Dose-response effect of 3-nitrooxypropanol on enteric methane emissions in dairy cows

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ABSTRACT

This experiment was designed to test the effect of inclusion rate of 3-nitrooxypropanol (3-NOP), a methane inhibitor, on enteric methane emissions in dairy cows. The study was conducted with 49 multiparous Holstein cows in a randomized complete block design in 2 phases; phase 1 was with 28 cows, and phase 2 with 21 cows. Cows were fed a basal total mixed ration ad libitum and were blocked based on days in milk, milk yield, and enteric methane emissions during a 14-d covariate period. Treatments were control (no 3-NOP) and 40, 60, 80, 100, 150, and 200 mg of 3-NOP/kg of feed dry matter. Following a 14-d adaptation period, enteric gaseous emissions (methane, carbon dioxide, and hydrogen) were measured using the GreenFeed system (C-Lock Inc., Rapid City, SD) over a 3-d period. Compared with the control, inclusion rate of 3-NOP quadratically decreased daily enteric methane emissions from 22 to 40%. Maximum mitigation effect was achieved with the 3 highest 3-NOP doses (with no statistical difference among 100, 150, and 200 mg/kg). The decrease in methane emission yield and emission intensity ranged from 16 to 36% and from 25 to 45%, respectively. Emissions of hydrogen quadratically increased 6- to 10-fold, compared with the control; the maximum increase was with 150 mg/kg 3-NOP. Treatment did not affect daily emissions of carbon dioxide, but a linear increase in carbon dioxide emission yield was observed with increasing 3-NOP doses. Dry matter intake and milk yield of the cows was not affected by 3-NOP. Milk fat concentration and yield were increased

by 3-NOP due to increased concentration of de novo synthesized short-chain fatty acids in milk. Inclusion of 3-NOP also tended to increase milk urea nitrogen but had no other effects on milk components. In this short-term experiment, 3-NOP decreased enteric methane emissions without affecting dry matter intake or milk yield and increased milk fat in dairy cows. Maximum mitigation effect was achieved at 100 to 200 mg/kg of feed dry matter.

Key words: methane, 3-nitrooxypropanol, dairy cattle

INTRODUCTION

The anti-methanogenic effect of 3-nitrooxypropanol (3-NOP) has been consistent in cattle and its enteric CH₄ mitigation potential has been confirmed in previous long-term studies with lactating dairy cows (Hristov et al., 2015a; Van Wesemael et al., 2019; Melgar et al., 2019, 2020) and beef cattle (Romero-Pérez et al., 2014, 2015). A meta-analysis concluded that inclusion level and administration technique appeared to affect the extent of CH₄ mitigation by 3-NOP in cattle (Dijkstra et al., 2018). Due to its molecular structure, 3-NOP is highly soluble and rapidly metabolized in the rumen (Duin et al., 2016). The extent of mitigation effect of 3-NOP on enteric CH₄ in dairy cows has been reported to range from 7%, when administered as a pulse-dose directly into the rumen via the fistula (Reynolds et al., 2014), to 60%, when mixed with the TMR and fed continuously with the feed (Haisan et al., 2014). Consistent with these reports, results from Hristov et al. (2015a) and 3 follow-up studies from our laboratory (Lopes et al., 2016; Melgar et al., 2019, 2020) demonstrated a persistent CH₄ mitigation effect of 3-NOP when administered through the TMR. In beef cattle, Romero-Pérez et al. (2015) observed a greater reduction in CH₄ emis-

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sions (59%) when 280 mg/kg of 3-NOP was mixed with the TMR than top-dressing 3-NOP at up to 345 mg/kg of feed DM in animals fed the same backgrounding diet (33%; Romero-Pérez et al., 2014). It has been shown that the antimethanogenic effect of 3-NOP appears to be smaller in beef than in dairy cattle (Dijkstra et al., 2018). Long-term experiments with 3-NOP in both beef (Romero-Pérez et al., 2015) and dairy cattle (unpublished data from Melgar et al., 2020) have also shown that enteric CH₄ emissions returned to basal levels within a week after 3-NOP administration was discontinued.

