Published May 1, 2015

Sustained reduction in methane production from long-term addition of 3-nitrooxypropanol to a beef cattle diet[1]

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ABSTRACT: The objective was to evaluate whether long-term addition of 3-nitrooxypropanol (NOP) to a beef cattle diet results in a sustained reduction in enteric CH_4 emissions in beef cattle. Eight ruminally cannulated heifers (637 ± 16.2 kg BW) were used in a completely randomized design with 2 treatments: Control (0 g/d of NOP) and NOP (2 g/d of NOP). Treatments were mixed by hand into the total mixed ration (60% forage, DM basis) at feeding time. Feed offered was restricted to 65% of ad libitum DMI (slightly over maintenance energy intake) and provided once per day. The duration of the experiment was 146 d, including an initial 18-d covariate period without NOP use; a 112-d treatment period with NOP addition to the diet, divided into four 28-d time intervals (d 1 to 28, 29 to 56, 57 to 84, and 85 to 112); and a final 16-d recovery period without NOP use. During the covariate period and at the end of each interval and the end of the recovery period, CH_4 was measured for 3 d using whole animal metabolic chambers. The concentration of VFA was measured in rumen fluid samples collected 0, 3, and 6 h after feeding, and the microbial population was evaluated using rumen samples collected 3 h after feeding on d 12 of the covariate period, d 22 of each interval within the treatment period, and d 8 of the recovery period. Average DMI for the experiment was 7.04 ± 0.27 kg. Methane emissions were reduced by 59.2% when NOP was used (9.16 vs. 22.46 g/kg DMI; $P < 0.01$). Total VFA concentrations were not affected ($P = 0.12$); however, molar proportion of acetate was reduced and that for propionate increased when NOP was added ($P < 0.01$), which reduced the acetate to propionate ratio (3.0 vs. 4.0; $P < 0.01$). The total copy number of the 16S rRNA gene of total bacteria was not affected ($P = 0.50$) by NOP, but the copy number of the 16S rRNA gene of methanogens was reduced ($P < 0.01$) and the copy number of the 18S rRNA gene of protozoa was increased ($P = 0.03$). The residual effect of NOP for most of the variables studied was not observed or was minimal during the recovery period. These results demonstrated that the addition of NOP to a diet for beef cattle caused a sustained decrease of methanogenesis, with no sign of adaptation, and that these effects were reversed once NOP addition was discontinued.

Key words: beef cattle, long term, methane, 3-nitrooxypropanol

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J. Anim. Sci. 2015.93:1780–1791
doi:10.2527/jas2014-8726

INTRODUCTION

Rumen microorganisms ferment feed and supply host animals with VFA and microbial protein as fermentation products. During this process, CH_4 is produced as a byproduct and released by respiration and eructation into the atmosphere (Mitsumori and Sun, 2008; Gerber et al., 2013). This represents a loss of energy to the animal ranging from 2 to 12% of GE intake (Johnson and Johnson, 1995). In addition, CH_4

[1]This project was supported by Agriculture and Agri-Food Canada and DSM Nutritional Products. Authors wish to acknowledge technical staff, postdoctoral fellows, and students at the Lethbridge Research Centre and the University of Alberta who helped conduct this research. Atmir Romero-Perez is supported by scholarships from the University of Alberta, CONACYT, and SEP-Mexico.

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Received November 14, 2014.
Accepted February 13, 2015.

is also a potent greenhouse gas with a global warming potential 28 times greater than CO_2 (Stocker et al., 2013). The estimated total greenhouse gas emission from livestock production is 7.1 Gt CO_2 equivalents per year, representing 14.5% of global anthropogenic emissions (Gerber et al., 2013). About 44% of this total is in the form of CH_4. Beef production contributes to 41% of livestock sector emissions globally, with enteric fermentation as the main source (Gerber et al., 2013).

3-Nitrooxypropanol (**NOP**), developed by Duval and Kindermann (2012), is a structural analog of methyl coenzyme M that is believed to act as an inhibitor of the enzyme methyl coenzyme M reductase (**MCR**) during the last step of methanogenesis (Romero-Perez et al., 2014). It reduced CH_4 emissions in vitro (Romero-Perez et al., 2013) and in vivo using sheep (Martínez-Fernández et al., 2014), dairy cows (Haisan et al., 2013, 2014; Reynolds et al., 2014), and beef cattle (Romero Perez et al., 2014). These studies report that the potential of NOP to reduce CH_4 corrected for DMI ranged from 6.7 to 59.6%, depending on the mode of providing NOP to the animals, that is, mixed with the feed, top-dressed, or dosed directly into the rumen. Overall, NOP addition is associated with a shift in ruminal fermentation where molar proportion of acetate is reduced and that of propionate increased with no effect on digestibility or animal productivity (Haisan et al., 2013, 2014; Martínez-Fernández et al., 2014; Reynolds et al., 2014; Romero-Perez et al., 2014). In contrast, compounds with inhibitory effect on MCR, such as bromoethanesulfonate (**BES**), showed adaptation after 4 d of supplementation (Immig et al., 1996).

Results of short-term experiments evaluating NOP to reduce CH_4 emissions have yielded promising results; however, no studies have been conducted to evaluate its long-term effect on methanogenesis. The objective of the present study was to evaluate whether adding NOP to a beef cattle diet for 112 d results in a sustained reduction in enteric CH_4 emissions.

MATERIALS AND METHODS

The experiment was conducted at Agriculture and Agri-Food Canada's Research Centre in Lethbridge, AB, Canada. Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (2009).

Diet and Animal Management

A uniform group of 8 mature Angus heifers (637 ± 16.2 kg of initial BW; 21 mo old) with rumen cannulas were fed daily at 1300 h with a total mixed ration (**TMR**; Table 1) consisting of 60% barley silage, 35% barley grain, and 5% vitamin–mineral supplement (DM basis) prepared daily using a feed mixer (Data Ranger; American Calan Inc., Northwood, NH). For the entire experiment, intake was restricted to 65% of the group average ad libitum DMI observed 10 d before beginning the experiment. The amount of feed supplied was calculated to slightly exceed NE required for maintenance based on the NRC recommendations (NRC, 1996). Feed restriction was necessary to avoid possible effects of ad libitum DMI on variables of interest but also to prevent heifers from excessive weight gain. Animals were housed in a heated research barn in individual tie stalls fitted with rubber mattresses and bedded with wood shavings. They were exercised daily for 1.5 h except when they were in the metabolic chambers.

