their rumen microbial community vary with diet, time after feeding and genotype. Br J Nutr. 2014;112(3):398–407. https://doi.org/10.1017/S0007114514000932.
12. Ungerfeld EM. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. Front Microbiol. 2015;6:37. https://doi.org/10.3389/fmicb.2015.00037.
13. Ellis JL, Dijkstra J, Kebreab E, Bannink A, Odongo NE, McBRIDE BW, et al. Aspects of rumen microbiology central to mechanistic modelling of methane production in cattle. J Agric Sci. 2008;146(2):213–33. https://doi.org/10.1017/S0021859608007752.
14. Janssen PH. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Anim Feed Sci Technol. 2010;160(1-2):1–22. https://doi.org/10.1016/j.anifeedsci.2010.07.002.
15. Guyader J, Ungerfeld EM, Beauchemin KA. Redirection of metabolic hydrogen by inhibiting methanogenesis in the rumen simulation technique (RUSITEC). Front Microbiol. 2017;8:393. https://doi.org/10.3389/fmicb.2017.00393.
16. Yang CJ, Mao SY, Long LM, Zhu WY. Effect of disodium fumarate on microbial abundance, ruminal fermentation and methane emission in goats under different forage : concentrate ratios. Animal. 2012;6(11):1788–94. https://doi.org/10.1017/S1751731112000857.
17. Li Z, Liu N, Cao Y, Jin C, Li F, Cai C, et al. Effects of fumaric acid supplementation on methane production and rumen fermentation in goats fed diets varying in forage and concentrate particle size. J Anim Sci Biotechnol. 2018;9(1):21. https://doi.org/10.1186/s40104-018-0235-3.
18. Tarkin JM, Kaski JC. Vasodilator therapy: nitrates and nicorandil. Cardiovasc Drugs Ther. 2016;30(4):367–78. https://doi.org/10.1007/s10557-016-6668-z.
19. Kotoda M, Ishiyama T, Mitsui K, Hishiyama S, Matsukawa T. Nicorandil increased the cerebral blood flow via nitric oxide pathway and ATP-sensitive potassium channel opening in mice. J Anesth. 2018;32(2):244–9. https://doi.org/10.1007/s00540-018-2471-2.
20. Li ZJ, Ren H, Liu SM, Cai CJ, Han JT, Li F, et al. Dynamics of methanogenesis, ruminal fermentation, and alfalfa degradation during adaptation to monensin supplementation in goats. J Dairy Sci. 2018;101(2):1048–59. https://doi.org/10.3168/jds.2017-13254.
21. NRC. Nutrient requirements of dairy cattle. 7th ed. Washington, D.C: National Academic Press; 2001.
22. AOAC. Official methods of analysis. 18th ed. Gaithersburg: Association of Official Analytical Chemists; 2005.

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23. Li F, Li Z, Li S, Ferguson JD, Cao Y, Yao J, et al. Effect of dietary physically effective fiber on ruminal fermentation and the fatty acid profile of milk in dairy goats. J Dairy Sci. 2014;97(4):2281–90. https://doi.org/10.3168/jds.2013-6895.

24. Shurubor YI, Cooper AJL, Isakova EP, Deryabina YI, Beal MF, Krasnikov BF. Simultaneous determination of tricarboxylic acid cycle metabolites by high-performance liquid chromatography with ultraviolet detection. Anal Biochem. 2016;503:8–10. https://doi.org/10.1016/j.ab.2016.03.004.

25. Shen J, Zheng L, Chen X, Han X, Cao Y, Yao J. Metagenomic analyses of microbial and carbohydrate-active enzymes in the rumen of dairy goats fed different rumen degradable starch. Front Microbiol. 2020;11:1003. https://doi.org/10.3389/fmicb.2020.01003.

26. Wear EK, Wilbanks EG, Nelson CE, Carlson CA. Primer selection impacts specific population abundances but not community dynamics in a monthly time-series 16S rRNA gene amplicon analysis of coastal marine bacterioplankton. Environ Microbiol. 2018;20(8):2709–26. https://doi.org/10.1111/1462-2920.14091.

