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The potential of 3-nitrooxypropanol to lower enteric methane emissions from beef cattle[1]

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ABSTRACT: This study evaluated if 3-nitrooxypropanol reduces enteric methane (CH_4) emissions when added to the diet of beef cattle. The effects of 3-nitrooxypropanol on related variables including diet digestibility, ruminal fermentation, and ruminal microorganisms were also investigated. Eight ruminally cannulated Angus heifers (549 ± 64.3 kg [mean BW ± SD]) were fed a high forage diet (backgrounding diet) supplemented with 4 levels of 3-nitrooxypropanol (0, 0.75, 2.25 and 4.50 mg/kg BW). The experiment was designed as a duplicated 4 × 4 Latin square with 2 groups of heifers and four 28-d periods. Methane emissions were measured during 3 consecutive days using metabolic chambers. Up to a 5.8% reduction in ad libitum DMI was observed when 2.5 mg/kg BW of 3-nitrooxypropanol was fed ($P = 0.03$). Increasing level of 3-nitrooxypropanol linearly ($P < 0.001$) reduced CH_4, with 33% less CH_4 (corrected for DMI) at the highest level of supplementation compared with the control. Feed energy lost as CH_4 was also reduced when 3-nitrooxypropanol was supplemented ($P < 0.001$). Molar proportion of acetate was reduced ($P < 0.001$) and that for propionate increased ($P < 0.001$) with increasing dose of 3-nitrooxypropanol, which in turn led to a reduction in the acetate to propionate ratio ($P < 0.001$). Total copy numbers of 16S ribosomal RNA (rRNA) genes for bacteria, methanogens, and 18S rRNA genes for protozoa in ruminal contents were not affected by 3-nitrooxypropanol supplementation ($P \geq 0.31$). There was no effect of 3-nitrooxypropanol on DM ($P = 0.1$) digestibility in the total tract. The use of 4.5 mg/kg BW of 3-nitrooxypropanol in beef cattle consuming a backgrounding diet was effective in reducing enteric CH_4 emissions without negatively affecting diet digestibility.

Key words: beef cattle, enteric methane emissions, 3-nitrooxypropanol

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INTRODUCTION

Ruminants produce methane (CH_4) as a byproduct of enteric fermentation (EPA, 2012) which represents a loss of energy to the animal that can vary from 2 to 12% of GE intake (Johnson and Johnson, 1995). Methane is an important greenhouse gas and livestock is responsible for 37% of global anthropogenic emissions (Steinfeld et al., 2006). Therefore, a reduction in CH_4 production is desirable from the standpoint of both animal productivity and environmental impact.

Research groups around the world are working to develop compounds with a mode of action similar to 2-bromo-ethane sulfonate (**BES**), bromochloromethane, and chloroform, which are potent inhibitors of methanogenesis, but without toxic or harmful effects on the environment (Hristov et al., 2013). Duval and Kindermann (2012) proposed the use of nitrooxy organic molecules in feed, such as 3-nitrooxypropanol (**NOP**; Fig. 1), to reduce enteric CH_4 emissions in ruminants. 3-Nitrooxypropanol is a structural analog to methyl-coenzyme M, a cofactor involved in the

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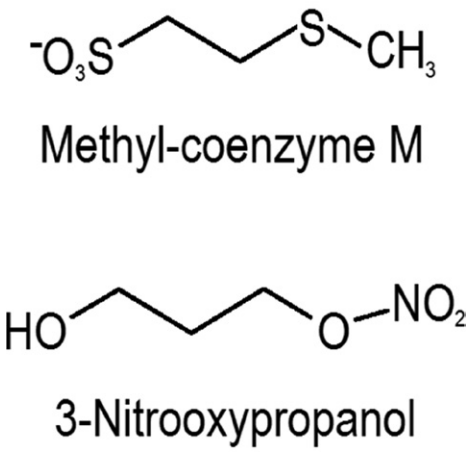

Figure 1. Structural formula of methyl-coenzyme M and 3-nitrooxypropanol. Adapted from Shima and Thauer (2005) and Duval and Kindermann (2012).

terminal step of methanogenesis that transfers a methyl group to methyl-coenzyme M reductase (**MCR**; Shima and Thauer, 2005). Therefore, NOP is thought to inhibit MCR, similarly to BES (Van Nevel and Demeyer, 1995). 3-Nitrooxypropanol has been shown to reduce CH_4 production both in vitro (Romero-Perez et al., 2013) and in vivo when supplemented to sheep (Martinez-Fernandez et al., 2013) and dairy cattle (Haisan et al., 2013, 2014; Reynolds et al., 2014) with no signs of animal toxicity. However, NOP has not yet been evaluated in beef cattle.

The objective of the present study was to evaluate the potential of NOP to reduce enteric CH_4 production from beef cattle fed a backgrounding diet and determine the effects on digestibility, ruminal fermentation, and the ruminal microbial community. We hypothesized that supplementation with NOP would reduce CH_4 emissions without detrimental effects on ruminal fermentation and digestibility.