Table 1. Ingredient and chemical composition of the basal diet

Item	Percent of DM
Ingredient[1]	
Barley silage[2]	60
Barley grain, dry rolled	35
Barley grain, ground	2.688
Calcium carbonate	1.374
Canola meal	0.500
Salt	0.158
Urea	0.110
Molasses, dried	0.108
Feedlot vitamin–mineral premix[3]	0.055
Vitamin E (500,000 IU/kg)	0.004
Flavoring agent	0.003
Chemical composition[4]	
DM	53.6 ± 2.18
OM, % of DM	92.5 ± 0.24
CP, % of DM	11.29 ± 0.63
NDF, % of DM	38.6 ± 1.35
ADF, % of DM	22.4 ± 1.72
Starch, % of DM	33.8 ± 0.57
Fat, % of DM	1.85 ± 0.13
GE, Mcal/kg	4.9 ± 0.53

[1]All ingredients except barley silage and dry-rolled barley grain were provided as part of a pelleted supplement.

[2]Composition: 42.4% DM, 10.5% CP, 49.9% NDF, and 33.1% ADF.

[3]Feedlot vitamin–mineral premix contained 35.01% $CaCO_3$, 10.37% $CuSO_4$, 28.23% $ZnSO_4$, 0.15% ethylenediamine dihydriodide (80% concentration), 5.01% selenium 1% (10,000 mg Se/kg), 0.1% $CoSO_4$, 14.54% $MnSO_4$, 1.71% vitamin A (500,000,000 IU/kg), 0.17% vitamin D (500,000,000 IU/kg), and 4.7% vitamin E (500,000 IU/kg).

[4]Mean ± SD; $n = 6$.

Experimental Design and Dietary Treatments

The experiment was designed as a completely randomized design with 2 treatments: Control (0 g of NOP animal^{-1} d^{-1}; DSM Nutritional Products, AG, Kaiseraugst, Switzerland) and NOP (2 g of NOP animal^{-1} · d^{-1}). The

treatments were hand mixed into the TMR daily at feeding time. Animals were randomly allocated to 2 groups of 4 heifers to facilitate the CH_4 measurement using metabolic chambers (n = 4) and randomly assigned to a treatment (2 heifers per treatment in each group). The duration of the experiment was 146 d, which included the following sampling periods: an initial 18-d covariate period without NOP use to establish a baseline for variables of interest; a 112-d treatment period with NOP added to the diet, divided into four 28-d intervals (d 1 to 28, 29 to 56, 57 to 84, and 85 to112); and a final 16-d recovery period in which NOP was not provided.

The NOP dose used in the present study was based on the levels used in a previous experiment (Romero-Perez et al., 2014) in which different doses of NOP were evaluated (0, 0.75, 2.25, and 4.50 mg/kg BW, equivalent to 0, 0.5, 1.4, and 2.8 g/d).

The amount of feed offered and refused was recorded daily for each heifer. Dry matter intake was calculated using the DM contents of the basal diet and refusal samples. If present, refusals were collected and composited by week for each animal and stored at –20°C until analyzed for DM. The basal diet and barley silage were sampled 3 times per week to monitor DM content. Dried samples of the basal diet and barley silage were pooled by sampling period or interval within the treatment period and stored for chemical analysis. Barley grain and the vitamin–mineral supplement were sampled once each week to monitor DM content. The basal diet was adjusted when the DM content of an ingredient varied by more than 3%. Animals (not fasted) were weighed at the beginning of the experiment and before and after the animals went into the metabolic chambers, which corresponded to the end of each sampling period or interval within the treatment period.

Enteric Gas Production

For the last 3 d of each sampling period or interval within the treatment period, heifers were moved into 4 metabolic chambers (1 heifer/chamber) where the production of CH_4 and CO_2 was continuously monitored. The 2 groups of heifers were staggered by 1 wk to allow gas measurements. Animals were conditioned to the chambers before the beginning of the experiment to minimize stress. Each chamber measured 4.4 m wide by 3.7 m long by 3.9 m high (63.5 m^3; model C1330; Conviron Inc., Winnipeg, MB, Canada). Within each chamber, the animal was housed in a stall equipped with a feeder and drinking bowl and fitted with a rubber mattress. Concentrations of CH_4 and CO_2 in the intake and exhaust air ducts were monitored using a CH_4 analyzer (model Ultramat 5E; Siemens Inc., Karlsruhe, Germany) and a CO_2/H_2O analyzer (model LI-7000; LI-COR Environmental, Lincoln, NE), respectively. Gas concentrations in the intake and exhaust air ducts of the chambers were monitored sequentially (3 or 4 min from the intake or from the exhaust ducts) for a total of 7 min/chamber, except for 1 chamber that was sampled for 6 min (3 min from the intake and 3 min from the exhaust duct). All chambers were sampled within 27 min, with an additional 3 min used to measure a zero reference gas (pure nitrogen gas). The gas sampling procedure was repeated every 30 min. Only the last minute of the 3- or 4-min sampling was retained for analysis to avoid any possible carryover contamination. The air flow in the intake and exhaust ducts was continually monitored using a duct mounted airflow measurement station (FE-1500-FX-12; Paragon Controls Inc., Santa Rosa, CA). The mass and airflow were used to calculate the amount generated.

Each chamber door was opened daily for about 5 min while the gas concentrations were not being monitored. The chambers were opened sequentially to allow feeding and cleaning. These interruptions had no effect on daily emissions because there was an 18- or 19-min reequilibration period before sampling resumed in which the chamber time constant was 4.4 min. To account for between-chamber differences, each chamber was calibrated before and after the experiment by releasing known amounts of CH_4 and CO_2 and calculating the recovered amount. Calibration factors were then generated to correct emissions for each gas. Details of the chamber design and the calculation of CH_4 and CO_2 emissions were reported by McGinn et al. (2004).

The recovery of metabolic hydrogen in the form of reduced protons (H^+) was estimated from $2H^+$ produced and $2H^+$ utilized to synthesize VFA and CH_4 during rumen fermentation (Mitsumori et al., 2012). Calculations were done using the ruminal concentrations of acetate, propionate, butyrate, isovalerate, and valerate. Recovered $2H^+$ in the form of VFA, CH_4, and H_2 was assumed to be 90% of $2H^+$ produced. The production of H_2 is expected to be minimal for the Control treatment and was not considered in the calculation. The metabolic hydrogen for H_2 synthesis during the treatment period was estimated based on CH_4 reduction rates, which were calculated from CH_4 production in respiration chambers as proposed by Mitsumori et al. (2012).