Dose-response effects of 3-NOP have been studied in beef cattle (Romero-Pérez et al., 2014; Vyas et al., 2016) and dairy cows (Reynolds et al., 2014; Hristov et al., 2015a). No dose-dependent effect of 3-NOP was reported in the Reynolds et al. (2014) study after administering 3-NOP directly into the rumen at a rate of 500 and 2,500 mg/d, representing 27 and 135 mg/kg of feed DM, respectively. Hristov et al. (2015a) evaluated 3 levels of inclusion of 3-NOP (40, 60, and 80 mg/kg of feed DM) in mid- to late-lactation cows and observed a similar decrease in enteric CH₄ emissions, yield, and intensity with all 3-NOP doses (although a statistically significant linear effect was reported). In the latter study, 3-NOP was administered through the TMR, which allowed continuous consumption of the compound throughout the day, whereas Reynolds et al. (2014) used a pulse-dose, which could explain the lack of dose effect of 3-NOP. None of the studies with dairy cows, however, have investigated response to 3-NOP included in doses of up to 200 mg/kg of TMR DM.

Therefore, the objective of this study was to investigate the effect of 3-NOP, administered through the TMR in doses of up to 200 mg/kg of feed DM, on enteric CH₄ emissions, DMI, and lactational performance of dairy cows. We hypothesized that within the range of application rates studied, 3-NOP would linearly decrease enteric CH₄ emissions without affecting DMI and lactational performance of the cows.

MATERIALS AND METHODS

Animals involved in this experiment were cared for according to the guidelines of The Pennsylvania State University Institutional Animal Care and Use Committee. The committee reviewed and approved the experiment and all procedures involving animals.

Experimental Design, Diet, and Treatment

The study was conducted with 49 multiparous Holstein cows (155 ± 7.7 DIM, 634 ± 8.1 kg of BW, and 44 ± 1.1 kg/d milk yield at the beginning of the experi-

ment) in a randomized complete block design at The Pennsylvania State University's Dairy Teaching and Research Center's tiestall barn (University Park, PA). Cows were blocked into 7 blocks of 7 animals each, based on DIM, milk yield, and enteric CH₄ emissions during a 14-d covariate period. The experiment was conducted in 2 phases due to limited tiestall space for the number of cows enrolled in the study; phase 1 was with 28 cows (blocks 1 to 4) and phase 2 was with 21 cows (blocks 5 to 7). Within block, cows were randomly assigned to 1 of 7 treatments: control (placebo, no 3-NOP) and 40, 60, 80, 100, 150, and 200 mg of 3-NOP/kg of feed DM (**40NOP**, **60NOP**, **80NOP**, **100NOP**, **150NOP**, and **200NOP**, respectively). Each phase consisted of 14-d covariate and 17-d treatment periods.

The ration was fed as TMR and 3-NOP was incorporated into the TMR through a premix containing (% DM basis) 60% ground corn grain, 5% soybean oil, 15% dry molasses, and 20% of an active or placebo supplement (DSM Nutritional Products, Basel, Switzerland). The active supplement contained 10.9% 3-NOP on SiO₂ and propylene glycol and the placebo supplement contained SiO₂ and propylene glycol only. Two premixes were prepared (i.e., control and 3-NOP premix) and 3-NOP treatment doses were achieved by mixing proportions of the 2 supplements (control/3-NOP supplement: 100/0, 80/20, 70/30, 60/40, 50/50, 25/75, and 0/100% for control, 40NOP, 60NOP, 80NOP, 100NOP, 150NOP, and 200NOP, respectively). The premixes were prepared twice weekly, kept at 4°C in sealed containers with no headspace, and mixed daily with the TMR before feeding to deliver the final 3-NOP application rate as indicated above.

Cows were housed in a tiestall barn, fed ad libitum once daily at approximately 0800 h targeting 10% refusals and had free access to drinking water. Cows were fed a basal TMR (Table 1) for 14 d before the beginning of the treatment period (i.e., covariate period). The basal TMR was formulated to meet or exceed the NE_L and MP requirements of a cow producing 44 kg/d of milk with 3.50% milk fat and 3.05% milk true protein and consuming 26 kg/d of feed DM according to NRC (2001). The composition of the basal TMR was the same in both phases of the experiment. Feed was pushed up to the cows 4 to 6 times daily. The basal TMR was prepared using a stationary mixer (Electra-Mix, model 1062, I. H. Rissler, Mohnton, PA) and separate mixers (Rissler Mobile TMR Mixer Model 1050, I. H. Rissler) were used to mix the control and 3-NOP TMR. The mixing order for the 6 TMR containing 3-NOP (i.e., 40NOP, 60NOP, 80NOP, 100NOP, 150NOP, and 200NOP) was from lowest to the highest concentration of 3-NOP. Cows were milked twice daily at 0600 and 1800 h. Milk from 3-NOP fed cows was discarded for

the duration of the study and for an additional 7 d upon completion of the study.