27. Ren H, Bai H, Su X, Pang J, Li X, Wu S, et al. Decreased amylolytic microbes of the hindgut and increased blood glucose implied improved starch utilization in the small intestine by feeding rumen-protected leucine in dairy calves. J Dairy Sci. 2020;103(5):4218–35. https://doi.org/10.3168/jds.2019-17194.

28. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7(5):335–6. https://doi.org/10.1038/nmeth.f.303.

29. Littell RC, Henry PR, Ammerman CB. Statistical analysis of repeated measures data using SAS procedures. J Anim Sci. 1998;76(4):1216–31. https://doi.org/10.2527/1998.7641216x.

30. Bray JR, Curtis JT. An ordination of the upland forest communities of southern Wisconsin. Ecol Monogr. 1957;27:326–49.

31. Waite R, White JCD, Robertson A. Variations in the chemical composition of milk with particular reference to the solids-not-fat: I. the effect of stage of lactation, season of year and age of cow. J Dairy Res. 1956;23(1):65–81. https://doi.org/10.1017/S0022029900008049.

32. Dijkstra J, Bannink A, France J, Kebreab E, van Gastelen S. Short communication: Antimethanogenic effects of 3-nitrooxypropanol depend on supplementation dose, dietary fiber content, and cattle type. J Dairy Sci. 2018;101(10):9041–7. https://doi.org/10.3168/jds.2018-14456.

33. Haisan J, Sun Y, Guan LL, Beauchemin KA, Iwaasa A, Duval S, et al. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. J Dairy Sci. 2014;97(5):3110–9. https://doi.org/10.3168/jds.2013-7834.

34. Melgar A, Welter KC, Nedelkov K, Martins CMMR, Harper MT, Oh J, et al. Dose-response effect of 3-nitrooxypropanol on enteric methane emissions in dairy cows. J Dairy Sci. 2020;103(7):6145–56. https://doi.org/10.3168/jds.2019-17840.

35. Reynolds CK, Humphries DJ, Kirton P, Kindermann M, Duval S, Steinberg W. Effects of 3-nitrooxypropanol on methane emission, digestion, and energy and nitrogen balance of lactating dairy cows. J Dairy Sci. 2014;97(6):3777–89. https://doi.org/10.3168/jds.2013-7397.

36. Romero-Pérez A, Okine EK, Guan LL, Duval SM, Kindermann M, Beauchemin KA. Effects of 3-nitrooxypropanol on methane production using the rumen simulation technique (Rusitec). Anim Feed Sci Technol. 2015;209:98–109. https://doi.org/10.1016/j.anifeedsci.2015.09.002.

37. Romero-Perez A, Okine EK, McGinn SM, Guan LL, Oba M, Duval SM, et al. The potential of 3-nitrooxypropanol to lower enteric methane emissions from beef cattle. J Anim Sci. 2014;92(10):4682–93. https://doi.org/10.2527/jas.2014-7573.

38. van Zijderveld SM, Dijkstra J, Perdok HB, Newbold JR, Gerrits WJJ. Dietary inclusion of diallyl disulfide, yucca powder, calcium fumarate, an extruded linseed product, or medium-chain fatty acids does not affect methane production in lactating dairy cows. J Dairy Sci. 2011;94(6):3094–104. https://doi.org/10.3168/jds.2010-4042.

39. Strobel HJ, Russell JB. Succinate transport by a ruminal selenomonad and its regulation by carbohydrate availability and osmotic strength. Appl Environ Microbiol. 1991;57(1):248–54. https://doi.org/10.1128/aem.57.1.248-254.1991.

40. Russell JB, Houlihan AJ. Ionophore resistance of ruminal bacteria and its potential impact on human health. FEMS Microbiol Rev. 2003;27(1):65–74. https://doi.org/10.1016/S0168-6445(03)00019-6.

41. Nisbet DJ, Martin SA. Effects of fumarate, l-malate, and an aspergillus oryzae fermentation extract on d-lactate utilization by the ruminal bacterium *Selenomonas ruminantium*. Curr Microbiol. 1993;26(3):133–6. https://doi.org/10.1007/BF01577366.