Ruminal Variables

Starting on d 12 of the initial covariate period, d 22 of each interval within the treatment period, and d 8 of the final recovery period, ruminal pH was continuously measured for 7 d using the LRCpH logger system (Dascor, Escondido, CA). The system was standardized using buffers pH 4 and 7 at the start and end of each measurement.

The shift in millivolt reading from the electrodes between the start and end standardizations was assumed to be linear and was used to convert millivolt readings to pH units as described by Penner et al. (2006).

On the initial day of pH measurement for the initial covariate period and intervals within the treatment period and final recovery period, samples of rumen contents from multiple sites within the rumen (atrium, dorsal, ventral, caudodorsal, and caudoventral sacs) were collected at 0 h (prefeeding) and at 3 and 6 h after feeding. Rumen contents were strained through a PECAP polyester screen (355-µm pore size; B & S H Thompson, Ville Mont-Royal, QC, Canada) and 5 mL of the filtered ruminal fluid was added to 1 mL of 25% meta-phosphoric acid (wt/vol) for VFA determination. Another 5 mL of filtered ruminal fluid was added to 1 mL of 1% sulfuric acid (vol/vol) for NH_3–N determination. These samples were immediately frozen after collection using liquid nitrogen and stored at –20°C until analyzed. Samples from whole rumen contents collected 3 h after feeding were also prepared, frozen with liquid nitrogen, and stored at –80°C until analyzed for protozoa, methanogens, and bacteria using quantitative PCR (**qPCR**).

Laboratory Analyses

Analyses were performed on each sample in duplicate; when the coefficient of variation was greater than 5%, the analysis was repeated. The DM content was determined by oven drying for 72 h at 55°C. Dried feed samples were ground in a Wiley mill (A.H. Thomas, Philadelphia, PA) through a 1-mm screen. Analytical DM was determined by drying at 135°C for 2 h (AOAC, 2005; method 930.15) followed by hot weighing. The OM was calculated as the difference between DM and ash (AOAC, 2005; method 942.05). The NDF and ADF contents were determined according to Van Soest et al. (1991) with heat-stable amylase and sodium sulfite used in the NDF procedure. Crude fat (AOAC, 2006; method 2003.05) was determined using ether extraction (Extraction Unit E-816 HE; BŰCHI Labortechnik AG, Flawil, Switzerland). The GE content was determined using a bomb calorimeter (model E2k; CAL2k, Johannesburg, South Africa). Samples ground through a 1-mm screen were reground using a ball grinder (Mixer Mill MM2000; Retsch, Haan, Germany) before determination of nitrogen and starch content. The nitrogen content was determined by flash combustion with gas chromatography and thermal conductivity detection (Carlo Erba Instrumentals, Milan, Italy). Starch content was determined by enzymatic hydrolysis of α-linked glucose polymers as described by Chung et al. (2011).

Ruminal VFA was quantified using GLC (model 5890; Hewlett-Packard, Wilmington, DE) with a capillary column (30 m by 0.32 mm by 1 µm; ZB-FFAP; Phenomenex Inc., Torrance, CA) and flame ionization detection. Crotonic acid was used as internal standard. The oven temperature was maintained at 150°C for 1 min, increased by 5°C/min to 195°C, and held at this temperature for 2.5 min. The injector temperature was 225°C, the detector temperature was 250°C, and the carrier gas was helium. Ruminal NH_3–N concentration was determined by the salicylate–nitroprusside–hypochlorite method using segmented flow analyzer (Rhine et al., 1998).

Frozen rumen contents (1 g) were 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 were measured at 260 and 280 nm by using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Total bacteria and methanogens in the rumen digesta were estimated by measuring the copy number of 16S rRNA genes using qPCR. The primer pair U2 (forward, 5′-ACTCCTACGGGAGGCAG-3′, and reverse, 5′-GACTACCAGGGTATCTAATCC-3′; Stevenson and Weimer, 2007) was used to detect the total copy number of bacterial 16S rRNA genes, and the primer pair uniMet (forward, 5′-CCGGAGATGGAACCTGAGAC-3′, and reverse, 5′-CGGTCTTGCCCAGCTCTTATTC-3′; Zhou et al., 2009) was used to detect the total copy number of methanogenic 16S rRNA genes. The qPCR was performed with SYBR Green chemistry (Fast SYBR Green master mix; Applied Biosystems, Foster City, CA), using the StepOnePlus real-time PCR system (Applied Biosystems). The amplification program included a fast cycle and a melting curve section. For total bacteria, the program consisted of 95°C for 5 min followed by 40 cycles at 95°C for 20 s and 62°C for 30 s. The program for total methanogens included 95°C for 20 s for initial denaturation and then 40 cycles of 95°C for 3 s followed by annealing/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 then an increase to 95°C with fluorescence collection at 0.1°C intervals. The standard curves were constructed using a serial dilution of plasmid DNA from clones containing the full length 16S ribosomal DNA insert of *Butyrivibrio hungatei* (Chen et al., 2011) and *Methanobrevibacter* sp. strain AbM4 (Zhou et al., 2009), respectively. The final copy number of 16S rRNA genes of targeted microbes per gram of rumen contents was calculated based on the formula according to Chen et al. (2012).

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

P-SSU-316F (5′-GCTTTCGWTGGTAGTGTATT-3′) andP-SSU-539R(5′-CTTGCCCTCYAATCGTWCT-3′; Sylvester et al., 2004) and using qPCR with SYBR Green chemistry. The standard curve was constructed using plasmid DNA containing a cloned sequence (223 bp) amplified by the same primer set, which was confirmed by basic local assignment search tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). The initial copy number for the standard curve were calculated based on the formula (NL × A × 10^{-9})/(660 × n), in which NL is the Avogadro constant (6.02 × 10^{23} molecules per mol), A is the molecular weight of the molecule in standard, and n is the length of the amplicon (bp). The serial dilutions were assigned from 10^{-3} to 10^{-8}. The qPCR was performed using a fast cycle and a melting curve section. The program was 95°C for 20 s for initial denaturation and then 40 cycles of 95°C for 3 s followed by annealing/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 then an increase to 95°C with fluorescence collection at 0.1°C intervals. Similarly, the final copy number of 18S rRNA genes of targeted microbes per gram of rumen content was obtained using the same method as described above.