Sampling and Measurements

Diet and Feed Ingredients. The amount of feed offered and refused was weighed individually and recorded for each cow at the time of feeding to measure daily as-fed intake during the entire experiment. Dry matter content of the TMR and refusals was determined weekly and used to calculate DMI from the as-fed TMR intake. Samples of the forages and concentrate feeds were collected once weekly and samples of the TMR and refusals were collected twice weekly and stored at -20°C . Feed samples were later dried for 72 h at 55°C in a forced-air oven and ground in a Wiley Mill (Thomas Scientific, Swedesboro, NJ) through a 1-mm sieve for further analysis. Forages and concentrate feeds were composited (equal DM weight basis) per phase and submitted to Cumberland Valley Analytical Services (CVAS, 2018) for wet chemistry analyses of CP (method 990.03; AOAC International, 2000), amylase-treated NDF (Van Soest et al., 1991), ether extract (method 2003.05; AOAC International, 2006), ADF (method 973.18; AOAC International, 2000), ash (method 942.05; AOAC International, 2000), minerals (method 985.01; AOAC International, 2000), and estimated NFC (NRC, 2001). Starch was analyzed as described in Hall (2009). The chemical composition of the basal diet (i.e., CP, NDF, ADF, starch, ash, Ca, and P) was calculated by using the analyzed chemical composition of the individual feed ingredients and their inclusion rate in the TMR (Table 1). Concentration of RDP, RUP, NE_L , and NFC and NE_L balance were estimated based on NRC (2001) using average DMI, milk yield, milk composition, and BW of the cows during the experiment.

During the enteric gas measurement week in both study phases, 2 samples of each treatment TMR, including the basal TMR without added premix, were collected and kept frozen at -20°C . These samples were composited by treatment and sent for analysis of 3-NOP concentration (DSM Nutritional Products, Global R&D Analytics, Kaiseraugst, Switzerland).

Enteric Gas Emissions. Enteric gaseous emissions (CH_4 , CO_2 , and H_2) were measured, in each experimental phase, during the last 3 d of the covariate period and in the last 3 d of the treatment period using the GreenFeed system (C-Lock Inc., Rapid City, SD). Measurements were conducted following the procedures recommended by Hristov et al. (2015a,b). Briefly, during each measurement period, spot gas emission data were collected over 3 d as follows: starting at 0900, 1500, and 2100 h (d 1), 0300, 1200, and 1700 h (d 2), and 0000

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

Item	Diet ¹
Feed ingredient, % of diet DM	
Corn silage ²	50.0
Alfalfa haylage ³	10.0
Ground corn grain ⁴	10.0
Soybean seeds, roasted ⁵	7.0
Canola meal ⁶	8.0
Cottonseed, whole ⁷	6.0
SoyPlus ⁸	4.0
Molasses ⁹	3.0
Mineral and vitamin premix ¹⁰	2.0
Composition, ¹¹ % of DM (or as indicated)	
CP ¹¹	16.5
RDP ¹²	10.3
RUP ¹²	6.2
NDF ¹¹	31.8
ADF ¹¹	21.3
Starch	24.3
Ether extract	5.8
NE_L , ¹¹ Mcal/kg	1.62
Average NE_L balance, ¹³ Mcal/d	0.70
Average MP balance, ¹³ g/d	89.0
NFC ¹²	42.3
Ash	5.92
Ca ¹¹	0.70
P ¹¹	0.40

¹Average diet composition for covariate and treatment phases 1 and 2.

²Corn silage was 37.8% DM and contained (DM basis) 7.9% CP, 40.2% NDF, and 31.7% starch.

³Haylage was 33.1% DM and contained (DM basis) 20.1% CP and 42.7% NDF.

⁴Ground corn grain contained (DM basis) 7.7% CP.

⁵Soybean seeds, roasted, contained (DM basis) 37.4% CP.

⁶Canola meal contained (DM basis) 42.4% CP.

⁷Cottonseed contained (DM basis) 23.4% CP.

⁸SoyPlus (Landus Cooperative, Ames, IA) contained (DM basis) 46.6% CP.

