The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation

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ABSTRACT

The objective of the current study was to determine the effects of adding 3-nitrooxypropanol to the diet of lactating Holstein cows on methane emissions, rumen fermentation, ruminal microbial profile, and milk production. Twelve ruminally cannulated Holstein cows in midlactation were used in a crossover design study with 28-d periods. Cows were fed a diet containing 38% forage on a dry matter basis with either 2,500 mg/d of 3-nitrooxypropanol (fed as 25 g of 10% 3-nitrooxypropanol on silicon dioxide) or 25 g/d of silicon dioxide (control). After a 21-d diet adaptation period, dry matter intake (DMI) and milk yield were recorded daily. Rumen fluid and digesta were collected on d 22 and 28 for volatile fatty acid analysis and microbial profiling. Enteric methane emissions were measured on d 23 to 27 using the sulfur hexafluoride tracer gas technique. Feeding 3-nitrooxypropanol did not affect DMI; however, methane production was reduced from 17.8 to 7.18 g/kg of DMI. No change in milk or milk component yields was observed, but cows fed 3-nitrooxypropanol gained more body weight than control cows (1.06 vs. 0.39 kg/d). Concentrations of total volatile fatty acids in ruminal fluid were not affected by treatment, but a reduction in acetate proportion and a tendency for an increase in propionate proportion was noted. As such, a reduction in the acetate-to-propionate ratio was observed (2.02 vs. 2.36). Protozoa counts were not affected by treatment; however, a reduction in methanogen copy count number was observed when 3-nitrooxypropanol was fed (0.95 vs. 2.69×10^8 /g of rumen digesta). The data showed that feeding 3-nitrooxypropanol to lactating dairy cows at 2,500 mg/d can reduce methane emissions without compromising DMI or milk production.

Key words: 3-nitrooxypropanol, enteric methane emission, milk production, methanogen

INTRODUCTION

Demand exists to reduce greenhouse gas emissions generated by the agricultural sector, in particular, the livestock industry. In recent years, it has been estimated that cattle alone are responsible for 11 to 17% of the methane generated globally (Beauchemin et al., 2009a). As methane has a global warming potential 21 times that of carbon dioxide (United Nations, 2013), the environmental importance of emissions is self-evident. Another consideration is that between 2 and 12% of the ingested gross energy of cattle can be lost to methane (Johnson and Johnson, 1995), a loss of energy that could potentially be used by the animal. Enteric methane emissions from cattle can be reduced through dietary techniques such as improving forage quality, higher inclusion of concentrates in the diet and feeding lipids (Martin et al., 2010; Eckard et al., 2012). Additionally, previous research has shown that natural compounds, such as tannins and saponins, and synthetic dietary compounds, such as ionophores, can reduce methane emissions from ruminants through inhibition of methanogenesis or by shifting fermentation pathways to promote alternative hydrogen sinks, such as propionate production, thus reducing methane emissions (McAllister and Newbold, 2008; Martin et al., 2010).

Recently, several molecules, substituted at various positions with at least one nitrooxy group, were identified as potential inhibitors of enteric methanogenesis (Duval and Kindermann, 2012). One such compound, 3-nitrooxypropanol (**NOP**), was developed from predecessor compounds (ethyl 3-nitrooxy propionate; WO2011/070133 and 3-azido-propionic acid ethyl ester), which were identified from an *in vitro* rumen simulation screening assay (Soliva et al., 2011). The NOP exhibited a significantly higher potential to reduce methanogenesis *in vitro* than the well-known model compound bromoethanesulfonate (Soliva et al., 2011). Bromoethanesulfonate is a coenzyme M analog (Gunsalus et al., 1978) with very specific activity against methanogens that inhibits the reduction of

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methyl-coenzyme M to methane during the last step of methanogenesis (Immig, 1996).

Research using a rumen simulation technique showed that NOP is capable of reducing methane production (Romero Perez et al., 2013b). Furthermore, when NOP was directly dosed into the rumen of sheep (Martinez-Fernandez et al., 2013) and lactating dairy cows (Reynolds et al., 2013) or fed once daily to beef cattle (Romero Perez et al., 2013a), methane emissions were reduced and an increase in propionate concentration was observed, suggesting a shift in rumen fermentation. However, the effect of NOP on methanogen numbers or microbial profile was not reported or consistently demonstrated in those previous studies.

