J. Dairy Sci. 99:5335–5344
http://dx.doi.org/10.3168/jds.2015-10832

Effect of 3-nitrooxypropanol on methane and hydrogen emissions, methane isotopic signature, and ruminal fermentation in dairy cows

J. C. Lopes,* L. F. de Matos,* M. T. Harper,* F. Giallongo,* J. Oh,* D. Gruen,† S. Ono,† M. Kindermann,‡ S. Duval,§ and A. N. Hristov*[1]
*Department of Animal Science, The Pennsylvania State University, University Park 16802
†Department of Earth, Atmospheric and Planetary Sciences, Massachusetts Institute of Technology, Cambridge 02139
‡DSM Nutritional Products, Animal Nutrition and Health, Basel, Switzerland CH-4002
§DSM Nutritional Products France, Research Centre for Animal Nutrition and Health, Saint Louis Cedex, France 68305

ABSTRACT

The objective of this crossover experiment was to investigate the effect of a methane inhibitor, 3-nitrooxypropanol (3NOP), on enteric methane emission, methane isotopic composition, and rumen fermentation and microbial profile in lactating dairy cows. The experiment involved 6 ruminally cannulated late-lactation Holstein cows assigned to 2 treatments: control and 3NOP (60 mg/kg of feed dry matter). Compared with the control, 3NOP decreased methane emission by 31% and increased hydrogen emission from undetectable to 1.33 g/d. Methane emissions per kilogram of dry matter intake and milk yield were also decreased 34% by 3NOP. Milk production and composition were not affected by 3NOP, except milk fat concentration was increased compared with the control. Concentrations of total VFA and propionate in ruminal fluid were not affected by treatment, but acetate concentration tended to be lower and acetate-to-propionate ratio was lower for 3NOP compared with the control. The 3NOP decreased the molar proportion of acetate and increase those of propionate, butyrate, valerate, and isovalerate. Deuterium-to-hydrogen ratios of methane and the abundance of $^{13}CH_3D$ were similar between treatments. Compared with the control, minor (4‰) depletion in the $^{13}C/^{12}C$ ratio was observed for 3NOP. Genus composition of methanogenic archaea (*Methanobrevibacter*, *Methanosphaera*, and *Methanomicrobium*) was not affected by 3NOP, but the proportion of methanogens in the total cell counts tended to be decreased by 3NOP. *Prevotella* spp., the predominant bacterial genus in ruminal contents in this experiment, was also not affected by 3NOP. Compared with the control, *Ruminococcus* and *Clostridium* spp. were decreased and *Butyrivibrio* spp. was increased by 3NOP. This experiment demonstrated that a substantial inhibition of enteric methane emission by 3NOP in dairy cows was accompanied with increased hydrogen emission and decreased acetate-to-propionate ratio; however, neither an effect on rumen archaeal community composition nor a significant change in the isotope composition of methane was observed.

Key words: methane, 3-nitrooxypropanol, rumen fermentation, dairy cow

INTRODUCTION

In the rumen, CH_4 is an end product of microbial fermentation of carbohydrates and AA. Methanogenesis is the major sink for hydrogen in the rumen, but enteric CH_4 represents also a net feed energy loss for the animal (Johnson and Johnson, 1995) and is a major contributor to agricultural greenhouse gas (**GHG**) emissions globally (IPCC, 2014).

Several reviews presented technical options for abatement of livestock GHG emissions (Boadi et al., 2004; McAllister and Newbold, 2008; Hristov et al., 2013). These strategies focus on feeding management practices such as fat supplementation, concentrate inclusion, processing low-quality feeds, and improving overall forage quality, as well as feed additives, such as alternative electron receptors, ionophoric antibiotics, plant bioactive compounds, enzymes, and CH_4 inhibitors.

Among CH_4 inhibitors, bromochloromethane, 2-bromo-ethane sulfonate, and chloroform are the most studied compounds in ruminants (Hristov et al., 2013). Both in vitro and in vivo experiments have demonstrated that these compounds were effective in reducing CH_4 emission without negatively affecting animal productivity (Sawyer et al., 1974; Goel et al., 2009; Tomkins et al., 2009; Abecia et al., 2012). Use of these compounds, however, is limited due to toxicity, rumen adaptation, or environmental regulation issues (Hristov et al., 2013). In response, natural or synthetic compounds with a similar mode of action are being

Received December 28, 2015.
Accepted March 8, 2016.
[1]Corresponding author: anh13@psu.edu

developed. The compound tested in this experiment, 3-nitrooxypropanol (**3NOP**), was designed to inhibit the activity of methyl coenzyme-M reductase (Duval and Kindermann, 2012), the enzyme responsible for microbial formation of CH_4 (Ermler et al., 1997). Recent studies showed that 3NOP consistently decreased enteric CH_4 emission in lactating dairy cows (Haisan et al., 2014; Reynolds et al., 2014). In a 12-wk study, Hristov et al. (2015a) reported persistent reduction of enteric CH_4 emission, whereas productive performance of high-producing dairy cows was not affected by 3NOP supplementation. In their study, however, the effect of 3NOP on ruminal fermentation could not be evaluated because the cows used were not ruminally cannulated.

Stable isotope compositions of CH_4 ($^{13}C/^{12}C$, D/H, and $^{13}CH_3D$, where D is a stable isotope of hydrogen with one extra neutron) reflect the isotope compositions of substrate ($^{13}C/^{12}C$ of feeds and D/H of rumen fluids), as well as isotope fractionation associated with microbial methanogenesis. The latter is shown to be a function of pathways, growth phase, and hydrogen levels (Whiticar et al., 1986; Burke, 1993; Valentine et al., 2004; Wang et al., 2015). For the CO_2 reduction pathway, thought to be dominant in the rumen (Hungate, 1966), large ^{13}C-depletion is associated with stationary growth, low metabolic rates, and low H_2 levels, whereas large D-depletion is associated with high metabolic rate and high H_2 levels (Burke, 1993; Zyakun, 1996; Valentine et al., 2004; Wang et al., 2015).

Therefore, one of the objectives of our study was to test if, in addition to decreased CH_4 emission, 3NOP affects CH_4 isotope compositions due to changes in physiology or environmental conditions for methanogenesis in lactating dairy cows. The study also investigated the effect of 3NOP on rumen fermentation, ruminal microbial profile, and production variables. We hypothesized that 3NOP would, similar to previous experiments, decrease acetate-to-propionate ratio in ruminal fluid and, due to the large reduction in CH_4 emission, would also affect the composition of ruminal archaea and the isotopic signature of enteric CH_4.

MATERIALS AND METHODS

Animals involved in these experiments were cared for according to the guidelines of the Pennsylvania State University Animal Care and Use Committee. The committee reviewed and approved the experiment and all procedures carried out in this study.