⁹Molasses (Westway Feed Products, Tomball, TX) contained (DM basis) 3.9% CP and 66% total sugar.

¹⁰The premix (Cargill Animal Nutrition, Cargill Inc., Roaring Spring, PA) contained (% as-is basis) trace mineral mix, 0.86; MgO (56% Mg), 8.0; NaCl, 6.4; vitamin ADE premix (Cargill Animal Nutrition, Cargill Inc.), 0.48; limestone, 37.2; selenium premix (Cargill Animal Nutrition, Cargill Inc.), 0.07; and dry corn distillers grains with solubles, 46.7. Ca, 14.1%; P, 0.39%; Mg, 4.59%; K, 0.44%; S, 0.39%; Se, 6.91 mg/kg; Cu, 362 mg/kg; Zn, 1,085 mg/kg; Fe, 186 mg/kg; vitamin A, 276,717 IU/kg; vitamin D, 75,000 IU/kg; and vitamin E, 1,983 IU/kg.

¹¹Values calculated using the chemical analysis (Cumberland Valley Analytical Services Inc., Waynesboro, PA) of the feed ingredients and their inclusion in the diets.

¹²Estimated based on NRC (2001).

¹³Estimated (total required minus total supply) based on NRC (2001) using actual DMI, milk yield, milk composition, and BW of the cows throughout the experiment.

and 0500 h (d 3) to obtain a representative sample of a 24-h feeding cycle. A pelletized cattle feed (Stoker Grower 14, Purina Animal Nutrition LLC, Shoreview, MN) was used as bait feed and was offered at each sampling event for a total of 4,000 g/cow over 3 d, and the amount was included in the DMI value during the

gas sampling week. Two GreenFeed units were used and all cows were sampled in less than 2 h. Individual breath gas samples were collected for 5 min followed by a 2-min background sample collection. The GreenFeed system is equipped with a head position sensor and gas emission data are rejected when the cow's head position criteria are not met. Each GreenFeed unit was calibrated following the manufacturer's recommendations. Enteric CH₄ emission yield (g/kg of DMI) and intensity (g/kg of milk yield or g/kg of ECM) were calculated based on the daily CH₄ emissions and DMI and ECM yield data during the gas measurement periods.

Milk Production, Composition, and BW. Milk production of the cows was recorded daily at each milking. Milk samples were collected from 2 consecutive milkings (p.m. and a.m.) during the last day of the covariate and the last day of the treatment periods (phase 1 and 2). An aliquot of each milk sample was placed in tubes with a preservative (2-bromo-2-nitropropane-1,3-diol) and submitted to Dairy One Cooperative Inc. (Ithaca, NY) for analysis of milk fat, true protein, lactose, SCC, and MUN using Milkoscan models 6000, FT+ and Fossomatic models 5000 of FC (Foss Electric A/S, Hillerød, Denmark). Milk composition data were weighted for the corresponding milk yield during each milk sampling event. An aliquot of the milk samples collected during the treatment periods was placed in tubes without preservative and stored at -20°C until analyzed for milk fatty acid composition as described in Rico and Harvatine (2013). Cow BW was recorded twice daily upon exiting the milking parlor using an AfiFarm 3.04E scale system (S.A.E. Afikim, Rehovot, Israel).

Statistical Analysis

All data were analyzed using the MIXED procedure of SAS, version 9.4 (SAS Institute Inc., Cary, NC). Data were tested for normality using the UNIVARIATE procedure of SAS. Statistical analysis of SCC was performed on log-transformed data. The statistical model included treatment, phase (phases 1 and 2), treatment × study phase interaction, and covariate response (for DMI, milk yield and composition, BW, and enteric CH₄ emission data). The effect of treatment × study phase was nonsignificant ($P \geq 0.05$) for any of the variables studied and was removed from the final model. Block and block × treatment were random effects and all others were considered fixed effects. Enteric gas emission data (CH₄, CO₂, and H₂, g/d per animal) were averaged by cow for the covariate and treatment periods and the average values were used in the statistical analysis. Feed DMI, milk yield, BW, and estimated feed efficiency data were averaged during the