Although use of NOP is a promising approach to reduce enteric methane emissions from ruminants, further studies are required to confirm its efficacy in reducing methane emissions while evaluating its effects on rumen fermentation and animal productivity. It was hypothesized that lactating dairy cows fed NOP would have reduced methane emissions; hence, more energy would be available for milk production. The objectives of the present study were to determine the effects of NOP on methane emissions, animal performance, rumen fermentation, and rumen microbial profile of lactating dairy cows.

MATERIALS AND METHODS

All procedures were preapproved by the Animal Care and Use Committee for Livestock at the University of Alberta and conducted according to the guidelines of the Canadian Council of Animal Care (Ottawa, ON, Canada). The use of the biochemical compound [10% NOP on silicon dioxide (SiO_2)], developed by DSM Nutritional Products Ltd. (Kaiseraugst, Switzerland) in the diet of lactating dairy cows at 25 g/d was preapproved by the Veterinary Drugs Directorate Division (Health Canada, Ottawa, ON, Canada) for research. According to their instructions, milk was discarded for the duration of the study and an additional 14-d milk withdrawal period was implemented upon completion of the study.

Experimental Design, Diet, and Treatment

Twelve lactating Holstein cows with ruminal cannulas (Bar Diamond Inc., Parma, ID) were used in a crossover design study with 28-d periods consisting of 21 d of adaptation and 7 d of data and sample collection. Cows were separated into 2 groups based on the pre-experiment DIM (means $\pm$ SD: Group 1 = 100 $\pm$ 5.4, Group 2 = 76 $\pm$ 10.1). Group 1 had 4 multiparous and 4 primiparous cows and group 2 had 2 multiparous

and 2 primiparous cows, and they were randomly assigned to the treatment sequence. Pre-experiment BW (means $\pm$ SD) were 591.5 $\pm$ 58.9 and 567.5 $\pm$ 93.5 kg for groups 1 and 2, respectively. The study was conducted using 2 groups of cows to facilitate methane measurement; however, we did not have sufficient supplies to measure methane emission for all cows at once, thus the whole study protocol was staggered by 7 d between the 2 groups. Cows were housed individually in tiestalls and milked twice daily in their stalls at 0400 and 1600 h.

All cows were fed the same diet as a TMR (Table 1), *ad libitum*, once daily at 0900 h, allowing for 5% refusals throughout the study, and had free access to water. The diet was formulated to provide adequate ME and MP for a 650-kg cow producing 40 kg of milk per day (NRC, 2001) and to ensure ME and MP intake did not limit milk production of all cows. Cows were fed either SiO_2 as a control (**CON**) or NOP at 25 g/d, resulting in 0 and 2,500 mg/d of 3-nitrooxypropanol, respectively. Both NOP and SiO_2 , in powder form, were hand-mixed with 80 g of ground barley grain, 50 g of wet molasses, and 40 g of canola oil to improve adhesion to feed particles and palatability. Cows were assigned to either treatment on d 1 of each period without incremental adaptation. To avoid contamination of feeding equipment at the farm, each treatment mixture was applied by hand-mixing into the TMR once daily, within 30 min of feeding. This protocol also simulated an on-farm feeding scenario, allowing for consumption of NOP throughout the day as feed is consumed.

Data and Sample Collection

The amount of feed offered and refused was recorded for individual cows at the time of feeding, and the amount of TMR fed was adjusted daily to maintain 5% refusals. The NOP content in refusals was not determined. Dietary ingredients were sampled (approximately 500 g) on d 25 to 27 and composited for each period to determine the chemical composition of the diet. All samples were dried for 72 h at 55°C in a forced-air oven and stored at 4°C until further analysis. Additionally, diets were adjusted weekly to maintain the same concentrate-to-forage ratio on a DM basis. Body weight was measured at the beginning and end of each period. Milk yield was recorded at every milking, and milk samples (approximately 50 mL) were taken from 6 consecutive milkings from d 25 to 27 and stored at 4°C with 2-bromo-2-nitropropane-1,3-diol until milk composition analysis.