Animals and Experimental Design

The experiment used 6 ruminally cannulated late-lactation Holstein cows in a 2 × 2 crossover design with 2 experimental periods of 14 d each. A 7-d washout period was allowed between the experimental periods. Cows were grouped by DIM and current milk yield in 2 squares of 3 cows each. Cows were 1.3 (SD = 0.52) lactations, 233 (SD = 45) DIM at the beginning of the experiment, had an average BW during the experiment of 610 (SD = 158) kg, and were fitted with soft plastic ruminal cannulas (10.2 cm internal diameter; Bar Diamond Inc., Parma, ID). Within a period, the first 10 d served as adaptation and the remaining 4 d were used for sample and production data collection. Cows received recombinant bST (Posilac, Elanco Co., Greenfield, IN; 500 mg/cow, i.m.) on d 1 of each experimental period. The following 2 treatments were tested: 0 mg of 3NOP/kg of dietary DM (control) and 60 mg of 3NOP/kg of DM (3NOP; DSM Nutritional Products, Basel, Switzerland). The 60-mg/kg of DM dose was selected based on a previous experiment with 3NOP (Hristov et al., 2015a). The basal diet was formulated to meet or exceed the NE_L and MP requirements of a Holstein cow (according to NRC, 2001) with 610 kg of BW, producing 32 kg of milk/d with 4.10% milk fat and 3.60% true milk protein, and consuming 24 kg/d of DMI (Table 1). Diets were fed as TMR once daily at 0800 h targeting 10% refusals. The 3NOP supplement contained 8.85% 3NOP on SiO_2 and propylene glycol; the placebo supplement contained SiO_2 and propylene glycol only (Hristov et al., 2015a). The supplements were mixed with the TMR to deliver the final 3NOP concentration as indicated above. Cows were milked twice daily at approximately 0600 and 1800 h and had continuous access to a fresh water source.

Sampling and Measurements

During the experiment, TMR offered and refusals were recorded daily. Samples of the forages were collected once weekly and samples of the TMR were collected twice weekly. Samples of the concentrate feeds were collected once per experimental period. Feed samples were dried for 48 h at 65°C in a forced-air oven and ground in a Wiley Mill (Thomas Scientific, Swedesboro, NJ) through a 1-mm sieve for further analysis.

Composite (equal DM weight basis) samples of the forages and concentrate feeds fed during the experiment were submitted to Cumberland Valley Analytical Services (Maugansville, MD) for wet chemistry analyses of CP, NDF, ADF, Ca, P, and estimated NE_L (CVAS, 2014). The analyzed composition of the feed ingredients and their inclusion in the TMR was used to compute the CP, NDF, ADF, Ca, and P concentration of the diets (Table 1). During the last 3 d of each period, CH_4, CO_2, and H_2 emission were measured using the GreenFeed system (C-Lock Inc., Rapid City, SD) 8 times in

3 d covering a 24-h period at 0900, 1500, and 2100 h (sampling d 1), 0300, 1200, and 1700 h (sampling d 2), and 0000 and 0500 h (sampling d 3). Gas measurements were performed as described by Hristov et al. (2015b). A rumen cannula extension (fistula attachment; C-Lock Inc.) was used to capture and direct through GreenFeed rumen gas potentially leaking through the cannula. The cannula extension consisted on an impermeable fabric covering the back and sides of the cow with a tubing attached to it in close proximity of the rumen cannula and extending from the cannula to the GreenFeed unit. The tubing was attached to a port that directed the collected gas toward GreenFeed sensors. Constant negative pressure was maintained by using a vacuum pump to withdraw gas from between the fabric sheet and the animal into the tubing. The gas was then routed from the tubing and released into the GreenFeed air collection pipe where total airflow and concentrations of CH_4, CO_2, and H_2 were continuously measured.

Table 1. Ingredient and chemical composition of the basal diet fed during the trial

Item	Measurement
Ingredient, % of DM	
Corn silage[1]	43.5
Alfalfa haylage[2]	12.0
Soybean seeds, whole heated[3]	8.7
Canola meal[4]	8.6
Corn grain, ground	6.0
Candy by-product meal[5]	6.5
SoyPLUS[6]	5.0
Cottonseed, hulls	3.2
Molasses[7]	3.5
Vitamin and mineral premix[8]	3.0
Composition, % of DM (or as indicated)	
CP[9]	16.1
RDP[10]	9.8
RUP[10]	6.9
NDF[9]	30.9
ADF[9]	23.2
NE_L, Mcal/kg[9]	1.69
Ca[9]	1.02
P[9]	0.39
NFC[10]	45.2

[1]Corn silage was 46.5% DM and contained (DM basis) 6.7% CP, 32.4% NDF, and 42.7% starch.

[2]Alfalfa haylage was 90.7% DM and contained (DM basis) 21.0% CP, and 43.7% NDF.

[3]Soybean seeds contained (DM basis) 40.0% CP.

[4]Canola meal contained (DM basis) 40.9% CP.

[5]Candy by-product meal (Graybill Processing, Elizabethtown, PA) contained (DM basis) 16.9% CP and 26.7% NDF.

[6]SoyPLUS (West Central Cooperative, Ralston, IA) contained (DM basis) 47.2% CP.

[7]Molasses (Westway Feed Products, Tomball, TX) contained (DM basis) 3.9% CP and 66% total sugar.

[8]The premix (Cargill Animal Nutrition, Cargill Inc., Roaring Spring, PA) contained (%, as-is basis) trace mineral mix, 0.86; MgO (56% Mg), 8.0; NaCl, 6.4; vitamin ADE premix (Cargill Animal Nutrition, Cargill Inc.), 0.48; limestone, 37.2; selenium premix (Cargill Animal Nutrition, Cargill Inc.), 0.07; and dry corn distillers grains with solubles; 46.7. Ca, 14.1%; P, 0.39%; Mg, 4.59%; K, 0.44%; S, 0.39%; Se, 6.91 mg/kg; Cu, 362 mg/kg; Zn, 1,085 mg/kg; Fe, 186 mg/kg, vitamin A, 276,717 IU/kg; vitamin D, 75,000 IU/kg; and vitamin E, 1,983 IU/kg.

[9]Values were calculated using the chemical analysis (Cumberland Valley Analytical Services Inc., Maugansville, MD) of the ingredients of the diet.

[10]Estimated by NRC (2001).

Separate spot rumen gas samples were collected during experimental period 2 through the rumen cannula using a sampling device described in Tekippe et al. (2011). Samples were collected once on d 13, between 3 and 4 h after feeding, in 250-mL Kimax glass serum bottles (Kimble Chase, Vineland, NJ) sealed with blue chlorobutyl stoppers (Bellco, Vineland, NJ) and stored at room temperature until analyzed for isotopic composition of CH_4 (Ono et al., 2014). Methane was extracted from rumen gas samples using preparatory gas chromatography system equipped with a packed column (Carboxen-1000, 152 cm length, 3.2 mm o.d.) at 30°C (Wang et al., 2015). Isotopologue ratios of purified CH_4 samples were measured using tunable infrared direct absorption spectroscopy. Tunable infrared direct absorption spectroscopy measures isotopologue specific absorption in the mid-infrared (~8.5 μm wavelength) region (corresponding C-H and C-D bending vibrations) using multipass (76 m pathlength) direct absorption cell and continuous wavelength quantum cascade lasers (Ono et al., 2014).