gas sampling week and the average values were used in the statistical analysis. The average milk yield and DMI during milk sampling weeks (i.e., last 7 d of the covariate or treatment periods) were used to calculate milk fat, true protein, lactose, and ECM yields; ECM was calculated according to Sjaunja et al. (1990): $\text{ECM (kg/d)} = \text{kg of milk} \times [(38.3 \times \% \text{ fat} \times 10 + 24.2 \times \% \text{ true protein} \times 10 + 16.54 \times \% \text{ lactose} \times 10 + 20.7) \div 3,140]$. Average DMI and milk and ECM yields during the gas sampling week were used to estimate CH₄ and CO₂ yield (i.e., g per kg of DMI) and intensity (i.e., g per kg of milk or ECM). Body weight change was calculated as the difference between average BW during the last week of each treatment period and the average BW during the last week of the respective covariate period. Data were analyzed using orthogonal and polynomial contrasts to evaluate 3-NOP treatments versus control and evaluate for linear and quadratic effects of 3-NOP. The IML procedure of SAS with the ORPOL function was used to derive contrast coefficients. All data, except milk fatty acid composition, are presented as covariate-adjusted least squares means. Statistical differences were considered significant at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$. Differences at $0.10 < P \leq 0.15$ were considered numerical trends.

RESULTS AND DISCUSSION

Dose levels for this study were selected based on previous research with 3-NOP in our laboratory (i.e., the Hristov et al., 2015a). In addition, we wanted to test higher doses, comparable to studies conducted with beef cattle (Romero-Pérez et al., 2014, 2015; Vyas et al., 2016) to determine maximum response to 3-NOP.

Measured concentration of 3-NOP in the treatment TMR were 0, 37, 55, 73, 92, 137, and 183 mg/kg feed DM for control, 40NOP, 60NOP, 80NOP, 100NOP, 150NOP, and 200NOP, respectively. Relative standard deviation was calculated and results were below 10% for each 3-NOP level set of samples.

Compared with the control (Table 2), the decrease ($P < 0.001$) in daily enteric CH₄ emissions by 3-NOP ranged from 22 to 40% (overall average of 31%). Expressed in relation to DMI, the decrease ($P < 0.001$) in CH₄ emission yield by 3-NOP ranged from 16 to 36% (overall average of 26%), and expressed on an ECM basis, the decrease ($P < 0.001$) in CH₄ emission intensity ranged from 25 to 45% (overall average was 33%). Daily CH₄ emissions, emission yield, and intensity were lower ($P \leq 0.03$) than the control for each targeted 3-NOP inclusion rate, except emission yield tended to be lower than the control for 60NOP and 80NOP ($P = 0.06$ and 0.10 , respectively; data not shown in Table 2). Numerically, maximum mitigation effect was achieved

with 150NOP, but no statistical difference was observed in daily CH₄ emissions ($P \geq 0.93$), CH₄ emission yield ($P \geq 0.67$), and intensity ($P \geq 0.33$) among 100NOP, 150NOP, and 200NOP treatments. Further, CH₄ emission yield for 40NOP was not different ($P \geq 0.75$) from 60NOP and 80NOP and that for 100NOP was not different ($P \geq 0.84$) from 150NOP and 200NOP (data not shown in Table 2). Based on these results, it can be concluded that maximum mitigation effect of 3-NOP was achieved with 150NOP but no statistical difference was observed among the 3 highest doses. A summary of all experiments conducted in our laboratory (Figure 1) shows an average CH₄ yield mitigation effect of 3-NOP of -27.2% with about a 10 percentage-unit improvement in reduction with doses higher than 60 mg/kg. It is noted that of the 2 studies in which 80 mg/kg 3-NOP was used, one (current experiment) appears to be an outlier (-15.9% reduction vs. -29.1% in Hristov et al., 2015a) and also data for the highest doses (≥ 100 mg/kg) are from a single (current) study.

The pattern in daily enteric CH₄ emissions over a 24-h feeding cycle in the current study is shown in Figure 2. Across 3-NOP application rates and sampling time, the highest CH₄ mitigation effect (46% decrease with 100NOP) was 2 h after feeding fresh TMR and the lowest (12% decrease with 40NOP) was at 21 h after feeding. These results are consistent with a report from a recent long-term study from our laboratory under similar feeding conditions, in which the mitigation effect of 3-NOP fed at 60 mg/kg DM basis was the lowest (5%) 2 h before feeding and the highest (45%) within 6 h after feeding (Hristov and Melgar, 2019).