42. Hungate RE. The rumen microbial ecosystem. Annu Rev Ecol Syst. 1975;6(1):39–66. https://doi.org/10.1146/annurev.es.06.110175.000351.

43. Morgavi DP, Forano E, Martin C, Newbold CJ. Microbial ecosystem and methanogenesis in ruminants. Animal. 2010;4(7):1024–36. https://doi.org/10.1017/S1751731110000546.

44. Kim S-H, Lee C, Pechtl HA, Hettick JM, Campler MR, Pairis-Garcia MD, et al. Effects of 3-nitrooxypropanol on enteric methane production, rumen fermentation, and feeding behavior in beef cattle fed a high-forage or high-grain diet. J Anim Sci. 2019;97(7):2687–99. https://doi.org/10.1093/jas/skz140.

45. Vyas D, Alemu AW, McGinn SM, Duval SM, Kindermann M, Beauchemin KA. The combined effects of supplementing monensin and 3-nitrooxypropanol on methane emissions, growth rate, and feed conversion efficiency in beef cattle fed high-forage and high-grain diets. J Anim Sci. 2018;96(7):2923–38. https://doi.org/10.1093/jas/sky174.

46. Madsen TG, Cieslar SRL, Trout DR, Nielsen MO, Cant JP. Inhibition of local blood flow control systems in the mammary glands of lactating cows affects uptakes of energy metabolites from blood. J Dairy Sci. 2015;98(5):3046–58. https://doi.org/10.3168/jds.2014-8200.

47. Götze A, Honnens A, Flachowsky G, Bollwein H. Variability of mammary blood flow in lactating Holstein-Friesian cows during the first twelve weeks of lactation. J Dairy Sci. 2010;93(1):38–44. https://doi.org/10.3168/jds.2008-1781.

48. Cai J, Wang D, Liu J. Regulation of fluid flow through the mammary gland of dairy cows and its effect on milk production: a systematic review: mammary gland fluid flow and milk production. J Sci Food Agric. 2018;98(4):1261–70. https://doi.org/10.1002/jsfa.8605.

49. Sánchez-Duarte S, Márquez-Gamiño S, Montoya-Pérez R, Villicaña-Gómez EA, Vera-Delgado KS, Caudillo-Cisneros C, et al. Nicorandil decreases oxidative stress in slow- and fast-twitch muscle fibers of diabetic rats by improving the glutathione system functioning. J Diabetes Investig. 2021;12(7):1152–61. https://doi.org/10.1111/jdi.13513.

50. Serizawa K, Yogo K, Aizawa K, Tashiro Y, Ishizuka N. Nicorandil prevents endothelial dysfunction due to antioxidative effects via normalisation of NADPH oxidase and nitric oxide synthase in streptozotocin diabetic rats. Cardiovasc Diabetol. 2011;10(1):105. https://doi.org/10.1186/1475-2840-10-105.

51. Remling N, Riede S, Lebzien P, Meyer U, Höltershinken M, Kersten S, et al. Effects of fumaric acid on rumen fermentation, milk composition and metabolic parameters in lactating cows. J Anim Physiol Anim Nutr. 2013;98(5):968–81. https://doi.org/10.1111/jpn.12152.

52. Dewanckele L, Toral PG, Vlaeminck B, Fievez V. Invited review: role of rumen biohydrogenation intermediates and rumen microbes in diet-induced milk fat depression: an update. J Dairy Sci. 2020;103(9):7655–81. https://doi.org/10.3168/jds.2019-17662.

53. Black AL, Kleiber M, Brown AM. Butyrate metabolism in the lactating cow. J Biol Chem. 1961;236(9):2399–403. https://doi.org/10.1016/S0021-9258(18)64010-4.

54. Urrutia N, Bomberger R, Matamoros C, Harvatine KJ. Effect of dietary supplementation of sodium acetate and calcium butyrate on milk fat synthesis in lactating dairy cows. J Dairy Sci. 2019;102(6):5172–81. https://doi.org/10.3168/jds.2018-16024.