Table 1. Ingredient and chemical composition of the basal diet

Item	% of DM
Ingredient[1, 2]	
Barley silage[3]	60
Barley grain, dry rolled	35
Barley grain, ground	2.688
Calcium carbonate	1.374
Canola meal	0.5
Salt	0.158
Urea	0.11
Molasses, dried	0.108
LRC feedlot vitamin–mineral premix[4]	0.055
Vitamin E (500,000 IU/kg)	0.004
Flavoring agent	0.003
Chemical composition[5]	
DM	46.7 ± 2.05
OM, % of DM	92.9 ± 0.43
CP, % of DM	11.7 ± 0.25
NDF, % of DM	37.6 ± 1.31
ADF, % of DM	20.6 ± .55
Starch, % of DM	31.8 ± 1.03
Fat, % of DM	2.7 ± 0.19

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

[2]Each heifer received 2.69 mg/d of melengesterol acetate as a pellet to suppress estrous activity. Pellet contained MGA-100 premix (Pfizer Canada Inc., Kirkland, QC, Canada), 0.45%; ground barley grain, 95.99%; dried molasses, 2.51%; and flavoring agent, 0.05% (DM basis). It was fed at 600 g/animal daily (as-is basis).

[3]Composition: DM, 34.5%; CP, 11.4%; NDF, 49.7%; ADF, 32.3%; starch, 16.1%; and GE, 5.6 Mcal/kg.

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

[5]Mean ± SD; $n = 4$.

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 (1993).

Diet and Animal Management

Eight ruminally cannulated Angus heifers (549 ± 64.3 kg [mean ± SD]) were used in this study. The basal diet (Table 1) consisted of 60% barley silage, 35% barley grain, and 5% vitamin–mineral supplement (DM basis). A backgrounding diet was used rather than a finishing diet because of the higher emissions associated with higher forage diets (Johnson and Johnson, 1995) and hence greater need for reduction. The diet was formulated to meet animal requirements for CP, minerals, and vitamins (NRC, 1996). Additionally, each heifer received 600 g/d of a pellet containing 2.6 mg of melengesterol acetate (**MGA**; MGA-100 premix; Pfizer Animal Health, Pfizer Canada Inc., Kirkland, QC, Canada) top dressed onto the diet to suppress estrous activity. The diet was prepared daily using a feed mixer (Data Ranger; American Calan Inc., Northwood, NH), and heifers were fed for ad libitum intake (at least 10% orts) once daily at 1300 h. When the animals were in the metabolic chambers and during digestibility measurements, the amount fed was restricted to 90% of ad libitum intake to avoid day-to-day variation in DMI within animal and to ensure the digestibility measurements were conducted at approximately similar intake as methane emissions. From previous studies (McGinn et al., 2004; Beauchemin and McGinn, 2005), we anticipated a reduction in intake of the cattle when confined in metabolic chambers for measurement of CH_4 emissions.

Cows were housed in a heated tie stall barn, fitted with rubber mattresses and bedded with wood shavings, except when CH_4 production (animals confined in a controlled environment facility) and digestibility measurements (tie stall barn with no bedding) took place. Heifers had access to an open dry lot for exercise daily.

Experimental Design and Dietary Treatments

The experiment was designed as a replicated 4 × 4 Latin square with 2 groups, four 28-d periods, and 4 treatments. Heifers were grouped based on their pre-experimental BW. Lighter heifers were allocated to group 1 (497.4 ± 31.7 kg [mean ± SD]) and heavier heifers were allocated to group 2 (600.0 ± 40.6 kg [mean ± SD]). Dietary treatments were levels of NOP (DSM Nutritional Products, AG, Kaiseraugst, Switzerland) fed at 0, 0.75, 2.25, and 4.50 mg/kg of BW.

Heifers were adapted to their dietary treatments gradually at the beginning of each period. Heifers receiving 4.5 mg NOP/kg of BW were supplemented with 0.75 mg NOP/kg of BW on d 1, 2.25 mg NOP/kg of BW on d 2, and the full amount from d 3. Heifers receiving 2.25 mg NOP/kg of BW received 0.75 mg NOP/kg of BW on d 1 and the full amount from d 2. Heifers supplemented with 0.75 mg NOP/kg of BW received the full amount starting d 1. The NOP was mixed with 13 g of a carrier containing 10 g ground barley, 2 g liquid molasses, and 1 g canola oil (as-fed basis), to improve palatability. The feed additive mixture was top dressed together with the MGA pellet onto the fresh total mixed ration daily at the feeding time. The NOP and carrier were consumed by animals within 10 min of presentation.

Ruminal fermentation was monitored by collecting ruminal contents at 0 (prefeeding), 3, 6, 9, and 12 h after feeding on d 14. The heifers were moved to metabolic chambers to measure enteric CH_4 and carbon dioxide (CO_2) production for 3 consecutive days starting on d 18. Ruminal pH was continuously measured for 7 d from d 14 to 21, which coincided with the measurements of ruminal fermentation and enteric gas production. The heifers were returned to the heated tie-stall barn and apparent total tract digestibility was measured for 4 d from d 24 to 28. Based on information from a previous in vitro experiment done in our lab (Romero-Perez, Okine, McGinn, Guan, Oba, Duval, and Beauchemin, personal communication), we expected any carryover effects of NOP would dissipate within 5 d.

Sampling

Feed offered and refused were recorded daily for individual heifers. Dry matter intake was calculated using the DM contents of the basal diet and refusal samples. Refusals were sampled from Monday to Friday, composited by week for each animal, and stored at –20°C until analyzed for DM and chemical composition. Sampling of the basal diet and barley silage was done 3 times per week to monitor DM content. Dried samples of the basal diet from wk 4 (digestibility measurements) were pooled and stored for analysis. Dried samples for barley silage were pooled by period and stored until analysis. Dry ingredients including barley grain, vitamin–mineral supplement, and MGA pellet were sampled weekly. A subsample was used to determine DM content. Another subsample was composited by period and stored at –20°C until chemical analysis. The proportion of the ingredients in the basal diet (as-fed basis) were adjusted when the DM content of an ingredient varied by >3%.