Calculations and Statistical Analysis

Animal was the experimental unit for all variables. Data for ruminal pH were summarized by day for minimum, maximum, and average pH. Protozoa, methanogens, and bacteria data were not normally distributed and so a $\log_{10}$ transformation was applied before analysis with the inverse $\log_{10}$ least squares mean reported. The daily CH_4 flux was determined for each chamber and expressed relative to DMI and GE intake using intake from the CH_4 measurement day. Data were analyzed using a mixed model procedure (SAS Inst. Inc., Cary, NC). Data were covariate adjusted for their baseline measurements. For the analysis of the treatment period, the model included the fixed effects of treatment (Control and NOP), interval within the treatment period, and their interactions. For the analysis of the recovery period, the model included the fixed effect of treatment. Group was considered a random effect. When appropriate, time, day, or interval was considered a repeated measure in the model.

The variance components were estimated using the REML method and degrees of freedom were adjusted using the Kenward–Roger option. The PDIFF option was used to separate means when necessary. The covariance structure was selected based on the lowest Akaike and Bayesian information criteria values. Hourly means (at 2-h intervals) of ruminal pH and CH_4 production were compared using a t test to determine differences in daily pattern between treatments. Least squares means are presented and treatment differences and trends were declared significant at $P \leq 0.05$ and $P \leq 0.10$, respectively.

RESULTS

The DMI during the experiment averaged 7.09 ± 0.15 kg and no significant effects were observed for treatment, interval, or treatment × interval interaction ($P \geq 0.11$; Table 2). All feed was consumed by the animals within 4 h after feeding. Animals gained weight over the study (0.31 ± 0.05 kg/d).

Minimum ruminal pH was higher for animals fed NOP both during the treatment period ($P = 0.01$) and during the recovery period ($P = 0.01$; Table 2). There was no effect of treatment, interval, or treatment × interval interaction for mean, maximum, and range of pH during the treatment or recovery periods ($P \geq 0.11$). The diurnal ruminal pH pattern during the treatment period (Fig. 1) showed a consistent reduction of pH after feeding, reaching nadir 5 to 7 h later for the Control treatment and 5 h later for the NOP treatment. Subsequently, pH started to increase until the next feeding. Mean ruminal pH of cattle fed NOP was greater than ($P \leq 0.05$) for the Control treatment at 3, 5, 7, 9, 11, 19, 21, and 23 h after feeding (Fig. 1).

Total ruminal VFA was not affected by treatment during the treatment period ($P = 0.12$) or the recovery period ($P = 0.36$) and no treatment × interval interaction was observed ($P = 0.5$; Table 2). Additionally, an interval effect was observed for total VFA ($P = 0.04$) in which d 29 to 56 was greater compared to d 1 to 28, 57 to 84, and 85 to 112 (116.1 vs. 102.6, 106.4, and 105.5 mM, respectively). Molar proportions of propionate, butyrate, valerate, and isovalerate increased with NOP use during the treatment period ($P \leq 0.04$) but did not differ from the Control treatment during the recovery period ($P \geq 0.2$) except for isovalerate, which remained greater for the NOP treatment ($P = 0.05$). There was no interval ($P \geq 0.11$) or treatment × interval interaction ($P \geq 0.19$) for propionate, butyrate, or valerate proportion; however, there was an interval effect for acetate ($P = 0.02$; 62.6, 64.2, 64.5, and 62.9 for d 1 to 28, 29 to 56, 57 to 84, and 85 to 112, respectively) and isovalerate ($P = 0.02$; 2.1, 1.5, 1.8, and 2.0 for d 1 to 28, 29 to 56, 57 to 84, and 85 to 112, respectively). The acetate to propionate ratio was reduced with NOP in the treatment period ($P < 0.01$) but no treatment effect was observed in the recovery period ($P = 0.33$). There was an interval effect ($P = 0.03$; 3.4, 3.6, 3.6, and 3.3 for d 1 to 28, 29 to 56, 57 to 84, and 85 to 112, respectively) but no effect for treatment × interval interaction ($P = 0.11$). Ammonia concentration in the rumen was not affected

Table 2. Dry matter intake, BW, and ruminal fermentation of beef cattle fed a backgrounding diet supplemented with 3-nitrooxypropanol (NOP)[1]

	Treatment period[2]			*P*-value[3]			Recovery period[4]			
Item	Control	NOP	SEM	Trt	Int	Trt × Int	Control	NOP	SEM	*P*-value
DMI, kg	7.15	7.14	0.01	0.52	0.11	0.85	6.94	6.93	0.1	0.65
BW, kg	666	665	3.68	0.97	<0.01	0.55	689	686	5.63	0.78
Ruminal pH										
Minimum	5.67	5.89	0.1	0.01	0.12	0.11	5.75	5.93	0.13	0.01
Mean	6.44	6.64	0.06	0.58	0.11	0.15	6.47	6.57	0.07	0.42
Maximum	7.06	7.11	0.43	0.15	0.67	0.69	6.99	7.08	0.04	0.36
Range[5]	1.4	1.2	0.08	0.67	0.75	0.66	1.3	1.2	0.07	0.25
Total VFA, m*M*	112.6	102.7	3.8	0.12	0.04	0.5	99.5	108.1	6.3	0.36
VFA, mol/100 mol										
Acetate (A)	66.6	60.6	0.61	<0.01	0.02	0.29	67.2	65.1	0.8	0.06
Propionate (P)	17.1	20.5	0.42	<0.01	0.17	0.19	17.3	17.5	0.66	0.86
Butyrate	11.7	13.9	0.68	0.04	0.49	0.94	11.2	12.3	0.53	0.2
Valerate	1.52	2.17	0.09	<0.01	0.39	0.19	1.57	1.61	0.09	0.7
Isobutyrate	1.09	1.08	0.06	0.91	0.11	0.56	1.08	1.17	0.11	0.38
Isovalerate	1.63	2.05	0.16	0.02	0.02	0.3	1.8	2.22	0.38	0.05
A:P ratio	4	3	0.08	<0.01	0.03	0.11	3.9	3.7	0.19	0.73
NH_3, m*M*	7.7	6.2	1.02	0.2	0.14	0.85	5.5	6.4	0.67	0.33

[1]Two grams of NOP per animal per day.

[2]112-d period with NOP added to the diet divided into four 28-d time intervals (d 1 to 28, 29 to 56, 57 to 84, and 85 to 112).

[3]Trt = treatment; Int = interval.

[4]16-d period without NOP added to the diet.

[5]Range = maximum ruminal pH – minimum ruminal pH.

by NOP during the treatment ($P = 0.2$) or recovery ($P = 0.33$) periods and there was no effect of interval ($P = 0.14$) or treatment × interval interaction ($P = 0.85$).