In beef cattle fed high-forage or high-grain diets, a dose-related CH₄ mitigation effect of 3-NOP was reported by Vyas et al. (2016). In that study, only the 2 highest 3-NOP inclusion levels (150 and 200 mg/kg feed DM) were statistically different from the control when expressed as daily CH₄ emissions. However, when expressed as emission yield, 3-NOP was most effective at 100 mg/kg feed DM (Vyas et al., 2016).

Previous studies in our laboratory (Hristov et al., 2015a; Lopes et al., 2016; Melgar et al., 2019) have shown that the inhibitory effect of 3-NOP on enteric CH₄ emissions in mid- to late-lactation cows persisted over the duration of the experiment, did not decrease overall emissions of CO₂, and was accompanied by increased H₂ emissions. Although it is expected that enteric CO₂ emissions will increase under CH₄ inhibition, CO₂ responses to 3-NOP have been inconsistent. In the current study, daily CO₂ emissions were unaffected by 3-NOP, but when expressed per unit of DMI or milk/ECM yield, tended to linearly increase with 3-NOP dose. In contrast, Melgar et al. (2020) reported a 5% decrease in daily CO₂ emissions in 3-NOP-fed early-

lactation cows. This was explained with the slightly lower DMI of the 3-NOP cows compared with control cows. Carbon dioxide emission yield, however, was not affected by 3-NOP in the Melgar et al. (2020) study.

Hydrogen emissions were quadratically increased ($P = 0.005$) by 3-NOP in the current study. The highest H₂ emissions were associated with the lowest CH₄ emissions with 150NOP treatment. Inhibition of methanogenesis causes accumulation of H₂ (Trei et al., 1972) in the liquid and gas phases of ruminal contents (Hungate, 1967; Janssen, 2010; Wang et al., 2014). Hydrogen gaseous emissions and dissolved H₂ concentration in ruminal fluid, when methanogenesis is inhibited by 3-NOP, are also affected by fermentation pattern and molar proportion of the major VFA (Reynolds et al., 2014; Lopes et al., 2016; Melgar et al., 2020). Similar increases in H₂ emissions have been observed using various enteric CH₄ inhibitors (Trei et al., 1972; Demeyer and Van Nevel, 1975; Russell and Martin, 1984).

Limitations of the current experiment include relatively small number of animals per treatment and short duration of the study. Nevertheless, findings were generally consistent with those of previous experiments with 3-NOP (Hristov et al., 2015a; Melgar et al., 2019, 2020; see also Figure 1). Overall, DMI was not different between 3-NOP and the control in the current study but increasing 3-NOP dose tended to linearly decrease ($P = 0.07$) DMI by 6% with the higher doses. Some studies reported a decrease in DMI with the inclusion of 3-NOP in beef and dairy cattle (Vyas et al., 2018; Melgar et al., 2020). In the Vyas et al. (2018) study, beef steers receiving 200 and 125 mg of 3-NOP/kg feed DM decreased their DMI by 7 and 5% during the backgrounding and finishing phase, respectively. In the Melgar et al. (2020) study, which was with cows in 3 to 112 DIM, the effect of feeding 60 mg of 3-NOP/kg of feed DM on DMI was attributed to the lower BW of the 3-NOP cows at the beginning of the experiment (DMI was not different from control when expressed on a BW basis). Other studies with dairy cows (Hristov et al., 2015a; Melgar et al., 2019; Van Wesemael et al., 2019) demonstrated that the mitigation effect of 3-NOP was not associated with negative effects on DMI, milk yield, and BW. In another short-term experiment, Melgar et al. (2018) supplemented up to 120 mg 3-NOP/kg feed DM to dairy cows and reported no effect of 3-NOP on TMR palatability and DMI. Compared with the control, 3-NOP did not affect ECM yield but linearly increased ($P = 0.004$) ECM feed efficiency.

Inclusion of 3-NOP in the basal diet did not affect milk yield or milk composition of the cows in the current experiment (Table 3), except it increased milk fat concentration and yield ($P \leq 0.01$). A trend for a linear decrease ($P = 0.10$) in milk yield was observed with

Table 2. Dose-response effects of 3-nitrooxypropanol (3-NOP) on enteric gas emissions, DMI, and milk production in lactating dairy cows