Rumen digesta were collected through a rumen cannula, from 5 locations in the rumen (cranial dorsal, cranial ventral, central rumen, caudal dorsal, and caudal

Table 1. Ingredients and nutrition composition of experimental diet

Item, % DM (unless otherwise noted)	Total
Ingredient	
Barley silage	37.9
Dry ground corn	31.4
Canola meal	11.3
Corn gluten meal	8.4
Beet pulp	6.2
Canola oil	1.4
Limestone	1.2
Salt	1.2
Calcium diphosphate	0.6
Magnesium oxide	0.3
Mineral and vitamin premix ¹	0.1
Nutrient composition	
DM, %	53.5
OM	90.8
CP	19.6
NDF	26.5
ADF	18.4
Starch	26.8
Ether extracts	5.0

¹Contained 17,413 KIU/kg of vitamin A, 1,714 KIU/kg of vitamin D₃, 57 KIU/kg of vitamin E, 579 mg/kg of Co, 28,586 mg/kg of Cu, 1,286 mg/kg of I, 51,429 mg/kg of Mn, 85,714 mg/kg of Zn, and 571 mg/kg of Se.

ventral) before feeding at 0830 h on d 22 and 28. Digesta samples were collected by grabbing a handful of rumen digesta and transferring to a 50-mL sterile tube for each location. The tubes were immediately frozen on dry ice and stored at -80°C until subsequent microbial analysis. A separate sample of rumen digesta was obtained at 0, 6, and 12 h after feeding on d 22 and 28. The rumen digesta were collected by grabbing a handful from the same 5 locations (approximately 100 mL from each location). These digesta samples were combined to one sample per cow per sampling time, and strained through 2 layers of perforated fabric (WeedBlock, Easy Gardener, Waco, TX) to obtain rumen fluid samples. Thus, a 15-mL sample of rumen fluid was obtained and placed on ice until centrifugation at $3,000 \times g$ for 20 min at 4°C and subsequently stored at -20°C . Rumen fluid samples were thawed and pooled to 1 sample per cow per period accounting for diurnal variation, and stored at -20°C until subsequent VFA profile analysis.

Enteric methane emissions from individual cows were measured consecutively for 5 d (d 23–27) using the sulfur hexafluoride (SF_6) tracer gas technique (Johnson et al., 1994), as modified by McGinn et al. (2006), using halters and polyvinyl chloride neck yokes (approximately 2 L of internal capacity). The halters and yokes were designed to allow for a 50% reduction in yoke vacuum over 24 h by an in-line stainless steel capillary tube. Brass permeation tubes (12.5×40 mm) containing pure SF_6 were sourced from A. Iwaasa and stored at 39°C for at least 3 mo before the study start

date to determine release rates. The mean release rates were 3.25 ± 0.41 and 3.20 ± 0.23 mg/d (mean $\pm$ SD) for groups 1 and 2, respectively. Permeation tubes were placed in the rumen of individual cows 1 wk before the beginning of the study. One week before methane collection, cows were adapted to halters and yokes placed around the neck for methane emission measurement.

For methane emission measurement, air was evacuated from yokes and they were placed on each animal at 0700 h on d 23, and yokes and halters were replaced every 24 h until d 27. Halters were checked daily for line blockages using pure nitrogen (N_2). Yokes were pressurized with N_2 and allowed to sit for 1 h, to obtain representative samples, and three 20-mL subsamples were taken from each yoke with a syringe, and injected into 6.8-mL containers for further analysis. Following sampling, yokes were cleaned 3 times by pressurizing with N_2 , sitting for 1 h, and depressurizing. Yokes were again pressurized and allowed to sit until the following day to check for leakages, and used again for sample collection if no leakages were confirmed. Background concentrations of methane and SF_6 in the animal facility were determined by suspending halters and yokes in front of and above cows during the sample collection period. Background yokes were treated in the same manner as yokes on cows but halters were not changed on a daily basis.

Sample Analysis

Dried feed samples were ground through a 1-mm screen with a Wiley mill (Thomas-Wiley, Philadelphia, PA) and sent to Cumberland Valley Analytical Services (Hagerstown, MD) for analysis. Dry matter was determined by drying samples at 135°C for 2 h (AOAC International, 2000; method 930.15) and OM was determined after 4-h combustion at 550°C (AOAC International, 2000; method 942.05). Crude protein concentration was determined by flash combustion with gas chromatography and thermal conductivity detection (AOAC International, 2000; method 990.03). The NDF concentration was determined using sodium sulfite and amylase (Van Soest et al., 1991), and ADF was determined by boiling samples in an acidic solution followed by filtration (AOAC International, 2000; method 973.18). Crude fat was determined using a Tecator Soxtec System HT 1043 extraction unit (Tecator, Eden Prairie, MN) according to AOAC method 2003.05 (AOAC International, 2006) and starch concentration was determined as described in Hall (2009). Milk samples were individually analyzed for concentrations of milk fat, CP, lactose, MUN, and SCC contents at the Alberta Central Milk Testing Laboratory (Edmonton, AB, Canada; AOAC International, 2002). Rumen

fluid samples were centrifuged at $12,000 \times g$ for 10 min and supernatant was collected and analyzed for VFA concentration by gas chromatography as described by Schlau et al. (2012).