Isotope ratios are reported in conventional delta notations:

$$\delta^{13}\mathrm{C} = \frac{\left(^{13}\mathrm{C} \div {}^{12}\mathrm{C}\right)_{\mathrm{sample}}}{\left(^{13}\mathrm{C} \div {}^{12}\mathrm{C}\right)_{\mathrm{PDB}}} - 1,$$

$$\delta\mathrm{D} = \frac{\left(\mathrm{D} \div \mathrm{H}\right)_{\mathrm{sample}}}{\left(\mathrm{D} \div \mathrm{H}\right)_{\mathrm{SMOW}}} - 1,$$

where PDB and SMOW refer to the reference ratios, Pee Dee Belemnite and Standard Mean Ocean Water, respectively. The clumped isotopologue abundance is reported as $\Delta^{13}CH_3D$, which represents the deviation from a random (stochastic) distribution (Ono et al., 2014):

$$\Delta^{13}\mathrm{CH_3D} = [^{13}\mathrm{CH_3D}][^{12}\mathrm{CH_4}] \div [^{13}\mathrm{CH_4}][^{12}\mathrm{CH_3D}] - 1.$$

At the time of gas collection, a sample of rumen fluid was collected from the ventral rumen, filtered through 4 layers of cheesecloth, transferred into 20-mL scintillation vials (Fisher Scientific, Pittsburgh, PA), and stored

at 4°C until analyzed for dissolved H_2 (Vaportech Services, Valencia, PA) according to Chapelle et al. (1997).

Rumen fluid and whole rumen contents samples were collected (as described in Hristov et al., 2011) on d 14 of each experimental period at 0 (before feeding), 2, 4, 6, and 8 h after feeding. Rumen fluid pH was analyzed immediately (pH meter 59000–60 pH Tester, Cole-Parmer Instrument Company, Vernon Hills, IL) and samples were further processed for analyses of ammonia and VFA (Hristov et al., 2011). Aliquots of the whole rumen contents were composited on an equal wet weight basis per cow and period and frozen immediately at −80°C for analysis of bacterial and archaeal order and genus distribution according to Oh et al. (2015). Briefly, DNA was extracted from 300 mg of material using the MO BIO Powersoil kit (MO BIO Laboratories Inc., Carlsbad, CA) following the manufacturer's instructions. The 16S rRNA gene standard V4 variable region PCR primers 515/806 were used in a single-step 30-cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen Inc., Valencia, CA) under the following conditions: 94°C for 3 min, followed by 28 cycles (5 cycles used on PCR products) of 94°C for 30 s, 53°C for 40 s, and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX) on an Ion Torrent PGM (Thermo Fisher Scientific Inc., Waltham, MA) following the manufacturer's guidelines. Bacterial quantification was performed using quantitative PCR based on a universal primer, as indicated above. A linear standard was used to calculate the estimated cell counts. Sequence data were processed using a standard taxonomic analysis pipeline (MR DNA). In short, sequences were depleted of barcodes and primers, then sequences <150 bp were removed and sequences with ambiguous base calls and with homopolymer runs exceeding 6 bp were also removed. Sequences were denoised and chimeras removed. Operational taxonomic units were defined by clustering at 3% divergence (97% similarity). Final operational taxonomic units were taxonomically classified using BLASTn against a database derived from RDPII (http://rdp.cme.msu.edu) and NCBI (www.ncbi.nlm.nih.gov).

Milk samples were collected at 2 consecutive milkings (evening and morning) in the last day of each experimental period. Samples were preserved with 2-bromo-2-nitropropane-1,3-diol and submitted for analysis of milk fat, true protein, lactose, and MUN (Dairy One, Ithaca, NY) using infrared spectroscopy (MilkoScan 4000, Foss Electric, Hillerød, Denmark). Morning and evening samples were analyzed separately so milk component concentration and yield could be weighed for morning and evening milk yields.

Statistical Analysis

All data were analyzed using the MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). The statistical model included treatment, experimental period, and treatment × period interaction. Square and cow within square were random effects and all others were fixed. Milk yield and DMI data for the last 4 d of each experimental period were averaged and the average values were used in the statistical analysis. Data for one cow in period 2 (control) were removed from the analysis due to low milk production (64% of her milk production in period 1 and 43% of the average milk production for all cows in period 2). Rumen gas emissions and fermentation data (except dissolved H_2) were analyzed as repeated measures. The model included treatment, experimental period, time of sampling, and treatment × period and treatment × time of sampling interactions. Means were separated by pairwise *t*-test (pdiff option of PROC MIXED). Statistical differences were considered significant at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$. Data in tables are presented as least squares means.

RESULTS AND DISCUSSION

Carbon dioxide emission was not affected by treatment, although we noted a numerical increase ($P = 0.15$) by about 4% for 3NOP (Table 2). Methane emission was decreased by 31% ($P < 0.001$), whereas H_2 emission increased substantially ($P < 0.001$) for 3NOP compared with the control. When expressed per unit of DMI or milk yield, CH_4 emission was, on average, about 34% lower ($P < 0.001$) for 3NOP compared with the control. These results are in agreement with our data from a larger production experiment where 3NOP decreased CH_4 emission in dairy cows by an average of 29% (Hristov et al., 2015a). Similar results, although of a different magnitude, were also reported for 3NOP by Reynolds et al. (2014) and Haisan et al. (2014). Reynolds et al. (2014) observed a reduction of 7 and 10% with the supplementation of 500 and 2,500 mg/d of 3NOP, respectively, when 3NOP was dosed directly into the rumen of lactating dairy cows fed diets containing 60% forage. Haisan et al. (2014) reported a 64% reduction in CH_4 emission in midlactating dairy cows supplemented with 2,500 mg/d of 3NOP top-dressed and fed a diet containing 38% forage. Studies testing 3NOP in beef cattle have also shown significant reduction in enteric CH_4 emission. In a short-term experiment with beef heifers, Romero-Pérez et al. (2014) reported CH_4 production was linearly decreased by 4, 13, and 38%, for heifers receiving 0.75, 2.25, and 4.5

mg of 3NOP/kg of BW (or 412, 1,235, and 2,470 mg/head per day), respectively. In a more recent long-term study (146 d) by the same group, 3NOP consistently decreased CH_4 emission by 59% through the experiment (Romero-Pérez et al., 2015a). It has been reported that method of delivery of 3NOP (direct rumen administration, top-dressed, or mixed with the diet) and animal type (beef, dairy, sheep) can influence CH_4 emission response (Romero-Pérez et al., 2014). Haisan et al. (2014) suggested that CH_4 emission response to 3NOP may be diet-dependent, with enhanced mitigation potential when cows are fed low-forage diets.

Methanogenesis is the primary pathway to remove excess of H_2 in the rumen, whereas propionate production is a competing alternative H_2 sink (Hungate, 1967; Moss et al., 2000). The increase in H_2 emissions by 3NOP was large, from undetectable in the control to 1.3 g/d, and similar to the results from our larger experiment (Hristov et al., 2015a). As pointed out by Hristov et al. (2015a), H_2 emitted from the rumen was only a fraction of the estimated H_2 available from the decrease in CH_4 production due to 3NOP. Increased concentration of dissolved H_2 (see following discussion), possible adaptation of the rumen ecosystem, decreased H_2 production, or redirection to alternative H_2 sinks (Ungerfeld, 2015) may be possible mechanisms to explain the fate of the excess H_2 resulting from 3NOP application.