Body Weight

Body weights (not fasted) were measured at the beginning of the experiment, before and after the animals went into the metabolic chambers, and at the end of each period. The average BW for the before and after chamber weights was used when expressing enteric CH_4 production on the basis of BW. Body weights that were obtained at the beginning of the experiment were used for calculating the amount of NOP needed for Period 1. Thereafter, BW obtained before the chamber measurements were used to calculate the amount of NOP needed for the following period.

Enteric Gas Production

On d 18 of each period, heifers were moved to metabolic chambers (1 heifer/chamber) and production of CH_4 and CO_2 was measured during 3 consecutive d. Because only 4 chambers were available at a time, 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 deep by 3.9 m tall (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 fitted with a rubber mattress.

Gas concentrations were measured using infrared gas analyzers (CH_4; model Ultramat 5E; Siemens Inc., Karlsruhe, Germany; CO_2/H_2O; model LI-7000; LI-COR Environmental, Lincoln, NE). Gas concentrations in the intake and exhaust air ducts of each chamber were monitored sequentially for approximately 3 consecutive min (total 6–7 min/chamber). All chambers were sampled within 27 min, with an additional 3 min used to measure the zero reference gas (pure nitrogen gas). The gas sampling procedure was repeated every 30 min.

Only the last 1 min of the 3-min sampling was retained for analysis to avoid any possible carryover contamination. The difference between the incoming and outgoing mass of CH_4 or CO_2 was used to calculate the amount generated in each chamber by the animal. A positive pressure inside the chamber (about 2 Pa) generated by the flow rates of the intake and the exhaust ducts prevented gas leakage into the chambers.

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 once the doors were closed the chamber reached steady state conditions for gas concentration before the next sampling. 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 used to correct the gas emissions. Details of the calculation of CH_4 emissions were reported by McGinn et al. (2004).

Ruminal Variables

Starting on d 14, ruminal pH was measured every minute for 7 d using the Lethbridge Research Centre Ruminal pH Measurement System (LRCpH; Dascor, Escondido, CA). The system was standardized using buffers pH 4 and 7 at the start and end of each measurement period. 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).

Five milliliters of filtered ruminal fluid was added to 1 mL of 1% sulfuric acid (vol/vol) for ammonia-N (NH_3–N) determination. Another 5 mL of the filtered ruminal fluid was added to 1 mL of 25% metaphosphoric acid (wt/vol) for VFA determination. These samples were stored at –20°C until analyzed. Whole rumen contents were also collected at 0 (prefeeding), 6, and 12 h after feeding, immediately frozen using liquid nitrogen, and stored at –80°C until analyzed for protozoa, methanogens, and bacteria.

Total Collection for Digestibility

Apparent total tract digestibility was estimated by total collection of feces. Heifers were fitted with urinary catheters (Bardex Lubricath Foley catheter, 75 cm^3 and 12 mm; Bard Canada Inc., Oakville, ON, Canada) to ensure separation of urine and feces. Samples (approximately 10% of total) of the daily feces were dried for 72 h at 55°C in a forced air oven to a constant weight and composited by pooling the dried daily feces by animal within period based on their respective DM contents. The composited fecal samples were analyzed for contents of ash, nitrogen, NDF, ADF, starch, and GE.

Laboratory Analyses

Analyses were performed on each sample in duplicate; when the coefficient of variation was >5%, the analysis was repeated. The DM content was determined by drying for 72 h at 55°C. The dried samples (feeds, refusals, and feces) 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). The 1-mm ground samples were reground using a ball grinder (Mixer Mill MM2000; Retsch, Haan, Germany) before determination of nitrogen and starch contents. 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 were 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 ruminal contents (0.5–1 g) were thawed on ice and processed for DNA extraction. The bead-beating method was used to extract total DNA from the ruminal 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 ruminal digesta were estimated by measuring the copy

numbers of 16S rRNA genes using using quantitative PCR (**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 used 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 used 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 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* (Li et al., 2009) and *Methanobrevibacter* sp. strain AbM4 (Zhou et al., 2009), respectively. The final copy numbers of 16S rRNA genes of targeted microbes per gram of ruminal contents were 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′-CTTGCCCTCYAATCGTWCT-3′) and P-SSU-539R (5′-GCTTTCGWTGCTAGTGTATT-3′) using qPCR with SYBRGreen Chemistry. The standard curve was constructed using plasmid DNA containing a cloned sequence (223 bp) amplified by the same primer set and the sequence was confirmed by basic local alignment search tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches. The initial copy numbers for the standard curve were calculated based on the formula $(NL \times A \times 10^{-9})/(660 \times n)$, in which NL is the Avogadro constant (6.02×10^{23} molecules/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 an increase to 95°C with fluorescence collection at 0.1°C intervals.

Calculations and Statistical Analysis

Heifer was the experimental unit for all variables. The daily CH_4 flux was determined for each heifer and expressed as a portion of GE and DE intake on the same day, assuming that energy content of CH_4 is 13.3 Mcal/kg. Data for ruminal pH were summarized by day for minimum, maximum, and average pH. Because protozoa, methanogens, and bacteria data were not normally distributed, a $\log_{10}$ transformation was applied before analysis with the inverse $\log_{10}$ least square mean reported. Data were analyzed using a mixed model procedure (SAS Inst. Inc., Cary, NC) that included the fixed effect of the treatment (0, 0.75, 2.25, or 4.5 mg NOP/kg of BW), sampling time (h or d), and their interactions. Group, period within group, and heifer within group were considered random effects. Time of sampling was considered a repeated effect in the model with heifer × group × period × treatment as the subject. Methane was expressed on the basis of DMI, GE intake, and DE intake.