Frozen rumen content (about 0.5–1 g) was thawed on ice and processed for DNA extraction. The bead-beating method was used to extract total DNA from the rumen digesta using the protocol outlined by Guan et al. (2008). After extraction, the concentration and quality of DNA was measured at A260 and A280 using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Following DNA extraction, DNA samples (50 ng/uL) were used for estimation of total populations of bacteria and methanogenic Archaea in rumen digesta by measuring the copy numbers of 16S rDNA gene using qPCR. The primer pair U2 (forward, 5'-ACTCCTACGGGAGGCAG-3'; reverse, 5'-GACTACCAGGGTATCTAATCC-3'; Stevenson and Weimer, 2007) was used to detect the copy numbers of total bacteria and the primer pair uniMet (forward, 5'-CCGGA-GATGGAACCTGAGAC-3'; reverse, 5'-CGGTCTTGCCCAGCTCTTATTC-3'; Zhou et al., 2009) was used to detect the copy numbers of total methanogenic Archaea. All qPCR was performed with SYBR green chemistry (Fast SYBR green master mix; Applied Biosystems, Burlington, ON, Canada) using the StepOnePlus real-time PCR system (Applied Biosystems). The amplification program included a fast cycle and a melting curve section. The program used for total bacteria was 95°C for 5 min, followed by 40 cycles at 95°C for 20 s and 62°C for 30 s, whereas the program for total methanogenic Archaea was 95°C for 5 s for initial denaturation and then 40 cycles of 95°C for 10 s, followed by annealing or extension for 30 s at 60°C. The final melting curve detection of both microbes were the same, with 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s with fluorescence collection at 0.1°C intervals. The standard curves were constructed using a serial dilution of plasmid 16S rDNA from clones *Butyrivibrio hungatei* (Li et al., 2009) and *Methanobrevibacter* spp. strain AbM4 (Zhou et al., 2010) for total bacteria and methanogenic Archaea, respectively. The final copy numbers of 16S rDNA of targeted microbes, per gram of rumen digesta, were calculated as described by Chen et al. (2012). The initial copy numbers of the standard curve were calculated based on the formula $(NL \times A \times 10^{-9}) / (660 \times n)$, where NL is the Avogadro constant (6.02×10^{23} molecules per mole), A is the molecular weight of the molecule in standard, and n is the length of the amplicon (bp). The serial dilution was assigned from 10^{-3} to 10^{-8} .

Total protozoa were estimated by analyzing the total copy number of 18S rRNA genes with primer pair

P-SSU-316F (5'-CTTGCCCTCYAATCGTWCT-3'; Huws et al., 2009) and P-SSU-539R (5'-GCTTTTCG-WTGCTAGTGTATT-3') using qPCR with SYBR-Green Chemistry. The standard curve was constructed using plasmid DNA containing a cloned sequence (223 bp) using the same primer set that has been confirmed by BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) searches against *Entodinium longinucleatum* 18S rRNA gene (Accession number AB481099). The qPCR was performed using a fast cycle and a melting curve section. The program was 95°C for 5 s for initial denaturation and then 40 cycles of 95°C for 10 s, followed by annealing or extension for 30 s at 60°C. The final melting curve detection of protozoa was 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s with fluorescence collection at 0.1°C intervals. Similarly, the copy number of total protozoa 18S rRNA genes per gram of rumen digesta was calculated using the same formula as described for total bacteria and methanogenic Archaea.

Gas samples taken from the yokes were analyzed at the Lethbridge Research Centre (Lethbridge, AB, Canada) for concentrations of SF₆ and methane using gas chromatography (model 5890, Agilent Technologies, Santa Clara, CA) with flame-ionization detection for methane and electron capture detection for sulfur hexafluoride using the method described by Chung et al. (2011). The SF₆-to-methane ratio, corrected for background levels, was determined for each sample according to McGinn et al. (2006). Daily methane emission data were averaged to report one value per cow per period, and data from cows with less than 2 d of successful measurements were excluded from statistical analysis.