Total copy number of bacterial 16S rRNA genes was not affected by NOP ($P = 0.5$), interval ($P = 0.13$), or treatment × interval interaction ($P = 0.78$) during NOP use. Total copy number of methanogenic 16S rRNA genes was reduced when NOP was offered ($P = 0.01$), but no interval ($P = 0.27$) or treatment × interval effect ($P = 0.64$) was observed. Total copy number of protozoa 18S rRNA genes was increased when NOP was offered ($P = 0.03$; Table 3). The treatment × interval interaction ($P = 0.85$) was not significant but an interval effect was observed ($P = 0.01$; 2.2, 4.6, 4.4, and 2.5 copies/g for d 1 to 28, 29 to 56, 57 to 84, and 85 to 112, respectively). Total copy number for rRNA genes of these ruminal microorganisms were not affected during the recovery period ($P \geq 0.27$).

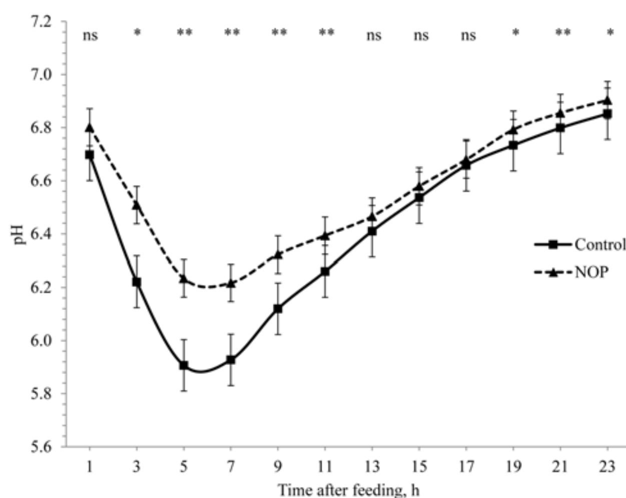

Figure 1. Mean daily pattern of ruminal pH averaged over the treatment period from beef cattle fed a backgrounding diet with or without 2 g/d of 3-nitrooxypropanol (NOP). Data were summarized by 2-h intervals and means are presented. Error bars indicate the SEM. Significance of the main effect of treatment is indicated for each time point by ns = not significant, $*P \leq 0.05$, and $**P \leq 0.01$.

Daily CH_4 produced per animal was reduced by 59.16% when NOP was offered ($P < 0.01$) with no interval ($P = 0.19$) or treatment × interval effect ($P = 0.29$; Table 4). The production of CH_4 corrected for DMI was 59.21% lower for the NOP treatment compared to the Control treatment ($P < 0.01$). Interval was not significant ($P = 0.13$) but treatment × interval interaction tended to be significant ($P = 0.06$; Fig. 2). Gross energy lost as CH_4 was reduced when NOP was offered ($P = 0.01$) and interval tended to differ ($P = 0.09$), but there was no treatment × interval interaction ($P = 0.16$). Use of NOP increased daily CO_2 produced per animal ($P = 0.01$) with no interval ($P = 0.15$) or treatment × interval interaction ($P = 0.94$).

The diel pattern of CH_4 production during the treatment period is presented in Fig. 3. For Control animals, CH_4 production rapidly increased, reaching peak production 4 h after feeding, and then a gradual

Table 3. Rumen microbial populations of beef cattle fed a backgrounding diet supplemented with 3-nitrooxypropanol (NOP)[1,2]

	Treatment period[3]			*P*-value[4]			Recovery period[5]			
Item	Control	NOP	SEM	Int	Int	Trt × Int	Control	NOP	SEM	*P*-value
Bacteria[6]	10.77	12.08	2.22	0.5	0.13	0.78	12.69	13.63	4.6	0.82
Protozoa[7]	2.7	4.17	2.59	0.03	0.01	0.21	3.46	1.66	2.7	0.27
Methanogens[8]	15.46	6.71	0.75	<0.01	0.27	0.64	17.24	10.18	3.64	0.49

[1]Two grams of NOP per animal per day.

[2]Data were $\log_{10}$ transformed before statistical analysis with the inverse $\log_{10}$ least squares mean reported.

[3]112-d period with NOP added to the diet divided into four 28-d time intervals (d 1 to 28, 29 to 56, 57 to 84, and 85 to 112).

[4]Trt = treatment; Int = interval.

[5]16-d period without NOP added to the diet.

[6]$\times 10^{10}$ copy number of the 16S rRNA gene per gram of rumen content.

[7]$\times 10^{8}$ copy number of the 16S rRNA gene per gram of rumen content.

[8]$\times 10^{5}$ copy number of the 18S rRNA gene per gram of rumen content.

reduction was observed for the rest of the day. When NOP was offered, a reduction in CH_4 emissions was observed immediately after feeding, with the lowest values observed from 2 to 4 h after feeding, and subsequently, a gradual increase was observed until prefeeding values were reached and remained for the rest of the day. Methane production of cattle fed NOP remained consistently lower at all times compared to the Control treatment.

Estimated molar proportion of $2H^+$ recovered as VFA, CH_4, and H_2 during the treatment period for the Control and NOP treatments was 31.9 ± 0.7 and 38.7 ± 1.1% for VFA, 68.1 ± 0.7 and 32.0 ± 4.9% for CH_4, and 0 and 29.3 ± 4.9% for H_2, respectively. The estimated H_2 production was 35.9 ± 4.4 g/d for the NOP treatment and assumed to be absent for the Control treatment.

DISCUSSION

Total CH_4 production and CH_4 production corrected for DMI or expressed as a percentage of GE intake were reduced when NOP was offered, consistent with results from previous studies (Haisan et al., 2013, 2014; Martínez-Fernández et al., 2014; Reynolds et al., 2014; Romero-Perez et al., 2014). The magnitude of the reduction in CH_4 emissions expressed in grams/kilogram DM (59.21%) was very similar to the 59.6% reduction observed by Haisan et al. (2014) but was greater than the 6.7% reduction observed by Reynolds et al. (2014), the 25.6% reduction reported by Martínez-Fernández et al. (2014), and the 33.1% reduction reported by Romero-Perez et al. (2014). In both the present study and the study by Haisan et al. (2014), NOP was mixed with the diet, which allowed NOP to be gradually introduced into the rumen as the animals consumed the feed. This synchronization between gradual NOP delivery into the rumen and feed fermentation may have improved the inhibitory potential of NOP. Synchronization between feed digestion and NOP consumption does not occur when NOP is dosed directly into the rumen through the rumen cannula as was the method used by Martínez-Fernández et al. (2014) and Reynolds et al. (2014) and only to a minor extent when NOP is top-dressed onto the feed as was done by Romero-Perez et al. (2014). Mixing NOP with feed appears to be an effective means of providing the compound to cattle. Although pure