The variance components were estimated using the REML method and degrees of freedom were adjusted using the Kenward-Roger option. The PDIFF option adjusted by the Tukey method was used to separate means. The covariance structure was selected based on the lowest Akaike and Bayesian information criteria values. Because NOP levels among treatments were not equally spaced, linear, quadratic, and cubic effects of treatments were tested using orthogonal contrasts. Treatment differences and trends were declared significant at $P \leq 0.05$ and $P \leq 0.10$, respectively.

RESULTS

Cubic effects were not statistically significant for any of the variables measured, and therefore only linear and quadratic effects are reported. There were no interactions between the effects of sampling time and NOP levels; therefore, only means for the main effect of treatment are presented.

Increasing levels of NOP reduced ad libitum DMI in a linear ($P = 0.02$) and quadratic ($P = 0.07$) manner (Table 2). However, reduction in DMI only differed from the control ($P = 0.03$) when 2.25 mg NOP/kg BW was supplemented. Despite the reduction in ad libitum DMI, BW and ADG were not affected by level of NOP ($P \geq 0.21$). The BW for heifers consuming 0, 0.75, 2.25, and 4.5 mg NOP/kg was 619, 618, 616, and 621 kg, respectively, while ADG was 1.03, 0.96, 0.87, and 0.87 kg/d, respectively.

Adding NOP to the diet tended ($P = 0.10$) to affect DM digestibility in a quadratic manner ($P = 0.05$), with slightly lower DM digestibility for 0.75 and 2.25 mg NOP/kg BW and slightly greater DM digestibility for 4.5 mg NOP/kg BW compared with the control (Table 2). Consequently, there was a trend for a quadratic ($P = 0.06$)

Table 2. Ad libitum DMI and nutrient digestibility of beef cattle fed a backgrounding diet supplemented with levels of 3-nitrooxypropanol

	Treatment[1]					*P*-value[2]		
Item	0	0.75	2.25	4.5	SEM	Trt	Lin	Quad
Ad libitum DMI,[3] kg/d	12.0[a]	11.7[ab]	11.3[b]	11.4[ab]	0.9	0.03	0.02	0.07
Digestibility, %								
DM	68.4	67.5	67.3	69.7	1.07	0.10	0.13	0.05
OM	70.8	70.1	69.7	71.9	1.02	0.14	0.17	0.06
CP	63.8	62.9	62.8	66.9	1.69	0.22	0.11	0.18
NDF	53.8	52.2	53.1	54.8	1.83	0.36	0.25	0.26
ADF	44.7	42.4	43.6	45.2	1.79	0.41	0.42	0.26
Starch	94.2	94.9	94.1	94.2	0.87	0.89	0.76	0.95
GE	66.4	65.6	65.4	68.2	1.19	0.10	0.08	0.07

[a,b]Within a row, means without a common superscript differ ($P < 0.05$).

[1]Expressed as milligrams of 3-nitrooxypropanol per kilogram of animal BW.

[2]Trt = treatment effect; Lin = linear effect; Quad = quadratic effect.

[3]Ad libitum intake from Day 1 to 16.

response in OM digestibility and a trend for a linear ($P = 0.08$) and quadratic ($P = 0.07$) response in GE digestibility to NOP. However, NOP supplementation had no effect ($P \geq 0.14$) on OM, CP, NDF, ADF, and starch digestibility.

Minimum ruminal pH linearly increased with increasing levels of NOP ($P = 0.02$) but only differed from control when 2.25 mg NOP/kg of BW was supplemented (Table 3). As a result, the pH range was linearly decreased ($P = 0.04$) with NOP supplementation. Mean and maximum pH remained unchanged with NOP inclusion. The diurnal ruminal pH pattern (Fig. 2) for treatments showed consistent reduction of pH after feeding reaching nadir 7 to 9 h later. Subsequently, pH started to increase with a slight drop 19 h after feeding. Ruminal pH was consistently greater than control when 2.25 and 4.5 mg NOP/kg of BW were supplemented. Total VFA linearly decreased ($P = 0.04$) when NOP was increased. Molar proportion of acetate decreased ($P < 0.001$) and that for propionate increased ($P < 0.001$) with increasing levels of NOP. Compared with the control, acetate proportion was reduced by 9 and 15% with 2.25 and 4.5 mg NOP/kg of BW, respectively, and propionate proportion was increased by 22% with the inclusion of 4.5 mg NOP/kg of BW. This change caused a reduction in the acetate to propionate ratio, which was reduced by 3, 17, and 38% when NOP was supplemented at 0.75, 2.25, 4.5 mg/kg of BW, respectively. The molar proportion of butyrate, valerate, and isovalerate linearly increased ($P \leq 0.04$) and isobutyrate tended to linearly increase ($P = 0.09$) with increasing levels of NOP. The NH_3–N concentration was unchanged ($P = 0.34$) when NOP was included.