Calculation and Statistical Analysis

Yield of 4% FCM was calculated according to the equation $4\% \text{ FCM} = [\text{milk yield (kg)} \times 0.4] + [\text{fat yield (kg)} \times 15]$, and ECM yield was calculated by an equation developed by Tyrrell and Reid (1965): $\text{ECM} = [\text{milk yield (kg)} \times 0.327] + [\text{fat yield (kg)} \times 12.95] + [\text{protein yield (kg)} \times 7.25]$. Feed efficiency was calculated as 4% FCM (kg) divided by kilograms of DMI. Energy expenditure to maintenance, milk production, change in BW, and methane was calculated according to NRC (2001). Gross energy intake from feed was calculated using the NRC (2001) formula: $\text{gross energy (Mcal/kg)} = [0.0415 \times \text{carbohydrate (\%)}] + [0.094 \times \text{fat (\%)}] + [0.057 \times \text{protein (\%)}]$.

Data were analyzed using the MIXED procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC) according to the model

$$Y_{ijkl} = \mu + P_i + T_j + R_k + C(R)_{l(k)} + e_{ijkl}$$

where Y_{ijkl} is the dependent variable, μ is overall mean, P_i is fixed effect of period, T_j is fixed effect of treatment, R_k is fixed effect of parity, $C(R)_{l(k)}$ is random effect of cow nested in parity, and e_{ijkl} is the residual error. Effects of group and parity $\times$ treatment interaction were originally included in the model, but were removed from the final statistical model, as their effects were not significant for primary response variables. Significance was declared when $P < 0.05$ and tendencies were discussed when $P < 0.10$.

RESULTS

The success rate for methane emission measurement was 64%. Of the unsuccessful measurements, 92% were caused by damages to the halters or yokes and the remaining 8% were due to inadequate amounts of SF_6 in gas subsamples, resulting in the inability to calculate methane emissions correctly. As the methane emission data with less than 2 d of successful measurements were excluded from statistical analysis, 5 data points were treated as missing values. Final n values were 8 and 11 for CON and NOP, respectively. Overall concentration of methane and SF_6 found in yokes placed on cows yielding a successful measurement was 36.8 ± 24.6 ppm and 49.2 ± 27.8 ppt (mean $\pm$ SD), respectively. In background yokes, concentrations of methane and SF_6 were 10.3 ± 4.6 ppm and 9.48 ± 5.92 ppt (mean $\pm$ SD), respectively. For successful measurements, coefficient of variation for daily methane emission data within cow was 23.6 and 22.7% for NOP and CON, respectively, and they did not differ ($P = 0.97$).

Feeding 2,500 mg/d of NOP did not affect DMI compared with CON (Figure 1A). Cows fed NOP had decreased methane emissions (132 vs. 372 g/d; SEM = 23.1; $P < 0.0001$), even when normalized for DMI (7.18 vs. 17.8 g/kg DMI; Figure 1B) or expressed as a percentage of gross energy intake (2.20 vs. 5.45%; SEM = 0.31; $P < 0.0005$) when compared with CON. Milk yield and milk component concentration were not affected when cows were fed NOP compared with CON (Table 2). Furthermore, when milk yield was corrected for milk fat or energy content, no differences between treatments were observed. However, BW gain was greater when cows were fed NOP compared with CON (1.06 vs. 0.39 kg/d; $P = 0.05$), but feed efficiency was not affected by treatment.

Feeding NOP did not affect total concentration of VFA; however, it reduced the molar proportion of acetate (52.1 vs. 55.5 mol/100 mol of VFA; $P = 0.04$; Table 3) and tended to increase the proportion of propionate (26.4 vs. 23.9 mol/100 mol of VFA; $P = 0.06$) when compared with CON. This resulted in a decreased acetate-to-propionate ratio when cows were fed

NOP (2.02 vs. 2.36; $P = 0.04$) compared with CON. No treatment effects were observed for other VFA. Total methanogen copy numbers were reduced with feeding of NOP (0.95 vs. 2.69×10^8 /g of rumen digesta; $P = 0.001$) compared with CON, but total bacteria and protozoa copy numbers were not affected by treatment. A tendency was observed for a positive relationship between methanogen count number and methane emissions normalized for DMI ($P = 0.08$; $y = 8.89 + 2.05 \times 10^8x$, $r^2 = 0.16$).

Figure 1. Effects of feeding 2,500 mg/d of 3-nitrooxypropanol (NOP) or 25 g/d of silicone dioxide (CON) to lactating dairy cows on DMI (A; SEM = 0.66; $P = 0.36$; $n = 12$ for both CON and NOP) and methane emissions (B; SEM = 0.95; $P < 0.001$; $n = 8$ and 11 for CON and NOP, respectively).