Table 4. Enteric CH_4 and CO_2 emissions of beef cattle fed a backgrounding diet supplemented with 3-nitrooxypropanol (NOP)[1]

	Treatment period[2]			*P*-value[3]			Recovery period[4]			
Item	Control	NOP	SEM	Trt	Int	Trt × Int	Control	NOP	SEM	*P*-value
CH_4 emissions										
CH_4, g/d	157.93	64.49	6.79	<0.01	0.19	0.29	156.14	159.3	5.06	0.49
CH_4, g/kg DMI	22.46	9.16	0.88	<0.01	0.13	0.06	22.97	23.43	1.01	0.72
CH_4, % of GE intake[5]	6.46	2.51	0.31	<0.01	0.09	0.16	6.81	6.58	0.14	0.49
CO_2, kg/d	6.24	6.45	0.06	0.01	0.15	0.94	6.21	6.29	0.14	0.65

[1]Two grams of NOP per animal per day.

[2]112-d period with NOP added to the diet divided into four 28-d time intervals (d 1 to 28, 29 to 56, 57 to 84, and 85 to 112).

[3]Trt = treatment; Int = interval.

[4]16-d period without NOP added to the diet.

[5]GE intake calculated from DMI in the chambers and GE content of the total mixed ration.

NOP is a volatile compound that becomes less stable with increasing temperature, the diluted formulation used herein (10% NOP in silicon dioxide) was effective in maintaining its CH_4 reducing potential; however, stability of the formulation needs to be assessed under more diverse scenarios. The assertion that the method of providing NOP to the animal has an effect on its response is made further evident by comparing the amount of NOP provided per kilogram of DMI in the present study (284 mg NOP/kg DMI) with a previous study using beef cattle fed a similar diet (345 mg NOP/kg DMI; Romero-Perez et al., 2014), relative to the reductions in CH_4 achieved. The results indicate that even when supplementing greater doses of NOP adjusted for DMI, the reduction in CH_4 emissions was smaller (33.0%; Romero-Perez et al., 2014) than in the present study (59.2%). The restricted amount of feed offered herein (7.04 ± 0.27 kg) could have increased the time that NOP remained in the rumen as a consequence of a reduced rate of passage. Dilution rate of the liquid phase is positively affected by DMI (Seo et al., 2006) wherein NOP is expected to be present (Romero-Perez. et al., 2014). However, even when supplementing only 129 mg NOP/kg DMI to dairy cows consuming 19.3 kg DM/d (Haisan et al., 2014), where a fast rate of passage would be expected, the observed reduction in CH_4 production was substantial (59.6%).

The diel CH_4 production profile in the present experiment differs from that reported by Reynolds et al. (2014) and Romero-Perez et al. (2014). The transitory effect observed in those studies was not present herein; in contrast, the reduction in CH_4 emissions was more prolonged and pronounced over the day in the present study, suggesting that the mode of supplementation (i.e., mixed with the feed, top-dressed, or dosed directly into the rumen) is an important factor to consider when using NOP. Gradual consumption of NOP and the synchronization of NOP and feed digestion achieved by mixing NOP with the feed could potentially improve the concentration of NOP in the rumen over time, especially at the time of maximum fermentation.

The treatment × interval interaction tended to be significant for CH_4 production (Fig. 2) and was caused by a greater CH_4 production with the NOP treatment during d 57 to 84 compared with d 1 to 28 and 29 to 56 ($P \leq 0.03$). However, the later reduction in CH_4 emissions during d 85 to 112 to a level similar to d 1 to 28 for the NOP treatment ($P = 0.14$) suggests there was no adaptation of animals to NOP over time. The inhibitory effect of NOP on CH_4 emissions was lost once the supplementation was stopped during the recovery period. In vitro continuous culture studies done in our lab (A. Romero-Perez, E. K. Okine, L. L. Guan, S. M. Duval, M. Kindermann, and K. A. Beauchemin, unpublished data) s where CH_4 production was reduced by 71% with NOP addition, showed that within 3 d after supplementation was discontinued, CH_4 inhibition was reduced on average 12% units per day (i.e. from 71% reduction to 35% reduction), suggesting that in vivo CH_4 production may be restored to baseline within less than 1 wk after withdrawal of NOP. Previous in vivo studies using enzymatic inhibitors of methanogenesis showed adaptation (i.e., lack of CH_4 inhibition) after short periods of supplementation. The supplementation of BES to sheep decreased CH_4 concentration in the rumen from 40% to less than 1%; however, after 4 d of administration, the rumen adapted to BES and CH_4 concentration in the rumen increased to 20% (Immig et al., 1996). When Knight et al. (2011) supplemented nonlactating dairy cows with chloroform, a known CH_4 inhibitor, they observed a drastic reduction in CH_4 emissions after 1 wk of supplementation but afterward CH_4 production gradually increased such that there were no differences between control and treated cows by d 39 of supplementation. Tomkins et al. (2009) supplemented beef cattle on a long-term basis with bromochloromethane (**BCM**), a halogenated CH_4 analog with inhibitory effect on methanogenesis, and reduced CH_4 production by 60, 35, and 40% on Days 30, 60, and 90, respectively. Although CH_4 production was reduced after 90 d of providing BCM, this compound cannot be recommended for commercial use because uncomplexed BCM has an ozone-depleting effect (Tomkins et al., 2009). The use of synthetic compounds such as NOP in animal production will depend on the ease of registration by regulatory officials within a particular country or region. For example, use of monensin as a feed additive to improve growth and milk production in beef and dairy cattle is not permitted in the European Union (EMEA, 2007); however, it is used extensively in Canada, the United States, Mexico, Australia, and New Zealand (Duffield et al., 2008). Additionally, the practical implementation of NOP as a CH_4 mitigation strategy under more extensive conditions, such as grazing animals, will depend on development of stable NOP formulations in a range of different environments and technologies to dose NOP to pastured cattle.