Treatment had no effect on rumen pH, total VFA, propionate, isobutyrate, and valerate concentrations (Table 3). There was no treatment × time of sampling interaction ($P \geq 0.41$) for the fermentation variables, except for isobutyrate and isovalerate concentrations ($P = 0.01$). Examination of the data, however, showed no apparent trends in the concentration of isobutyrate across sampling points and the concentration of isovalerate was higher for 3NOP than the control for all sampling times. Supplementation of 3NOP tended to decrease ($P = 0.08$) the concentration of acetate and to increase ($P = 0.08$) that of butyrate. The concentration of isovalerate was increased ($P < 0.01$), and acetate-to-propionate ratio was decreased ($P < 0.01$) by 3NOP. The molar proportion of acetate was decreased ($P < 0.001$) and those of propionate, butyrate, valerate, and isovalerate were increased ($P \leq 0.005$) by 3NOP compared with the control. Concentration of ammonia in ruminal fluid was decreased ($P < 0.02$) and that of dissolved H_2 appeared to be numerically increased ($P = 0.27$) by 3NOP. Methanogen and bacterial cell counts in whole ruminal contents were not different ($P \geq 0.19$) between treatments but as proportion of the total counts, bacteria tended to be increased ($P = 0.10$) and methanogens to be decreased ($P = 0.07$) by 3NOP.

Table 2. Effect of 3-nitrooxypropanol (3NOP) on carbon dioxide (CO_2), methane (CH_4), and hydrogen (H_2) emissions in lactating dairy cows

Item	Treatment[1]		SEM[2]	*P*-value
	CON	3NOP		
CO_2, g/d	14,303	14,905	623.5	0.15
CH_4, g/d	487	335	40.1	<0.001
CH_4, g/kg of DMI[3]	20.7	13.6	1.40	<0.001
CH_4, g/kg of milk[3]	18.0	11.9	2.35	<0.001
H_2, g/d	0.0[4]	1.3	0.13	<0.001

[1]Treatments were control (CON), and 60 mg of 3NOP/kg of feed DM (3NOP).

[2]Largest SEM published in table; n = 61 for all variables (n represents the number of observations used in the statistical analysis).

[3]Based on milk yield and DMI data during the gas measurement periods.

[4]0.005 g/d.

Similar to our results, both Haisan et al. (2014) and Reynolds et al. (2014) observed a decrease in acetate concentration and acetate-to-propionate ratio when cows were supplemented with 3NOP. Reynolds et al. (2014) observed also increased propionate concentration and a tendency for decreased rumen pH when cows were fed 500 mg of 3NOP/d and reported increased butyrate concentration with the 2,500-mg of 3NOP/d dose. In contrast with our data, however, Reynolds et al. (2014) reported decreased isovalerate concentration, but this pattern was not observed in beef steers (Romero-Pérez et al., 2014, 2015a). Propionate production is a competitive mechanism to remove excess H_2 in the rumen (Ellis et al., 2008; McAllister and Newbold, 2008). Considering that 3NOP decreased CH_4 emission and the incomplete recovery of reducing equivalents in emitted H_2, the observed increase in molar proportion of propionate and the decrease in acetate in this study was expected. A decrease in rumen ammonia concentration with 3NOP was also reported by Reynolds et al. (2014) for dairy cows, but not in the experiments with beef cattle by Romero-Pérez et al. (2014, 2015a). Decreased ammonia concentration in the rumen may be an indication of decreased proteolysis or increased ammonia uptake by the rumen bacteria. Urinary N excretion and MUN concentration (similar to the current data) were not affected by 3NOP in our larger experiment (unpublished data from Hristov et al., 2015a).

The dissolved H_2 data were extremely variable (Table 3) in this experiment and, similar to the isotope data (discussed below), were obtained during experimental period 2 only. Concentration of dissolved H_2 in rumen fluid did not differ ($P = 0.27$) between control and 3NOP. Hydrogen is an important intermediate in CH_4 formation in the rumen and concentration of dissolved

Table 3. Effect of 3-nitrooxypropanol (3NOP) on rumen fluid pH, VFA, ammonia, and dissolved hydrogen concentrations, and rumen bacteria and methanogens in lactating dairy cows

Item	Treatment[1]		SEM[2]	*P*-value[3]
	CON	3NOP		
pH	6.35	6.41	0.082	0.66
Total VFA, mmol/L	90.0	85.8	2.96	0.43
Acetate	59.1	52.9	2.18	0.08
Propionate	17.4	17.4	1.30	0.99
Butyrate	10.1	11.3	0.43	0.08
Isobutyrate	0.51	0.49	0.049	0.51
Valerate	1.91	1.96	0.073	0.66
Isovalerate	1.01	1.76	0.147	<0.01
Acetate-to-propionate ratio	3.51	3.12	0.277	<0.01
Molar proportions, %				
Acetate	65.7	61.7	1.24	<0.001
Propionate	19.3	20.3	1.38	0.03
Butyrate	11.1	13.1	0.33	<0.001
Isobutyrate	0.57	0.57	0.054	0.95
Valerate	2.12	2.29	0.071	0.005
Isovalerate	1.12	2.05	0.157	<0.001
Ammonia, mmol/L	2.93	1.94	0.404	0.02
Dissolved hydrogen, mg/L	0.2	1.2	0.55	0.27
Bacteria				
ECC,[4] $\times 10^6$/g of ruminal contents	10.6	11.5	0.78	0.45
% of total counts	98.8	99.2	0.19	0.10
Methanogens				
ECC, $\times 10^6$/g of ruminal contents	0.13	0.09	0.018	0.19
% of total counts	1.16	0.77	0.18	0.07

[1]Treatments were control (CON), and 60 mg of 3NOP/kg of feed DM (3NOP).

[2]Largest SEM published in table; n = 59 for all variables, except dissolved hydrogen, for which n = 6 (n represents the number of observations used in the statistical analysis).

[3]Treatment × time of sampling interaction, $P \geq 0.41$, except isobutyrate and isovalerate ($P = 0.01$).

[4]Estimated cell counts.

H_2 has been suggested as limiting methanogenesis (Hungate, 1967). Due to its rapid metabolism, however, H_2 does not accumulate in the rumen liquid phase. Robinson et al. (1981), for example, observed a rapid increase in dissolved H_2 concentration in the rumen of a cow 1 h after feeding, reaching 15 μmol/L, and then declining to about 3 μmol/L at 4 h after feeding. Similar dissolved H_2 concentration pattern after feeding was reported earlier by Czerkawski and Breckenridge (1971). Hegarty and Gerdes (1999) estimated that less than 0.1% of the H_2 in the rumen of a sheep was H_2 gas, with 92% being in water, 6% in feed, and 2% in the rumen microbes. Hydrogen balance in the rumen is highly dynamic and the low dissolved H_2 concentrations observed in the current experiment are in line with other published data. Although dissolved H_2 concentrations correlate poorly to H_2 concentration in the rumen gas space (Hegarty and Gerdes, 1999; Wang et al., 2014), the increased H_2 emission and the numerical trend for increased dissolved H_2 in the current experiment suggest increased H_2 accumulation in the rumen of the 3NOP-treated cows. This increase was accompanied with a shift in relative VFA concentrations, as would be expected based on rumen stoichiometry (Wolin, 1960; Hegarty and Gerdes, 1999). In a recent meta-analysis of in vitro data, rumen stoichiometry could not completely explain H_2 balance when methanogenesis was inhibited, and it was suggested that H_2 may have been increasingly incorporated into formate, microbial biomass, or reductive acetogenesis (Ungerfeld, 2015).