Total copy numbers of protozoa, methanogens, and bacteria were not affected by inclusion level of NOP ($P \geq 0.31$; Table 4). Time had a significant effect on total copy number of bacteria and methanogens ($P < 0.03$); however, the interaction between NOP and time was not significant for any of the microorganisms studied ($P \geq 0.38$).

No effect on DMI was observed when animals were in the chambers ($P = 0.21$; Table 5). However, DMI was 27% lower in the chambers than when animals were fed ad libitum (Table 2) due to the reduced energy expenditure and stress associated with isolation in the chambers.

Regardless of the manner in which CH_4 emissions were expressed, a reduction was observed when 4.5 mg NOP/kg of BW was included in the diet (Table 5). Total CH_4 emissions per animal (g/d) were linearly ($P < 0.001$) reduced by 3, 13, and 38% with the inclusion of 0.75, 2.25, and 4.5 mg NOP/kg of BW, respectively, compared with the control. The CH_4 production expressed on the basis of DMI or as a percentage of GE or DE intake was significantly reduced by 33, 33, and 35%, respectively, when the greatest level (4.5 mg NOP/kg of BW) of NOP was included ($P < 0.001$). Total CO_2 production was not affected by NOP ($P = 0.82$), however; CO_2 production 2 h after feeding was linearly increased with increasing level of NOP.

In addition to examining the total enteric CH_4 production, the diurnal CH_4 production pattern was examined (Fig. 3). For control animals, enteric CH_4 production rapidly increased 1 h after feeding. It continued to gradually increase reaching peak production 6 to 8 h after feeding and then it declined over the rest of the day. A negative transitory effect on CH_4 production was evident for a period of 2 h after feeding the different NOP dose levels. Thereafter, CH_4 production followed a similar postprandial CH_4 production pattern as the control, such that 13 h after feeding, CH_4 production for animals receiving 0.75 and 2.25 mg NOP/kg of BW was similar to that for control. This was not the case for the highest level of supplementation (4.5 mg NOP/kg BW),

Table 3. Ruminal fermentation of beef cattle fed a backgrounding diet supplemented with levels of 3-nitrooxypropanol

	Treatment[1]					*P*-value[2]		
Variable	0	0.75	2.25	4.5	SEM	Trt	Lin	Quad
Ruminal pH								
Minimum	5.83[b]	6.00[ab]	6.04[a]	6.06[a]	0.10	0.04	0.02	0.14
Mean	6.46	6.54	6.57	6.55	0.05	0.28	0.19	0.17
Maximum	6.96	6.98	6.97	6.91	0.06	0.70	0.35	0.52
Range[3]	1.13	0.99	0.93	0.85	0.09	0.08	0.02	0.38
Total VFA, m*M*	160.5	159.1	148.4	147.7	9.13	0.16	0.04	0.41
VFA, mol/100 mol								
Acetate (A)	61.8[a]	60.8[a]	56.3[b]	52.6[c]	0.99	<0.001	<0.001	0.57
Propionate (P)	19.3[b]	19.4[b]	21.4[b]	26.1[a]	1.16	<0.001	<0.001	0.23
Butyrate	12.3[b]	12.7[b]	15.2[a]	13.8[ab]	0.60	0.003	0.01	0.004
Valerate	1.74[b]	1.83[ab]	1.85[ab]	2.10[a]	0.09	0.03	0.004	0.52
Isobutyrate	1.01	1.01	1.08	1.06	0.04	0.15	0.09	0.17
Isovalerate	1.95[b]	2.27[ab]	2.256[ab]	2.30[a]	0.15	0.04	0.04	0.13
A:P	3.33[a]	3.23[ab]	2.74[b]	2.08[c]	0.21	<0.001	<0.001	0.76
NH_3, m*M*	5.62	5.67	5.76	5.05	0.56	0.68	0.34	0.46

[a–c]Within a row, means without a common superscript differ ($P < 0.05$).

[1]Expressed as milligrams of 3-nitrooxypropanol per kilogram of animal BW.

[2]Trt = treatment effect; Lin = linear effect; Quad = quadratic effect.

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

for which CH_4 production remained consistently lower at all times compared to the other treatments.

DISCUSSION

This study demonstrates that enteric CH_4 production by beef cattle fed a backgrounding diet was reduced when NOP was supplemented to the diet. The reduction in CH_4 production is consistent with the development of NOP as an inhibitor of methanogenesis due to inhibition of MCR. Information about the use of NOP is limited; however, the literature indicates significant reductions of enteric CH_4 emissions when supplemented to ruminants. The first experiment conducted to evaluate the effectiveness of NOP in food producing animals was done by Martinez-Fernandez et al. (2013). They supplemented 100 mg of NOP per day into the rumen of sheep consuming a diet consisting of alfalfa and oats in a 60:40 forage to concentrate ratio. On d 14, NOP significantly reduced CH_4 emissions by 24.7% and 2 wk later, on d 30, there was still a tendency to reduce emissions. Haisan et al. (2014), using Holstein lactating cows, observed a