Carbon dioxide is a substrate for CH_4 formation; therefore, inhibition of CH_4 production can theoretically increase CO_2 escape from the rumen, as was observed in the present experiment and by Romero-Perez et al. (2014). An increase in ruminal fermentation can also increase CO_2 production; however, this is less probable herein because NOP has been reported to slightly reduce VFA concentration in the rumen (Romero-Perez et al., 2014) without affecting total tract digestibility (Reynolds et al., 2014; Romero-Perez et al., 2014). Such reduction in VFA concentration without effect on

total tract digestibility could be explained by greater postruminal digestion or by an increased rate of VFA absorption or passage from the rumen. Although CO_2 was increased in the present experiment, this increment was more than offset by the reduction in CH_4, when calculated based on CO_2 equivalents. Additionally, for greenhouse gas inventory, the Intergovernmental Panel on Climate Change (2006) assumes that the carbon released by animals as CO_2 during respiration is zero because CO_2 photosynthesized by plants is returned to the atmosphere as respired CO_2.

The increase in minimum ruminal pH is consistent with previous studies in which NOP was offered (Reynolds et al., 2014; Romero-Perez et al., 2014) and coincides with a reduction in total VFA concentration in the rumen. In the present study, a numerical reduction of total VFA concentration when NOP was offered was also observed. The diurnal ruminal pH pattern observed herein is similar to that reported by Romero-Perez et al. (2014), whereby a consistent reduction of pH occurred after feeding, reaching a nadir 7 to 9 h later, with the lowest pH values ranging from approximately 6.2 to 6.4 for the Control and NOP (4.5 mg/kg BW) treatments, respectively. In the present experiment, the reduction in pH was more severe, reaching nadir 6 h after feeding, with values ranging from approximately 5.8 to 6.2 for the Control and NOP treatments, respectively. The diet used in the present experiment was similar to that used by Romero-Perez et al. (2014), and therefore, the greater reduction in pH in this experiment can be related to the short time that animals expended to consume the entire feed allotment (approximately 4 h). This consumption behavior can be related to the feed restriction to which animals were subjected (Munksgaard et al., 2005). Consequently, more feed reached the rumen in a shorter time, increasing fermentation and VFA production in the rumen with a rapid reduction in pH.

The molar proportions of individual VFA were affected by inclusion of NOP in the diet. Providing NOP to cattle is proven to reduce acetate proportion, increase propionate and butyrate proportions, and reduce the acetate to propionate ratio (Martínez-Fernández et al., 2014; Reynolds et al., 2014; Romero-Perez et al., 2014). Both CH_4 and propionate synthesis in the rumen are net sinks for H_2. When methanogenesis is inhibited, propionate synthesis is the primary alternative route for H_2 disposal. However, when CH_4 production is inhibited by using enzymatic inhibitors, it is also possible that H_2 simply escapes from the rumen without contributing to propionate formation (Mitsumori et al., 2012; Leng, 2014). The molar proportion of valerate and isovalerate were higher for the NOP treatment whereas that for isobutyrate remained unchanged. The increase in isovalerate proportion suggests that NOP can affect the deamination process in the rumen; this is because deamination of leucine is expected to result in CO_2, NH_3, and isovalerate (Russell, 2002). Adding NOP to the diet had no effect on ruminal NH_3 concentration, which may be related to very low concentrations of isovalerate. The increase in molar proportion of valerate observed in the present study can be explained on the basis that valerate can be a net sink for $2H^+$ (Russell, 2002); however, due to its relatively low concentration in the rumen, this pathway may be of minor importance for $2H^+$ disposal (Gerber et al., 2013).

As previously mentioned, a reduction in CH_4 production can increase the release of CO_2 from the rumen but may also increase H_2 emissions if not efficiently redirected to other metabolic pathways, such as propionate synthesis. In the present study the proportion of $2H^+$ captured in CH_4 was reduced by 36.1% units (i.e., from 68.1% for the Control treatment to 32.0% for the NOP treatment) and that for VFA was increased by 6.8% units (i.e., from 31.9% for the Control treatment to 38.7% for the NOP treatment). The remaining $2H^+$ (29.3% units) not used for CH_4 synthesis when NOP was added to the diet was assumed to be used for H_2 synthesis (35.9 ± 4.4 g/d). The H_2 production obtained with the hydrogen balance calculation in the present study is an estimation and needs further verification by direct H_2 measurements. We have observed that H_2 production and concentration in the gas mixture were increased with NOP added to feed when using Rusitec fermenters (A. Romero-Perez, E. K. Okine, L. L. Guan, S. M. Duval, M. Kindermann, and K. A. Beauchemin, unpublished data). This H_2 increase has also been observed in vivo when other CH_4 inhibitors such as BCM (Mitsumori et al., 2012) or BES (Immig et al., 1996) were used. Surprisingly, total tract digestibility in dairy cows fed diets containing NOP (51.2% forage diet; Reynolds et al., 2014), beef cattle fed NOP (60% forage diet; Romero-Perez et al., 2014), and goats fed BCM (50% forage diet; Mitsumori et al., 2012) has not been negatively affected. Additionally, no effect was observed for milk (Haisan et al., 2013, 2014; Reynolds et al., 2014; Romero-Perez et al., 2014) or meat production (Mitsumori et al., 2012; Romero-Perez et al., 2014), contrary to the expectation that an increase in H_2 partial pressure would result in a negative feedback effect on the regeneration of reduced cofactors (i.e., NADH and the reduced form of nicotinamide adenine dinucleotide phosphate) and, consequently, on feed digestion and animal production. Leng (2014) proposed that if formate is produced within the biofilm and then released to the external fluid and transformed to H_2 and CO_2, the partial pressure of H_2 within the biofilm would remain low, allowing for the oxidation of reduced cofactors and fermentation to continue.

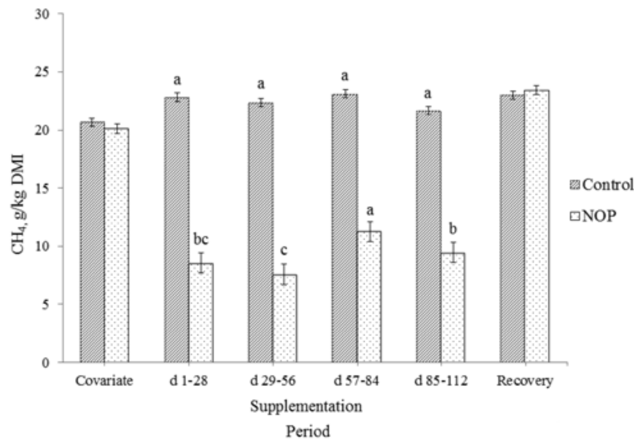

Figure 2. Methane production from beef cattle fed a backgrounding diet with or without 2 g/d of 3-nitrooxypropanol (NOP) added during the treatment period. 3-Nitrooxypropanol was not supplemented during the covariate period or during the recovery period. The treatment period was divided into four 28-d time intervals (d 1 to 28, 29 to 56, 57 to 84, and 85 to 112). A tendency for treatment × interval interaction was observed during the treatment period ($P = 0.06$). [a–c]For the treatment period, intervals with different letters, within treatments, differ ($P \leq 0.05$).