There was no difference in δD_{CH4} and $\Delta^{13}CH_3D$, but a trend for decreased ($P = 0.08$) $\delta^{13}C_{CH4}$ was observed for 3NOP compared with the control (Table 4). Isotope compositions of CH_4, similar to other fractionation processes in nature, reflect the source substrate as well as isotope fractionation associated with microbial methanogenesis. As an example, ammonia N volatilized from manure is highly depleted in the heavier isotope of N, ^{15}N, and the resulting manure becomes increasingly enriched in ^{15}N as ammonia is being emitted. These relationships were used to model and predict ammonia losses from manure (Hristov et al., 2006, 2009). Similarly, microbial CH_4 formation produces CH_4 that is relatively depleted in ^{13}C and D compared with source CO_2 (or acetate) and water, therefore more negative $\delta^{13}C_{CH4}$ and δD_{CH4} values (Whiticar, 1999). The source of carbon and H_2, metabolic substrate, and methanogenesis pathway influence the composition of conven-

tional isotopes of CH_4 (Valentine et al., 2004; Conrad, 2005). Moreover, growth phase and availability of environmental H_2 may affect the isotopologue compositions of CH_4 (Burke, 1993; Valentine et al., 2004; Wang et al., 2015). Therefore, in the current study we hypothesized that if the rumen archaeal population shifts due to effects associated with the addition of 3NOP, such as the inhibition of methyl coenzyme-M reductase, we may see a shift in the isotopic composition of CH_4. According to Vogel (1980), $\delta^{13}C_{CH4}$ of enteric CH_4 is strongly dependent on $\delta^{13}C$ of the feeds in the diet. Rust (1981) evaluated the isotopic composition of CH_4 in steers, dairy cows, and wethers fed diets that were mostly based on C_3 (alfalfa, soybean, and temperate grass species) or C_4 (corn-based diets) plants. That study reported that $\delta^{13}C_{CH4}$ of ruminants fed C_3-based diets averaged −63.7%, whereas animals fed C_4-based diets averaged −50.3%. Levin et al. (1993) investigated the isotopic composition of enteric CH_4 and different sources of dairy wastes (liquid manure, biogas) and reported $\delta^{13}C_{CH4}$ values for enteric CH_4 of −65.1% for cows fed 100% C_3 diets and −55.6% when cows were fed 60 to 80% C_4 diets. These differences are explained by the carbon isotope compositions of feeds, as C_3 plants produces larger $^{13}C/^{12}C$ fractionations compared with C_4 plants (e.g., Farquhar et al., 1989).

In a recent work by Wang et al. (2015), the $\delta^{13}C_{CH4}$ of rumen CH_4 from dairy cows (of the Pennsylvania State University herd) ranged from −52 to −54%. The $\delta^{13}C_{CH4}$ values reported in our study for the control group (average of −54%) are similar to those reported in Wang et al. (2015). The $\delta^{13}C_{CH4}$ values for the 3NOP group were slightly (4%) more depleted in ^{13}C. Although $\delta^{13}C$ value of the feeds were not measured, the same feeds were used for both 3NOP and control. Given the range observed by Vogel (1980) of 13.4% for source variation, the observed shift $\delta^{13}C_{CH4}$ of 4% would require major changes in feed composition. Therefore, the change in $\delta^{13}C_{CH4}$ value likely reflects the change in physiology of methanogens and is consistent with low methanogenesis rate under 3NOP treatment, as large ^{13}C-depletion is associated with low methanogenesis rates under stationary phase cultures (Botz et al., 1996; Zyakun, 1996).

Although it was not sampled at the same time, previous analysis of water in rumen yielded δD value of −32% (supplementary material, Table S4 of Wang et al., 2015). Although it was hypothesized that environmental H_2 levels affect δD_{CH4} and $\Delta^{13}CH_3D$ values (Burke, 1993; Wang et al., 2015), we did not observe changes in the values of δD_{CH4} and $\Delta^{13}CH_3D$.

Genus distribution of methanogenic archaea (*Methanobrevibacter*, *Methanosphaera*, and *Methanomicrobium* spp.) in whole ruminal contents was not affected ($P > 0.44$) by 3NOP (Table 5). *Methanobrevibacter* spp. was the dominant archaea genus, which was in agreement with methanogens distribution in the rumen of lactating dairy cows fed TMR diets (Whitford et al., 2001). Similar to our data, both Haisan et al. (2014) and Romero-Pérez et al. (2015a) reported a decreased number of methanogens when dairy or beef cattle were treated with 3NOP. In the current study, the predominant bacterial orders were *Clostridiales* and *Bacteroidales. Clostridiales* tended to be decreased ($P = 0.09$), whereas both *Selenomonadales* and *Fibrobacterales* were increased ($P = 0.04$, and 0.02, respectively) by 3NOP. *Prevotella* spp., the predominant bacterial genus in ruminal contents, was not affected by 3NOP. *Ruminococcus*, *Succiniclasticum, Clostridium*, and *Sarcina* spp. were decreased ($P \leq 0.03$), whereas *Butyrivibrio* and *Fibrobacter* spp. were increased ($P = 0.01$) by 3NOP compared with the control. Data on the effect of 3NOP on rumen microbial diversity are generally lacking or are inconsistent. Haisan et al. (2014), for example, reported a trend for decreased total bacterial 16S rDNA gene copy numbers and no effect on protozoal (18S rRNA) copy numbers in ruminal contents from 3NOP-treated cows. Romero-Pérez et al. (2015a) observed no effect of 3NOP on bacterial gene copy numbers but a large increase in protozoal copy numbers in beef cattle, whereas in another study from the same group, no effect was observed on both bacterial and protozoal copy numbers (Romero-Pérez et al., 2014). In yet another study by Romero-Pérez et al. (2015b) using the rumen stimulation technique RUSITEC, the total copy number of 16S rRNA genes for methanogens and bacteria in the liquid phase were not affected by 3NOP, but there was a large decrease in the solid associated methanogens. Likewise, no effect of 3NOP on rumen protozoal counts

Table 4. Effect of 3-nitrooxypropanol (3NOP) on isotopic composition of enteric methane in lactating dairy cows

Item	Treatment[1]		SEM[2]	*P*-value
	CON	3NOP		
$\delta^{13}C_{CH4}$, %	−54.5	−58.3	1.14	0.08
δD_{CH4}, %	−347.4	−347.4	1.79	0.99
$\Delta^{13}CH_3D$, %	1.66	1.41	1.063[3]	0.87

[1]Treatments were control (CON), and 60 mg of 3NOP/kg of feed DM (3NOP).