Table 2. The effects of feeding 3-nitrooxypropanol (NOP) to lactating dairy cows on body weight change, milk yield, and milk components

Variable	Treatment ¹		SE	P-value
	CON	NOP		
BW change, kg/d	0.39	1.06	0.21	0.05
Milk yield, kg/d	35.6	34.5	1.32	0.30
Milk fat, ² %	3.31	3.63	0.22	0.13
Milk CP, %	3.13	3.12	0.07	0.73
Milk lactose, %	4.65	4.65	0.03	0.98
4% FCM yield, ² kg/d	31.5	32.3	1.25	0.42
ECM yield, ² kg/d	34.6	35.0	1.20	0.56
Feed efficiency, ² 4% FCM/DMI	1.60	1.69	0.06	0.26

¹Control (CON) = 25 g/d of silicone dioxide; NOP = 2,500 mg/d of 3-nitrooxypropanol; n = 12 for both CON and NOP, unless otherwise noted.

²One data point for milk fat content was removed due to being an outlier, resulting in n = 12 and 11 for CON and NOP, respectively.

DISCUSSION

One of the primary objectives of the current study was to determine the effects of NOP on methane emissions of lactating dairy cows without moving them to metabolic chambers. By using the SF₆ tracer gas technique, we found that feeding 2,500 mg/d of NOP to lactating dairy cows reduced methane emissions by 60% without a reduction in DMI. The observed reduction in methane emission is slightly lower than that reported in vitro (Romero Perez et al., 2013b) but far greater than previously observed when the compound was tested in vivo (Martinez-Fernandez et al., 2013; Reynolds et al., 2013). Romero Perez et al. (2013b) measured in vitro methane production using a rumen simulation technique and found that methane production was reduced by 75%. However, it is important to note that this reduction was seen in an in vitro system where 5 mg/d of NOP was added to 10 g/d of feedstuff

compared with 2,500 mg/d of NOP in 19.5 kg of DMI in an in vivo setting. In comparison to other in vivo studies, when 100 mg/d of NOP was dosed directly in the rumen of sheep fed a 60% forage diet, methane emission was reduced by 23% (Martinez-Fernandez et al., 2013). Similarly, when 2,500 mg/d was dosed directly into the rumen of lactating dairy cattle, methane emission was reduced by 10% (Reynolds et al., 2013). When 2,720 mg/d was top-dressed on to a 60% forage TMR fed to beef heifers, methane emission was reduced by 33% (Romero Perez et al., 2013a).

All previous in vivo studies evaluating NOP were conducted using chambers to measure methane emissions, whereas the current study used the SF₆ tracer gas technique. However, greater reduction in methane emission from lactating dairy cows in the current study cannot be explained by difference in methodology alone because we used a crossover design in which each animal serves as their own control. Any potential bias or

Table 3. The effects of feeding 3-nitrooxypropanol (NOP) to lactating dairy cows on VFA profile of rumen fluid and bacterial profile counts

VFA	Treatment ¹		SE	P-value
	CON	NOP		
Total, mM	103	109	5.29	0.39
Acetate, mol/100 mol of VFA	55.5	52.1	1.08	0.04
Propionate, mol/100 mol of VFA	23.9	26.4	0.85	0.06
Butyrate, mol/100 mol of VFA	14.3	14.8	0.85	0.59
Isobutyrate, mol/100 mol of VFA	1.10	1.08	0.06	0.82
Valerate, mol/100 mol of VFA	2.25	2.57	0.19	0.27
Isovalerate, mol/100 mol of VFA	1.84	2.02	0.14	0.15
Caproate, mol/100 mol of VFA	1.17	1.06	0.25	0.62
Acetate-to-propionate ratio	2.36	2.02	0.15	0.04
Total bacteria copy numbers, × 10 ¹⁰ /g of rumen digesta	34.5	20.5	5.62	0.11
Methanogen copy numbers, × 10 ⁸ /g of rumen digesta	2.69	0.95	3.01	0.001
Protozoa copy numbers, × 10 ⁵ /g of rumen digesta	1.69	1.35	0.25	0.25

¹Control (CON) = 25 g/d of silicone dioxide; NOP = 2,500 mg/d of 3-nitrooxypropanol; n = 12 for both CON and NOP.

errors leading to less accurate methane measurement would therefore affect both NOP treatment and CON, and, as such, would not affect the evaluation of the effects due to treatment. However, the coefficient of variation for daily methane measurements using SF₆ tracer gas technique exceeded 20% in the current study, suggesting that this technique would not be appropriate to evaluate minor treatment effects on methane emissions. It has been suggested that cannulated animals not be used with the SF₆ tracer gas technique due to an increase in variability (Beauchemin et al., 2012). However, we were able to detect significant treatment effects of NOP on methane emissions with SF₆ tracer gas technique partly because of the great magnitude of reduction. Despite the inherent technical problem, the SF₆ tracer gas technique was considered advantageous for our study, as it allowed for animals to stay in their normal environment, and treatment effects on DMI and animal performance were determined while methane emissions were being measured.