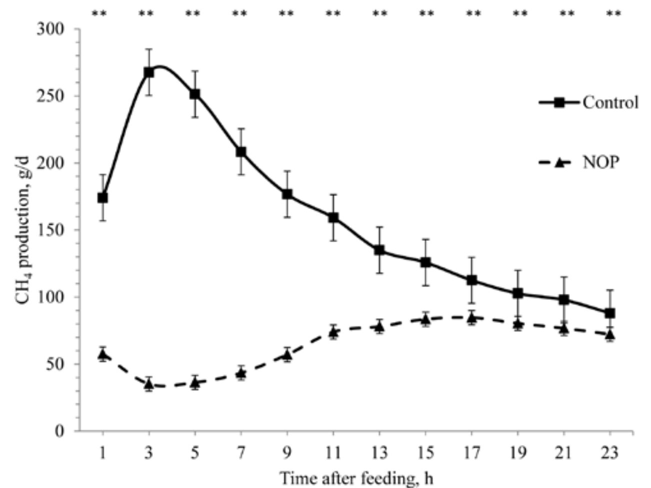

Figure 3. Mean daily pattern of CH_4 production averaged over the treatment period for beef cattle fed a backgrounding diet with or without 2 g/d of 3-nitrooxypropanol (NOP) during the treatment period. Data were summarized by 2-h intervals and means are presented. Error bars indicate the SEM. Treatment significance for each time point is indicated by $^{**}P \leq 0.01$.

Effects of NOP on the microbial community reported in the literature are inconsistent. Martínez-Fernández et al. (2014), Haisan et al. (2013), and Romero-Perez et al. (2014) observed no changes in total copy number of 16S and 18S rRNA genes from different microbes (bacteria, methanogens, and/or protozoa) when providing different doses of NOP to sheep or dairy or beef cattle, respectively, whereas Haisan et al. (2014) reported a reduction in total copy number of 16S rRNA genes of methanogens. The reduction in CH_4 emissions without a direct effect on total number of methanogens has been explained on the basis that NOP could possibly affect the activity of individual species rather that the total number of methanogens, as previously observed (Zhou et al., 2011). However, under in vivo conditions in which the provision of NOP drastically reduced (59.6%) CH_4 emissions (Haisan et al., 2014), a tendency for a positive relationship between number of methanogens and CH_4 production was observed. In the present study, the 59.2% reduction in CH_4 emissions (g CH_4/kg of DM intake) was consistent with the 56.6% reduction in total copy number of methanogens and suggests that a reduction of total copy number of methanogens when NOP is offered may occur when there is a relatively greater CH_4 mitigation effect of NOP. Although the various experiments evaluating NOP have reported reductions in CH_4 emissions, reasons for the inconsistent effects on rumen microorganisms, especially the methanogens, is unknown. It is possible that not all individuals within a herd respond in the same manner to NOP and the possibility that some animals may exhibit adaptation over time cannot be discounted. When evaluating the effects of providing goats with BCM, Mitsumori et al. (2012) observed increases up to 6-fold in *Prevotella* spp. In that study, an increase in H_2 production together with an increase in propionate concentration was reported with increasing doses of BCM, and it was proposed that the rumen adapted to increased $2H^+$ levels by shifting fermentation to propionate via *Prevotella* spp. In the current study, the observed increase in propionate molar proportion when NOP was provided indicates a redirection of $2H^+$ in the rumen and suggests the need to evaluate individual species of ruminal bacteria when NOP is added to cattle diets. Rumen methanogens living in association with protozoa are responsible for 9 to 25% of methanogenesis in rumen fluid (Newbold et al., 1995). Protozoa are H_2 producers and have a symbiotic relationship with methanogens conducting interspecies H_2 transfer (Vogels et al., 1980; Finlay et al., 1994; Morgavi et al., 2010). Consequently, defaunation has been proposed as a mitigation strategy with a potential to reduce CH_4 emissions by about 10.5% (Morgavi et al., 2010). Total copy number of protozoa in the present study was increased with NOP use; however, methanogenesis and copy number of methanogens were reduced. Soliva et al. (2011) evaluated the synthetic compound 3-azidopropionic acid ethyl ester (**APEE**), a structural analog of methyl coenzyme M, using the rumen simulation technique. They observed a drastic reduction in CH_4 emissions (98%) together with a 3-fold increase in entodiniomorph protozoal number and a 7.5-fold increase in H_2 production. Soliva et al. (2011) suggested that the additional H_2 supplied by these protozoa did not compensate for the adverse action of APEE on methanogenesis. The mechanism whereby rumen protozoa copy number in-

creased when NOP was added to the diet in the present study is not clear and requires further investigation.

Most of the ruminal fermentation variables evaluated during the recovery period were not different between the Control and NOP treatments, except for minimum pH and acetate and isovalerate proportions. The lack of effect during the recovery period for most ruminal fermentation variables is in agreement with the lack of effect on CH_4 emissions during this period. The recovery for CH_4 production seems to be faster than the recovery for certain VFA such as acetate or isovalerate and suggests that methanogens may adapt faster to new ruminal conditions after NOP provision is discontinued than microorganisms producing acetate and isovalerate.

Adding NOP to a backgrounding diet by mixing it into the TMR at the time of feeding reduced CH_4 emissions per kilogram of DMI by 59.2% over 112 d with no signs of adaptation. Rumen fermentation was affected by the addition of NOP. Most notably, minimum pH was increased, molar proportion of acetate was reduced, and molar proportion of propionate was increased in cattle fed NOP indicating a redirection of $2H^+$ to propionate formation. The reduction of total number of methanogens is in agreement with the reduction in CH_4 emissions and suggests that a substantial reduction in CH_4 emissions with NOP addition to diets may partially occur as a result of a reduced methanogen population. Residual effects of NOP on the variables studied were either nonexistent or minimal during the recovery period (16 d) when supplementation was discontinued. The synchronization of NOP availability in the rumen and feed digestion achieved by mixing NOP with the feed can potentially improve the synchronization between NOP concentration in the rumen and rumen fermentation, thereby maximizing the CH_4 inhibitory potential of NOP.

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