Item	Treatment ¹							P value ³			
	CON	40NOP	60NOP	80NOP	100NOP	150NOP	200NOP	SEM ²	C vs. Trt	L	Q
CH ₄											
CH ₄ , g/d	441	345	336	336	269	265	266	25.7	<0.001	<0.001	0.01
CH ₄ per DMI, g/kg	17.0	13.4	14.0	14.3	11.3	10.9	11.6	1.15	<0.001	<0.001	0.08
CH ₄ per milk yield, g/kg	9.7	7.9	7.9	7.8	6.7	6.0	6.7	0.64	<0.001	<0.001	0.06
CH ₄ per ECM yield, g/kg	10.9	8.2	8.1	8.0	7.0	6.0	6.7	0.69	<0.001	<0.001	0.01
CO ₂											
CO ₂ , g/d	13,704	13,397	13,735	13,971	13,940	13,521	13,849	616.5	0.96	0.83	0.89
CO ₂ per DMI, g/kg	544	522	550	574	589	568	604	27.4	0.39	0.04	0.97
CO ₂ per milk yield, g/kg	308	311	313	322	333	317	339	12.7	0.29	0.07	0.98
H ₂											
H ₂ , g/d	0.26	1.50	1.48	1.93	2.23	2.40	2.00	0.425	<0.001	<0.001	0.005
DMI, kg	25.3	26.0	25.2	24.4	24.0	24.2	23.3	1.12	0.50	0.07	0.97
Milk yield, kg	44.9	44.1	45.8	44.3	42.5	43.5	42.2	1.40	0.43	0.10	0.86
Feed efficiency, kg/kg	1.78	1.72	1.79	1.84	1.76	1.82	1.80	0.043	0.88	0.40	0.79
ECM, kg/d	39.6	41.5	44.9	43.2	40.6	43.5	42.3	2.02	0.16	0.46	0.29
ECM feed efficiency, kg/kg	1.56	1.65	1.75	1.79	1.66	1.80	1.79	0.067	0.005	0.004	0.16

¹Treatments were control (CON) and 3-NOP mg/kg feed DM (40, 60, 80, 100, 150, and 200 for 40NOP, 60NOP, 80NOP, 100NOP, 150NOP, and 200NOP, respectively). Measured concentration of 3-NOP in TMR were 0, 37, 55, 73, 92, 137, and 183 mg/kg feed DM for each dose level, respectively. Data are covariate-adjusted LSM.

²Largest SEM published in table; n = 49 (n represents number of observations used in the statistical analysis).

³Contrasts: C vs. Trt, control vs. all 3-NOP treatments; L, linear effect of 3-NOP dose; Q, quadratic effect of 3-NOP dose.

⁴Data for DMI and milk yield are for the week of enteric gas emissions measurements.

⁵Milk yield ÷ DMI.

⁶Energy-corrected milk (kg/d) = kg of milk × [(38.3 × % fat × 10 + 24.2 × % true protein × 10 + 16.54 × % lactose × 10 + 20.7) ÷ 3,140]; Sjaunja et al. (1990).

⁷ECM yield ÷ DMI.

increasing 3-NOP dose, but ECM yield was not affected by treatment. The observed lack of effect of 3-NOP on lactational performance in the current study is consistent with both long-term production studies (Hristov et al., 2015a; Melgar et al., 2019, 2020; Van Wesemael et al., 2019) as well as short-term experiments (Haisan et al., 2014; Reynolds et al., 2014; Lopes et al., 2016; Haisan et al., 2017). In the current study, increasing the dose of 3-NOP linearly increased ($P \leq 0.004$) milk fat concentration and milk fat yield. Similarly, milk fat concentration and yield were increased in a recent study with mid-lactation cows (Melgar et al., 2019). In contrast, Hristov et al. (2015a) did not report changes in milk fat concentration and yield with 3-NOP supplemented at 40, 60, and 80 mg/kg feed DM. Compared with control, 3-NOP did not affect milk true protein and lactose concentration, or milk true protein and milk lactose yield. Concentration of MUN was linearly increased ($P = 0.005$) by 3-NOP dose. The increased

MUN in cows receiving 3-NOP is consistent with previously reported data in long-term, randomized block design experiments with early-lactation (Melgar et al., 2020) and mid-lactation cows (Melgar et al., 2019). Others, however, have reported no effect of 3-NOP on MUN in dairy cows (Reynolds et al., 2014; Haisan et al., 2017). In the Melgar et al. (2020) study, the observed increase in MUN could be partially explained by the increased CP digestibility by 3-NOP. Melgar et al. (2020) also reported a decrease in plasma concentration of some essential (e.g., Arg) and nonessential (e.g., Cit) AA with 3-NOP, which could have contributed to the effect of 3-NOP on MUN in that study and suggests that hepatic oxidation of AA could be a significant source of MUN (Nousiainen et al., 2004). Digestibility and urinary N excretion or plasma AA data were not collected in the current experiment.