One possible explanation for a higher reduction in methane emissions in the current study, compared with previous *in vivo* trials, could be that the compound was mixed into the TMR. This approach might have allowed the cows to consume NOP throughout the day as feed was ingested compared with dosing into the rumen. When NOP was dosed into the rumen of dairy cattle, Reynolds et al. (2013) found that methane production dropped substantially immediately after dosing, but the effect was only sustained for 1 to 2 h. It was hypothesized that this fast decrease in efficacy was likely linked to the very high solubility of the compound that would rapidly leave the rumen, particularly in high-producing dairy cows with decreased rumen retention time. Additionally, the reduction in methane emission after dosing may coincide with the period that methanogens are expected to be highest. Leedle and Greening (1988) found that when steers were fed a 25% forage diet, methanogen counts increased shortly after feeding, dropped, and then continued to rise throughout the day (3.7×10^8 /g of ruminal contents at 4 h postfeeding vs. 8.3×10^8 /g of ruminal content at 20 h postfeeding). Allowing for continual consumption of NOP over a 24-h period, as was done in the current study, could potentially lead to a sustained reduction of methane production throughout the day. As cows were fed a TMR *ad libitum*, allowing for 5% refusal in the current study, actual NOP intake could have been lower than 2,500 mg/d for some animals. Although actual NOP intake and consistency of NOP supply to the animal were not determined in the current study, our data suggest that mixing the compound in TMR is more effective at reducing methane emissions compared with dosing into the rumen directly.

In the current study, a tendency was observed for a positive relationship between number of methanogens and methane production (Figure 2); however, a direct cause-and-effect relationship between methanogen abundance and methane production has not been confirmed in the literature. Contrary to our results, previous work by Martinez-Fernandez et al. (2013) found no difference in microbial profile in sheep dosed with NOP; similarly, Romero Perez et al. (2013a) reported that total methanogen counts were not affected by NOP. In both cases, the diet fed to the animals was 60% forage (DM basis) and they reported less reduction in methane emissions due to NOP compared with the current study, in which a 38% forage diet was fed. Hook et al. (2011) showed that the species of methanogens present in the rumen was different for a high-concentrate diet compared with a high-forage diet fed to nonlactating dairy cattle, whereas the level of concentrate did not affect total methanogen counts. Similarly, Zhou et al. (2010) found that, regardless of dietary energy content, the total population of methanogens present in the rumen of steers was not different; however, the predominant species differed. These previous data indicate that efficacy of NOP in reducing methane emissions might be diet-dependent, and that NOP may affect specific species of methanogens present to a greater extent in low-forage diets, which may partly explain greater animal responses to NOP in the current study compared with the other *in vivo* studies (Martinez-Fernandez et al., 2013; Reynolds et al., 2013; Romero Perez et al., 2013a).

In addition, ecto- and endosymbiotic relationships exist between protozoa and some methanogens, and their contribution is estimated at up to 37% of total methane production in the rumen (Dohme et al., 1999; Hegarty, 1999; McAllister and Newbold, 2008). A relationship between methane production, methanogens, and protozoa counts often exists. In particular, when effects of feeding lipids are assessed, a reduction in protozoa is often observed along with a reduction in methane (Dohme et al., 1999; Beauchemin et al., 2009b). In the current study, no change in protozoa counts was observed when NOP was fed; as such, the reduction in methanogens may not be attributed to changes in protozoa, but to the direct effects of NOP on methanogen counts or their function. The current data further support the suggestion that NOP reduces methane formation through impeding methanogenesis, thus reducing methanogen counts. This effect could be diet-dependent, therefore allowing for a species-specific response, which would explain the discrepancy in results between the current study and other studies using NOP *in vivo*. As such, further research is warranted to determine whether abundance of specific methanogen

Figure 2. Relationship between methanogen counts and methane emission per kilogram of DMI when 2,500 mg/d of 3-nitrooxypropanol (NOP) or 25 g/d of silicone dioxide (CON) was added to the diet of lactating dairy cows ($n = 8$ and 11 for CON and NOP, respectively). A tendency was observed for the overall relationship between methanogens and methane emissions ($P = 0.08$; $y = 8.89 + 2.05 \times 10^8$, $r^2 = 0.16$).

species is affected when NOP is supplemented in the diet.