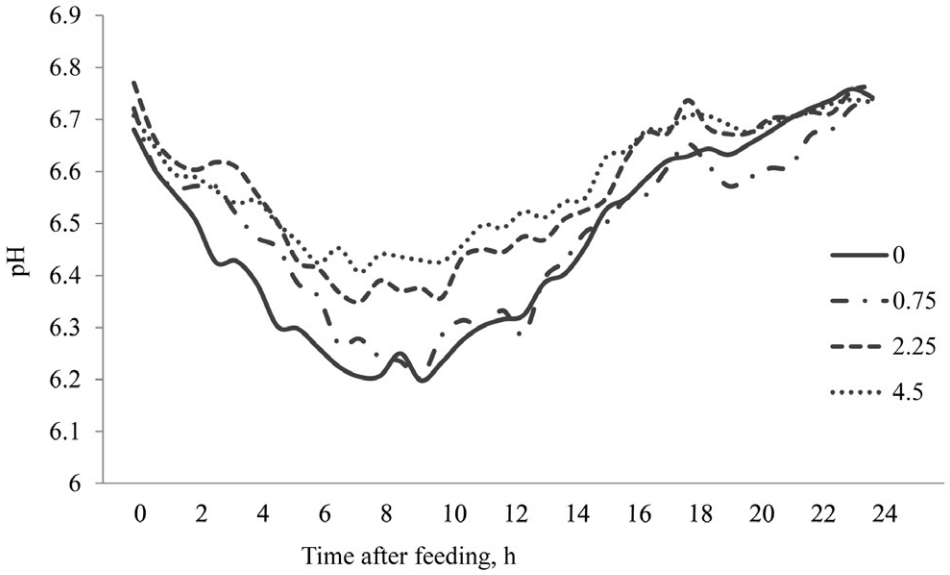

Figure 2. Daily pattern of ruminal pH from beef cattle fed a backgrounding diet supplemented with 0, 0.75, 2.25, and 4.5 mg of 3-nitrooxypropanol per kilogram of animal BW.

Table 4. Ruminal microorganisms of beef cattle fed a backgrounding diet supplemented with levels of 3-nitrooxypropanol

	Treatment[1]					*P*-value[2]		
Variable	0	0.75	2.25	4.5	SEM	Trt	Time	Trt × Time
Protozoa, × 10^5 copies/g	3.98	5.56	4.11	5.39	1.11	0.94	0.17	0.97
0 h	4.94	6.14	4.14	6.67	1.71			
6 h	3.73	5.75	4.63	6.17	1.71			
12 h	3.28	4.8	3.57	3.35	1.71			
Bacteria, × 10^{10} copies/g	1.34	1.25	1.98	1.26	0.57	0.55	<0.01	0.38
0 h	2.16	1.88	3.14	1.35	0.54			
6 h	0.84	0.85	1.52	0.57	0.54			
12 h	1.02	1.03	1.27	1.86	0.54			
Methanogens × 10^8 copies/g	6.2	6.92	7.42	3.66	1.58	0.31	0.03	0.42
0 h	7.74	6.52	7.46	4.24	1.21			
6 h	4.07	6.56	7.6	2.37	1.21			
12 h	6.81	7.65	7.2	4.39	1.21			

[1]Expressed as milligrams of 3-nitrooxypropanol per kilogram of animal BW.

[2]Trt = treatment effect.

substantial reduction of 60% in CH_4 production when 2.5 g/d of NOP were supplemented to dairy cows consuming a 38% forage diet (DM basis). In another study, a reduction of 41.5% was observed when the same dose was supplemented to dairy cows consuming a 60% forage diet (Haisan et al., 2013). In both dairy studies, NOP was mixed into the ration by hand. The reduction in CH_4 production (33%) obtained with the highest dose in our study was lower when compared to the reduction reported by Haisan et al. (2014), even though the dose used in our study was similar (4.5 mg NOP/kg of BW equivalent to 2.7 g/heifer) and DMI was lower (8.1 vs. 19.3 kg/d) than in their study. The dose of NOP supplemented per kilogram of feed was 2.5 times higher for our beef experiment and suggests that factors such as the method that NOP is supplemented to animals (e.g., mixed with the feed or top dressed) as well as animal type (e.g., beef, dairy, sheep) can affect the CH_4 response.

In a study conducted by Reynolds et al. (2014) using dairy cows fed a 43% forage diet (DM basis) supplemented with 0.5 or 2.5 g NOP/animal directly into the rumen, a significant reduction of 4.5 and 6.7%, respectively, in CH_4 production corrected for DMI was observed. The 4.5% reduction obtained when supplementing 0.5 g NOP/animal was similar to the reduction observed in our study (4.3%) when supplementing the lowest NOP dose (0.75 mg NOP/kg of BW equivalent to 0.45 g/animal); however, in our study, the reduction at the low supplementation level was not significant when compared with the control.

The marked reduction in CH_4 emissions observed 2 h after feeding in our study was similar to that observed by Reynolds et al. (2014) with 2.5 g NOP/d supplemented to dairy cows. They observed a pronounced reduction (2–3 h) in CH_4 concentration in exhaust air (inferring reduced emissions) when dosing NOP before

Table 5. Enteric CH_4 and CO_2 emissions of beef cattle fed a backgrounding diet supplemented with levels of 3-nitrooxypropanol

	Treatment					*P*-value[1]		
Item	0	0.75	2.25	4.5	SEM	Trt	Lin	Quad
DMI,[2] kg/d	8.5	8.7	8.6	8.1	0.63	0.52	0.21	0.46
CH_4 intensity								
CH_4, g/d	206.8[a]	199.2[ab]	180.2[b]	129.1[c]	22.9	<0.001	<0.001	0.25
CH_4, g/kg DMI	24.62[a]	23.54[a]	22.33[a]	16.48[b]	1.80	<0.001	<0.001	0.21
CH_4,[3] % of GE intake	6.49[a]	6.20[a]	5.89[a]	4.34[b]	0.47	<0.001	<0.001	0.20
CH_4,[4] % of DE intake	9.77[a]	9.32[a]	9.02[a]	6.34[b]	0.69	<0.001	<0.001	0.12
CO_2, kg/d	7.37	7.24	7.41	7.35	0.55	0.80	0.82	0.90
CO_2 2 h postfeeding, kg	0.57[b]	0.60[ab]	0.61[ab]	0.70[a]	0.67	<0.007	<0.001	0.59

[a–c]Within a row, means without a common superscript differ ($P < 0.05$).