[2]Largest SEM published in table; n = 6 for all variables (n represents the number of observations used in the statistical analysis). Values for $\delta^{13}C$, δD, and $\Delta^{13}CH_3D$, where D is deuterium, are reported relative to Pee Dee Belemnite (PDB), Standard Mean Ocean Water (SMOW), and the stochastic distribution, respectively as described in Wang et al. (2015).

[3]Large SEM caused by small sample size of 2 of the 3NOP samples.

Table 5. Effect of 3-nitrooxypropanol (3NOP) on bacterial and archaeal order and genus composition (as % of total sequence reads[1]) in whole ruminal contents of lactating dairy cows

Item	Treatment[2]		SEM[3]	*P*-value
	CON	3NOP		
Archaeal genus				
Methanobrevibacter	94.9	95.0	1.03	0.87
Methanosphaera	3.8	3.8	1.01	0.98
Methanomicrobium	1.2	0.8	0.39	0.44
Bacterial order				
Clostridiales	48.8	43.5	2.76	0.09
Bacteroidales	32.6	34.9	1.84	0.32
Selenomonadales	7.1	8.7	1.41	0.04
Fibrobacterales	1.3	2.5	0.32	0.02
Bifidobacteriales	1.8	1.8	0.52	0.86
Lactobacillales	1.2	1.7	0.29	0.25
Rhodocyclales	1.1	1.1	0.09	0.85
Actinomycetales	0.9	1.2	0.16	0.10
Bacterial genus				
Prevotella	22.0	23.2	1.62	0.55
Ruminococcus	8.2	6.5	0.62	<0.01
Succiniclasticum	8.1	6.6	1.34	0.03
Blautia	5.1	5.4	0.34	0.45
Clostridium	6.2	4.1	0.49	0.03
Butyrivibrio	3.6	4.8	0.33	0.01
Barnesiella	3.8	4.0	0.29	0.31
Pseudobutyrivibrio	3.2	3.1	0.18	0.57
Acetitomaculum	3.5	2.6	0.39	0.05
Alistipes	2.6	3.3	0.39	0.31
Coprococcus	2.8	2.4	0.37	0.28
Dorea	2.3	1.9	0.37	0.44
Sarcina	2.6	1.5	0.24	0.02
Fibrobacter	1.3	2.5	0.32	0.02
Bacteroides	1.8	1.9	0.12	0.45
Saccharofermentans	1.9	1.7	0.34	0.47
Bifidobacterium	1.7	1.8	0.56	0.96
Flavonifractor	1.6	1.8	0.19	0.35
Xylanibacter	1.1	1.2	0.13	0.56
Roseburia	1.1	1.2	0.13	0.64
Azospira	1.2	1.1	0.09	0.85
Streptococcus	0.9	1.1	0.11	0.38

[1]The percentage represents the percentage of the total sequences analyzed within the sample.

[2]Treatments were control (CON), and 60 mg of 3NOP/kg of feed DM (3NOP).

[3]Largest SEM published in table; n = 12 for all variables (n represents the number of observations used in the statistical analysis).

Table 6. Effect of 3-nitrooxypropanol (3NOP) on DMI, milk production, and feed efficiency in lactating dairy cows

Item	Treatment[1]		SEM[2]	*P*-value
	CON	3NOP		
DMI, kg/d	24.1	24.8	0.88	0.37
Milk yield, kg/d	28.2	30.3	2.63	0.27
ECM,[3] kg/d	32.9	32.0	2.57	0.87
Feed efficiency,[4] kg/kg	1.18	1.22	0.102	0.53
Milk fat, %	4.05	4.35	0.27	0.05
Milk fat, kg/d	1.13	1.32	0.10	0.08
Milk protein, %	3.56	3.58	0.13	0.80
Milk protein kg/d	1.02	1.10	0.10	0.40
Milk lactose, %	4.74	4.73	0.12	0.80
Milk lactose, kg/d	1.33	1.46	0.15	0.17
MUN, mg/dL	11.7	11.1	0.77	0.29

[1]Treatments were control (CON), and 60 mg of 3NOP/kg of feed DM (3NOP).

[2]Largest SEM published in table; n = 11 for all other variables (n represents the number of observations used in the statistical analysis). Data for one cow in period 2 (control) were removed from the analysis due to low milk production (see Materials and Methods).

[3]Energy-corrected milk calculated as ECM (kg) = milk production (kg) × (383 × fat % + 242 × protein % + 165.4 × lactose % + 20.7) ÷ 3,140 (Sjaunja et al., 1990).

[4]Milk yield ÷ DMI.

was noted in that study. No effect of 3NOP on bacterial and protozoal (as well as methanogenic archaea) gene copy numbers was also reported by Martínez-Fernández et al. (2014) in sheep. The distinct decrease in fibrolytic bacteria such as *Ruminococcus* spp. in the current study is in line with the decreased acetate concentration (and molar proportion) in ruminal fluid with 3NOP. Similarly, the increase in *Butyrivibrio* spp. is in agreement with the trend for increased butyrate concertation and molar proportion with 3NOP. The increase in *Selenomonadales*, an order that includes major propionate-producing bacteria such as *Selenomonas ruminantium* (Paynter and Elsden, 1970), is in agreement with the increased relative proportion of propionate in ruminal VFA. The prevalence of specific genera within this order, however (e.g., *Succiniclasticum* spp., bacteria converting succinate to propionate; van Gylswyk, 1995), was lowered by 3NOP. Some rumen clostridia have strong proteolytic and deaminative (Paster et al., 1993; Krause and Russell, 1996) activities (McSweeney et al., 1999), and the decrease in *Clostridium* spp. with 3NOP in the current experiment is in line with the observed decrease in rumen ammonia concentration.

Dry matter intake and milk yield were not different between 3NOP and the control (Table 6). Energy-corrected milk yield and feed efficiency were also not affected by treatment. Milk fat concentration was increased ($P = 0.05$) and milk fat yield tended to be increased ($P = 0.08$) by 3NOP compared with the control. A numerical increase in milk fat concentration with 3NOP was also reported by Haisan et al. (2014) and may be reflective of increased feed energy availability due to decreased CH_4 production. Milk protein and lactose concentrations and yields and MUN were not affected by treatment in the current experiment. The lack of effect of 3NOP on milk yield in the current experiment is in agreement with data from our larger production experiment (Hristov et al., 2015a) and other published studies (Haisan et al., 2014; Reynolds et al., 2014). Production data from the current experiment, however, should be interpreted with caution due to the low number of experimental units and the advanced DIM of the cows.

CONCLUSIONS

The CH_4 inhibitor tested in this experiment, 3-nitrooxypropanol, decreased enteric CH_4 emission by 31% and decreased acetate-to-propionate ratio, increased molar proportions of propionate and butyrate, and decreased ammonia concentration in ruminal fluid of lactating dairy cows. The inhibitor had no effect on rumen archaea composition, but tended to decrease the proportion of methanogen cell counts in whole ruminal contents. In line with the lack of effect on rumen archaeal genus distribution, the isotopic composition of CH_4 was similar between treatments.