The decrease in acetate concentration, tendency toward increased propionate concentration in ruminal fluid, and resulting reduction in acetate-to-propionate ratio is consistent with the inverse relationship reported for methane and propionate (Mathison et al., 1998). Promoting propionate production in the rumen can reduce methane production, as it acts as an alternate hydrogen sink (Kobayashi, 2010). Alternatively, a reduction in methane can cause an increase in propionate production, and the lower acetate-to-propionate ratio observed in the current study supports that propionate production acted at least partly as an alternate pathway for hydrogen. Similarly, other research has found a reduction in the acetate to propionate ratio when NOP is fed [3.89 vs. 4.91 (Martinez-Fernandez et al., 2013), 2.74 vs. 2.93 (Reynolds et al., 2013), and 2.1 vs. 3.3 (Romero Perez et al., 2013a)], suggesting fermentation in the rumen shifted toward propionate production. The authors acknowledge that the tendency for an increase in propionate concentration for NOP compared with CON would not exclusively explain the metabolic fate of available hydrogen, which is expected to increase from a 60% reduction in methane emissions. In an *in vitro* system, Immig (1996) studied the effects of 2-bromoethanesulfonic acid, a structural analog to coenzyme M that blocks the final step of methane formation, which is similar to the expected mode of action of NOP. They found a discrepancy between hydrogen produced during fermentation and recovered from the system through end products when methane was reduced. Those re-

sults suggested additional hydrogen sinks, and through stoichiometric calculations they determined that an accumulation of formate, a precursor for methanogenesis, had occurred. As formate is readily metabolized in the rumen and difficult to measure *in vivo* (Hungate et al., 1970), further research is warranted to look at the effects of NOP on rumen fermentation pathways and alternate hydrogen sinks.

Contrary to our hypothesis, milk yield and milk component concentration were not affected, despite a substantial reduction in methane; however, cows fed NOP gained more BW compared with CON (1.06 vs. 0.39 kg/d). When Reynolds et al. (2013) dosed 2,500 mg/d of NOP to midlactation cows, FCM yield was not affected and BW change was not reported. The results found in the current study could be due to the fact we used cows after peak lactation, and it has been found that partitioning of ME intake toward milk energy output decreases as lactation stage proceeds, whereas partitioning toward body tissue energy increases (Kirkland and Gordon, 2001).

To quantify the additional net energy provided by reduced methane emissions, energy outputs were calculated and compared between NOP and CON cows. When energy expenditures were calculated according to NRC (2001), net energy outputs toward maintenance (9.43 vs. 9.48 Mcal/d) and lactation (23.8 vs. 23.6 Mcal/d) were similar between NOP and CON cows, respectively; however, NOP cows had more net energy output toward BW gain compared with CON cows (5.86 vs. 2.12 Mcal/d). Assuming that energy outputs for heat, urine, and feces were not affected by treatment, the reduction in energy lost as methane (1.75 vs. 4.94 Mcal/d for CON and NOP, respectively) accounted for approximately 80% of the difference in total energy expenditures.

Given that cows used in the present study were in midlactation and the TMR fed was formulated to exceed their nutrient requirements for lactation, it is reasonable that excess ME, due to a reduction in methane production, would go toward BW gain rather than lactation. As such, in future studies, it would be necessary to evaluate the effects of feeding NOP on milk production and energy partitioning of cows in early lactation. In moving forward with establishing the efficacy of feeding NOP to reduce methane emissions from ruminants, its effects on nutrient digestibility and metabolism should also be evaluated. In addition, the current study evaluated effects of NOP for relatively short periods (i.e., 28 d) using a crossover design, in which possible confounding carryover effects of treatment cannot be excluded. A long-term study is warranted to determine effects of feeding NOP on rumen fermentation and animal performance more accurately.

CONCLUSIONS

Feeding 2,500 mg/d of NOP reduced enteric methane emissions from lactating dairy cows by 60% without affecting DMI, and the reduction in methane emission was associated with decreased methanogen counts and acetate-to-propionate ratio in the rumen. In addition, although milk production was not affected by treatment, cows fed NOP increased BW gain, indicating that the reduction in methane emissions increased energy availability to animals.

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