[1]Trt = treatment effect; Lin = linear effect; Quad = quadratic effect.

[2]Intake during CH_4 measurements.

[3]Gross energy intake calculated from DMI in the chambers (Conviron Inc., Winnipeg, MB, Canada) and GE content of the total mixed ration.

[4]Digestible energy intake calculated from DMI in the chambers and digestibility of GE measured during the digestibility phase.

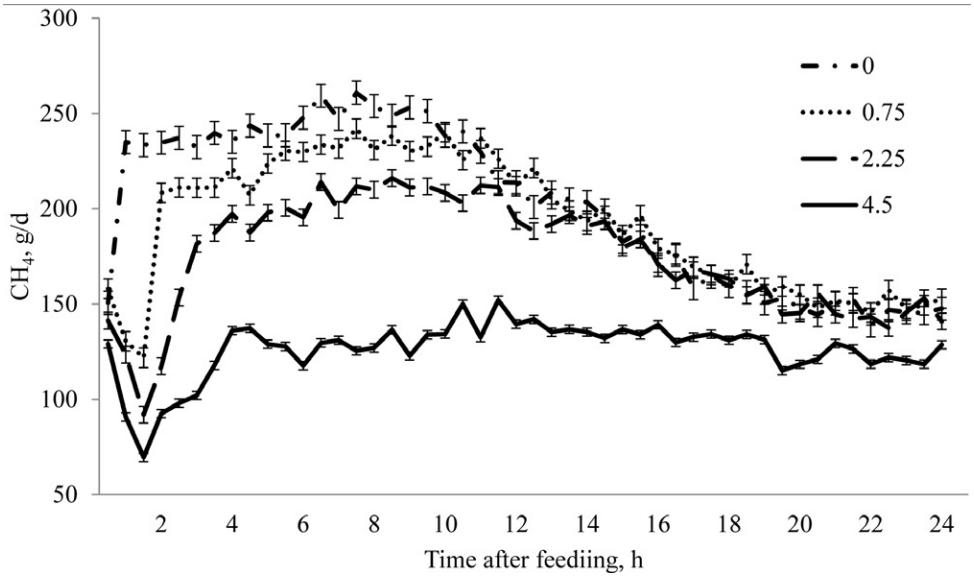

Figure 3. Daily pattern of CH_4 production from beef cattle fed a backgrounding diet supplemented with 0, 0.75, 2.25, and 4.5 mg of 3-nitrooxypropanol per kilogram of animal BW. Error bars indicate SEM.

the afternoon feeding and a delay in the increase of CH_4 production after the morning feeding in comparison with the control treatment. Although the reductions in CH_4 were pronounced in that study, their transitory nature suggests there is need of a continuous infusion of NOP into the rumen when supplemented to dairy cows. The authors in that study hypothesized that the compound may be absorbed, metabolized, or washed out of the rumen. The washout hypothesis from the rumen is very likely; however, washout would be expected to be more pronounced in dairy than in beef cattle. Based on the higher DMI observed in dairy experiments where NOP was supplemented, a higher dilution rate is expected compared to beef cattle. Dilution rate of the liquid phase is positively affected by DMI (Seo et al., 2006) wherein NOP is expected to be present.

The more pronounced effect of NOP on reducing CH_4 production during the first 2 h after feeding and the rapid increase in CH_4 after this time is in agreement with the suggestion that NOP is absorbed, metabolized, and/or washed out from the rumen. Therefore, continuous supplementation, rather than the pulse dose approach used in this study, may help prolong the effect of this compound on CH_4 reduction. Thoroughly mixing NOP with the diet, instead of top dressing the compound, could improve the potential benefits of feeding NOP by synchronizing the availability of the compound in the rumen with ruminal fermentation.

The percentage of GE intake lost as CH_4 for the control diet was similar to the value of 6.5% used by the International Panel on Climate Change Tier 2 methodology (IPCC, 2006). Beauchemin and McGinn (2005) reported that loses of CH_4 with backgrounding diets were higher than for finishing diets (7.4 vs. 3.4% of GE intake, respectively). In our study, cattle supplemented with the highest level of NOP had CH4 emissions, expressed as kg CH4/kg GE intake, typical of levels observed for finishing diets.

The daily CO_2 emissions in our study are greater than previous values reported by our group. McGinn et al. (2004) reported an average daily production of 3.44 kg of CO_2 per animal whereas Beauchemin and McGinn (2005) reported an average of 3.86 kg of CO_2 per animal. However, the animals used in those studies consumed less DM and BW was lower compared with the observation in our study. McGinn et al. (2004) observed a greater CO_2 production for beef cattle during the finishing phase compared to animals in the backgrounding phase and attributed this to greater energy intake and metabolic rate of the heavier cattle. The linear increase of CO_2 production 2 h after feeding with increasing levels of NOP is in agreement with the linear reduction observed for CH_4 emissions. Carbon dioxide and H_2 are substrates for methanogenesis. A reduction in CH_4 formation could cause an increase in CO_2 and hydrogen if alternate metabolic pathways cannot use these gases as substrates. This has been previously demonstrated for H_2 when goats were supplemented with bromochloromethane (Mitsumori et al., 2012).