ACKNOWLEDGMENTS

The authors thank the staff of the Department of Animal Science, Pennsylvania State University's Dairy Center for their conscientious care of the experimental cows. The study was partially funded by DSM Nutritional Products. Danielle Sarah Gruen was supported by Royal Dutch Shell (The Hague, the Netherlands)-Massachusetts Institute of Technology Energy Initiative graduate fellowship. J. C. Lopes was supported by CAPES Foundation, Ministry of Education of Brazil (Brasília, Brazil).

REFERENCES

Abecia, L., P. G. Toral, A. I. Martín-García, G. Martínez, N. W. Tomkins, E. Molina-Alcaide, C. J. Newbold, and D. R. Yáñez-Ruiz. 2012. Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. J. Dairy Sci. 95:2027–2036.

Boadi, D., C. Benchaar, and J. Chiquette. 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: Update review. Can. J. Anim. Sci. 84:319–335.

Botz, R., H.-D. Pokojski, M. Schmitt, and M. Thomm. 1996. Carbon isotope fractionation during bacterial methanogenesis by CO_2 reduction. Org. Geochem. 25:255–262.

Burke, R. A. 1993. Possible influence of hydrogen concentration on microbial methane stable hydrogen isotopic composition. Chemosphere 26:55–67.

Chapelle, F. H., D. A. Vroblesky, J. C. Woodward, and D. R. Lovely. 1997. Practical considerations for measuring hydrogen concentrations in groundwater. Environ. Sci. Technol. 31:2873–2877.

Conrad, R. 2005. Quantification of methanogenic pathways using stable carbon isotopic signatures: A review and a proposal. Org. Geochem. 36:739–752.

CVAS (Cumberland Valley Analytical Services). 2014. Resources–Lab Procedures. Accessed Nov. 23, 2015. http://www.foragelab.com/Resources/Lab-Procedures/.

Czerkawski, J. W., and G. Breckenridge. 1971. Determination of concentration of hydrogen and some other gases dissolved in biological fluids. Lab. Pract. 20:403–405., 413.

Duval, S., and M. Kindermann. 2012. Use of nitrooxy organic molecules in feed for reducing enteric methane emissions in ruminants, and/or to improve ruminant performance. World Intellectual Property Organization. International Patent Application WO 2012/084629 A1.

Ellis, J. L., J. Dijkstra, E. Kebread, A. Bannink, N. E. Odongo, B. W. McBride, and J. France. 2008. Aspects of rumen microbiology central to mechanistic modelling of methane production in cattle. J. Agric. Sci. 146:213–233.

Ermler, U., W. Grabarse, S. Shima, M. Goubeaud, and R. K. Thauer. 1997. Crystal structure of methyl-coenzyme M reductase: The key enzyme of biological methane formation. Science 278:1457–1462.

Farquhar, G. D., J. R. Ehleringer, and K. T. Hubick. 1989. Carbon isotope discrimination and photosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40:503–537.

Goel, G., H. P. S. Makkar, and K. Becker. 2009. Inhibition of methanogens by bromochloromethane: Effects on microbial communities and rumen fermentation using batch and continuous fermentations. Br. J. Nutr. 101:1484–1492.

Haisan, J., Y. Sun, L. L. Guan, K. A. Beauchemin, A. Iwaasa, S. Duval, D. R. Barreda, and M. Oba. 2014. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. J. Dairy Sci. 97:3110–3119.

Hegarty, R. S., and R. Gerdes. 1999. Hydrogen production and transfer in the rumen. Rec. Adv. Anim. Nutr. Austr. 12:37–44.

Hristov, A. N., L. Campbell, and J. H. Harrison. 2006. Evolution of ^{15}N abundance in cattle manure in relation to cumulative ammonia losses. J. Dairy Sci. 89:357. (Abstr.).

Hristov, A. N., C. Lee, T. Cassidy, M. Long, K. Heyler, B. Corl, and R. Forster. 2011. Effects of lauric and myristic acids on ruminal fermentation, production, and milk fatty acid composition in lactating dairy cows. J. Dairy Sci. 94:382–395.

Hristov, A. N., J. Oh, F. Giallongo, T. Frederick, H. Weeks, P. R. Zimmerman, R. A. Hristova, S. R. Zimmerman, and A. F. Branco. 2015b. The use of an automated system (GreenFeed) to monitor enteric methane and carbon dioxide emissions from ruminant animals. J. Vis. Exp. 103:e52904.

Hristov, A. N., J. Oh, F. Giallongo, T. W. Frederick, M. T. Harper, H. L. Weeks, A. F. Branco, P. J. Moate, M. H. Deighton, S. R. O. Williams, M. Kindermann, and S. Duval. 2015a. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. Proc. Natl. Acad. Sci. USA 112:10663–10668.

Hristov, A. N., J. Oh, C. Lee, R. Meinen, F. Montes, T. Ott, J. Firkins, A. Rotz, C. Dell, A. Adesogan, W. Yang, J. Tricarico, E. Kebreab, G. Waghorn, J. Dijkstra, and S. Oosting. 2013. Mitigation of greenhouse gas emissions in livestock production—A review of technical options for non-CO2 emissions. P. J. Gerber, B. Henderson, and H. P. S. Makkar, ed. FAO Animal Production and Health Paper No. 177. FAO, Rome, Italy.

Hristov, A. N., S. Zaman, M. Vander Pol, L. Campbell, P. Ndegwa, and S. Silva. 2009. Nitrogen losses from dairy manure estimated through nitrogen mass balance or using markers. J. Environ. Qual. 38:2438–2448.

Hungate, R. E. 1966. The Rumen and its Microbes. Academic Press, New York, NY.

Hungate, R. E. 1967. Hydrogen as an intermediate in the rumen fermentation. Arch. Mikrobiol. 59:158–164.

IPCC. 2014. Climate change 2014: Impacts, adaptation, and vulnerability. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge Univ Press, Cambridge, UK.

Johnson, K. A., and D. E. Johnson. 1995. Methane emissions from cattle methane emissions from cattle. J. Anim. Sci. 73:2483–2492.

Krause, D. O., and J. B. Russell. 1996. An rRNA approach for assessing the role of obligate amino acid-fermenting bacteria in ruminal amino acid deamination. Appl. Environ. Microbiol. 62:815–821.

Levin, I., P. Bergamaschi, H. Dörr, and D. Trapp. 1993. Stable isotopic signature of methane from major sources in Germany. Chemosphere 26:161–177.

Martínez-Fernández, G., L. Abecia, A. Arco, G. Cantalapiedra-Hijar, A. I. Martín-García, E. Molina-Alcaide, M. Kindermann, S. Duval, and D. R. Yáñez-Ruiz. 2014. Effects of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol on ruminal fermentation, microbial abundance, and methane emissions in sheep. J. Dairy Sci. 97:3790–3799.

McAllister, T. A., and C. J. Newbold. 2008. Redirecting rumen fermentation to reduce methanogenesis. Aust. J. Exp. Agric. 48:7–13.