When NOP was supplemented to dairy cattle by Haisan et al. (2013) using 2.5 g per cow per day, no effect was observed on DMI or milk production; however, BW gain was increased. In a subsequent study by the same research team (Haisan et al., 2014), feeding 1.25 or 2.5 g NOP per cow did not affect DMI, milk production, or BW. Results from Martinez-Fernandez et al. (2013) showed no reduction in DMI when NOP was supplemented. Therefore, the decline in feed in-

take observed in our study is inconsistent with observations in dairy cows and sheep. 3-Nitrooxypropanol needs to be evaluated not only in metabolism studies, such as the present experiment, but also in production studies, to better assess its effects on the energy balance of ruminants.

The reduction in CH_4 production with NOP supplementation did not correspond to changes in diet digestibility. The reduction in ad libitum DMI when NOP was supplemented may indicate a potential palatability issue with NOP or could have resulted from negative feedback from the end products of digestion in the rumen. The linear reduction of total VFA concentration, acetate proportion, and acetate to propionate ratio, together with a linear increase in propionate, butyrate, valerate, isobutyrate, and isovalerate proportion with increasing levels of NOP, indicates changes in ruminal fermentation as a result of NOP supplementation. As observed in our study, antimethanogenic compounds reduce the acetate to propionate molar ratio in the rumen (Abecia et al., 2012). Similarly, a shift in fermentation pattern was observed in previous studies that evaluated NOP (Haisan et al., 2013, 2014; Martinez-Fernandez et al., 2013). A decrease in acetate proportion together with an increase in propionate proportion and a decrease in acetate to propionate ratio has been reported with no changes in total VFA. Based on the hypothesis that NOP would act as a competitive inhibitor of the enzyme MCR, thus inhibiting the reduction of methyl-coenzyme M to CH_4 plus a coenzyme M-coenzyme B complex, a reduction in CH_4 production was expected together with the enhancement of an alternative metabolic pathway to utilize H_2. Considering that propionate is the principal alternative H_2 sink after CH_4 (McAllister and Newbold, 2008), a redirection of H_2 to propionate synthesis was expected.

There was a surprising increase in minimum ruminal pH that needs further verification. Typically, CH_4 production and ruminal pH are positively associated across diets (Janssen, 2010), although within an animal, pH and CH_4 production are inversely related because pH drops and CH_4 production increases after feed consumption. In some studies, an increase in ruminal pH was observed (Nagaraja et al., 1981; Burrin and Britton, 1986; Melendez et al., 2004) when using monensin, an ionophore reported to reduce CH_4 emissions (McGinn et al., 2004; Odongo et al., 2007). Burrin and Britton (1986) related the increase in pH to a reduction in total VFA concentration. Recently, Reynolds et al. (2014) observed an increase in minimum ruminal pH together with a reduction in CH_4 and total VFA when 2.5 g of NOP were supplemented to dairy cattle. The linear reduction in total VFA concentration in the rumen observed in the present study could partially explain the increase in minimum ruminal pH when NOP was supplemented.

The lack of effect of NOP on the numbers of protozoa, bacteria, and methanogens is inconsistent with the observed reduction in CH_4 emissions. Haisan et al. (2013) and Martinez-Fernandez et al. (2013) did not observe changes in total bacteria and/or methanogens, even though a numeric reduction was observed for copy numbers of methanogens. Understanding the interactions between H_2 producers (e.g., bacteria, protozoa, and fungi) and consumers (e.g., methanogens and acetogens) is important to analyze the process of CH_4 production in the rumen (Cieslak et al., 2013). Morgavi et al. (2010) reported protozoa play an important role in methanogenesis because they are considered H_2 producers with a very close physical association with methanogens, favoring the transfer of H_2 between them. Protozoa are also important in ruminal nitrogen metabolism, because they phagocyte bacteria and contribute to protein turnover and NH_3 production (Morrison and Mackie, 1996). The lack of effect of NOP on ruminal NH_3 concentration in the present study can be explained by the absence of effects on protozoa numbers (Ivan et al., 2001).

Reductions in CH_4 production do not always correspond to reductions in abundance of total protozoa, methanogens, or bacteria (Tekippe et al., 2011; Chung et al., 2012; Abecia et al., 2012). It follows that NOP may alter the function of specific microorganisms rather than the microbial community itself. Zhou et al. (2011) analyzed ruminal content samples from Chung et al. (2012) for total methanogens at various sampling times from dairy cows fed different enzyme treatments. There was no correlation between copy numbers of methanogens and CH_4 production, but some changes in the methanogen community profile were reported leading to the conclusion that particular species and metabolic activity of methanogens rather than the total methanogenic population itself may be responsible for CH_4 production.

Conclusions

The addition of NOP to a backgrounding diet decreased daily enteric CH_4 emissions from beef cattle without negatively affecting diet digestibility. The shift in end products of digestion (i.e., increased propionate proportion) in the rumen indicated that NOP changed microbial fermentation of feed. The small reduction (up to 5.8%) in DMI observed when NOP was fed could potentially negatively affect animal performance if the reduction in energy lost as CH_4 is not captured as ME. Further research is needed to confirm the net effects of NOP supplementation in beef cattle diets on CH_4 production and growth performance over a longer feeding period.

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