McSweeney, C. S., B. Palmer, R. Bunch, and D. O. Krause. 1999. Isolation and characterization of proteolytic ruminal bacteria from sheep and goats fed the tannin-containing shrub legume *Calliandra calothyrsus.* Appl. Environ. Microbiol. 65:3075–3083.

Moss, A. R., J.-P. Jouany, and J. Newbold. 2000. Methane production by ruminants: Its contribution to global warming. Ann. Zootech. 49:231–253.

NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Sci., Washington, DC.

Oh, J., F. Giallongo, T. Frederick, J. Pate, S. Walusimbi, R. J. Elias, E. H. Wall, D. Bravo, and A. N. Hristov. 2015. Effects of dietary *Capsicum oleoresin* on productivity and immune responses in lactating dairy cows. J. Dairy Sci. 98:6327–6339.

Ono, S., D. T. Wang, D. S. Gruen, B. Sherwood Lollar, M. S. Zahniser, B. J. McManus, and D. D. Nelson. 2014. Measurement of a doubly substituted methane isotopologue, $^{13}CH_3D$, by tunable infrared laser direct absorption spectroscopy. Anal. Chem. 86:6487–6494.

Paster, B. J., J. B. Russell, C. M. J. Yang, J. M. Chow, C. R. Woese, and R. Tanner. 1993. Phylogeny of ammonia-producing ruminal bacteria, P*eptostreptococcus anaerobius, Clostridium sticklandii*, and *Clostridium aminophilum* sp. nov. Int. J. Syst. Bacteriol. 43:107–110.

Paynter, M. J. B., and S. R. Elsden. 1970. Mechanism of propionate formation by *Selenomonas ruminantium*, a rumen micro-organism. J. Gen. Microbiol. 61:1–7.

Reynolds, C. K., D. J. Humphries, P. Kirton, M. Kindermann, S. Duval, and W. Steinberg. 2014. Effects of 3-nitrooxypropanol on methane emission, digestion, and energy and nitrogen balance of lactating dairy cows. J. Dairy Sci. 97:3777–3789.

Robinson, J. A., R. F. Strayer, and J. M. Tuedje. 1981. Method for measuring dissolved hydrogen in anaerobic ecosystems: Application to the rumen. Appl. Environ. Microbiol. 41:545–548.

Romero-Pérez, A., E. K. Okine, S. M. McGinn, L. L. Guan, M. Oba, S. M. Duval, M. Kindermann, and K. A. Beauchemin. 2014. The potential of 3-nitrooxypropanol to lower enteric methane emissions from beef cattle. J. Anim. Sci. 92:4682–4693.

Romero-Pérez, A., E. K. Okine, S. M. McGinn, L. L. Guan, M. Oba, S. M. Duval, M. Kindermann, and K. A. Beauchemin. 2015a. Sustained reduction in methane production from long-term addition of 3-nitrooxypropanol to a beef cattle diet. J. Anim. Sci. 93:1780–1791.

Romero-Pérez, A., E. K. Okine, L. L. Guan, S. M. Duval, M. Kindermannd, and K. A. Beauchemina. 2015b. Effects of 3-nitrooxypropanol on methane production usingthe rumen simulation technique (Rusitec). Anim. Feed Sci. Technol. 209:98–109.

Rust, F. 1981. Ruminant methane delta($^{13}C/^{12}C$) values: Relation to atmospheric methane. Science 211:1044–1046.

Sawyer, M. S., W. H. Hoover, and C. J. Sniffen. 1974. Effects of a ruminal methane inhibitor on growth and energy metabolism in the ovine. J. Anim. Sci. 38:908–914.

Sjaunja, L. O., L. Baevre, L. Junkkarinen, J. Pedersen, and J. Setälä. 1990. A Nordic proposal for an energy corrected milk (ECM) formula. Pages 156–157 in 27th Session of the International Commission for Breeding and Productivity of Milk Animals, Paris, France. Wageningen Academic Publishers, Wageningen, the Netherlands.

Tekippe, J. A., A. N. Hristov, K. S. Heyler, T. W. Cassidy, V. D. Zheljazkov, J. F. S. Ferreira, S. K. Karnati, and G. A. Varga. 2011. Rumen fermentation and production effects of *Origanum vulgare* L. leaves in lactating dairy cows. J. Dairy Sci. 94:5065–5079.

Tomkins, N. W., S. M. Colegate, and R. A. Hunter. 2009. A bromochloromethane formulation reduces enteric methanogenesis in cattle fed grain-based diets. Anim. Prod. Sci. 49:1053–1058.

Ungerfeld, E. M. 2015. Shifts in metabolic hydrogen sinks in the methanogenesis inhibited ruminal fermentation: A meta-analysis. Front. Microbiol. 6:37.

Valentine, D. L., A. Chidthaisong, A. Rice, W. S. Reeburgh, and S. C. Tyler. 2004. Carbon and hydrogen isotope fractionation by moderately thermophilic methanogens. Geochim. Cosmochim. Acta 68:1571–1590.

van Gylswyk, N. O. 1995. *Succiniclasticum ruminis* gen. nov., sp. nov., a ruminal bacterium converting succinate to propionate as the sole energy-yielding mechanism. Int. J. Syst. Bacteriol. 45:297–300.

Vogel, J. C. 1980. Fractionation of the Carbon Isotopes During Photosynthesis. Springer, Berlin, Germany.

Wang, D. T., D. S. Gruen, B. S. Lollar, K.-U. Hinrichs, L. C. Stewart, J. F. Holden, A. N. Hristov, J. W. Pohlman, P. L. Morrill, M. Könneke, K. B. Delwiche, E. P. Reeves, C. N. Sutcliffe, D. J. Ritter, J. S. Seewald, J. C. McIntosh, H. F. Hemond, M. D. Kubo, D. Cardace, T. M. Hoehler, and S. Ono. 2015. Methane cycling. Nonequilibrium clumped isotope signals in microbial methane. Science 348:428–431.

Wang, M., X. Z. Sun, P. H. Janssen, S. X. Tang, and Z. L. Tan. 2014. Responses of methane production and fermentation pathways to the increased dissolved hydrogen concentrationgenerated by eight substrates in in vitro ruminal cultures. Anim. Feed Sci. Technol. 194:1–11.

Whitford, M. F., R. M. Teather, and R. J. Forster. 2001. Phylogenetic analysis of methanogens from the bovine rumen. BMC Microbiol. 1:5.

Whiticar, M. J. 1999. Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. Chem. Geol. 161:291–314.

Whiticar, M. J., E. Faber, and M. Schoell. 1986. Biogenic methane formation in marine and freshwater environments: CO_2 reduction vs. acetate fermentation-Isotope evidence. Geochim. Cosmochim. Acta 50:693–709.

Wolin, M. J. 1960. A theoretical rumen fermentation balance. J. Dairy Sci. 43:1452–1459.

Zyakun, A. M. 1996. Potential of $^{13}C/^{12}C$ variations in bacterial methane in assessing origin of environmental methane. Pages 341–352 in Hydrocarbon Migration and its Near-Surface Expression: AAPG Memoir 66D. D. Schumacher and M. A. Abrams, ed. The American Association of Petroleum Geologists, Tulsa, OK.