Body weight and BW change of the cows were not affected by 3-NOP in the current experiment. The ef-

Figure 1. Average methane mitigation effect of dietary supplementation with 3-nitrooxypropanol (3-NOP) in dairy cows. Inclusion doses were control and 3-NOP included at 40, 60, 80, 100, 150, and 200 mg/kg of feed DM, and mitigation effects were -24.3 , -26.5 , -22.5 , -33.5 , -35.9 , and -31.8% for 40, 60, 80, 100, 150, and 200 mg/kg of 3-NOP, respectively. Enteric methane emissions were measured using the GreenFeed system (C-Lock Inc., Rapid City, SD). Data are average methane yield (g of CH_4 /kg of DMI) reduction, compared with a control, and error bars represent SEM (data for 100, 150, and 200 mg/kg 3-NOP are from a single experiment, and therefore no error bars are associated with these doses); $n = 236$ for 40 and 80 mg/kg (187 individual cow observations from Hristov et al., 2015a; and 49 from the current study), $n = 649$ for 60 mg/kg (187 observations from Hristov et al., 2015a; 61 from Lopes et al., 2016; 160 from Melgar et al., 2019; 192 from Melgar et al., 2020; and 49 from the current study), $n = 49$ for 100, 150, and 200 mg/kg from the current study (n represents the number of observations used in the statistical analysis in each experiment).

Figure 2. Diurnal pattern in enteric methane emissions of lactating dairy cows fed 3-nitrooxypropanol (3-NOP). Treatments were control and 3-NOP mg/kg of feed DM (40, 60, 80, 100, 150, and 200; 40NOP, 60NOP, 80NOP, 100NOP, 150NOP, and 200NOP, respectively). Methane emissions were measured using the GreenFeed system (C-Lock Inc., Rapid City, SD). Data are treatment covariate-adjusted LSM and bars represent SE; $n = 7$ (number of independent data points for each mean value). Main effect of treatment ($P < 0.001$). Treatment $\times$ study phase interaction, $P = 0.32$. The average decrease ($P \leq 0.03$), across 3-NOP application rates, compared with the control for each measurement time point (see Materials and Methods) was 26, 26, 26, 35, 33, 30, 31, and 28% for 0000, 0300, 0500, 0900, 1200, 1500, 1700, and 2100 h, respectively.

fect of 3-NOP on BW appeared to differ between beef (Romero-Pérez et al., 2015; Martinez-Fernandez et al., 2018; Vyas et al., 2018) and dairy cattle (Haisan et al., 2014; Hristov et al., 2015a; Melgar et al., 2019, 2020), but the current short-term experiment was not designed to elucidate effects of 3-NOP on BW.

Inclusion dose of 3-NOP quadratically increased ($P = 0.02$) the sum of de novo synthesized fatty acids in milk (Table 4). In contrast, no effects on milk fatty acids profile was reported by Reynolds et al. (2014) when 3-NOP was dosed directly into the rumen where they observed a transient CH_4 inhibition effect. In dairy cows, 50% of milk fatty acids are formed from de novo synthesis in the mammary gland (Bauman and Davis, 1974). Thus, the increase in milk fat concentration and yield observed in the current experiment and in Melgar et al. (2019) likely resulted from a shift in rumen fermentation toward increased production of short-chain milk fatty acids precursors, such as butyrate (Black et al., 1961; Ellis et al., 2008; Dijkstra et al., 2011). If rumen VFA absorption rate is not affected by treatment, it is expected that VFA concentrations would

be approximately representative of VFA production rates (Sutton et al., 2003). Several studies had reported increased molar proportion of butyrate in rumen fluid of dairy cows fed 3-NOP (Lopes et al., 2016; Haisan et al., 2017; Melgar et al., 2020). Although Melgar et al. (2020) did not report an effect of 3-NOP inclusion on molar proportion of propionate in early-lactation cows, it is important to indicate that in line with the theory of changes in fermentation pattern when methanogenesis is inhibited (Wolin, 1960; Marty and Demeyer, 1973), an increase in propionate was observed by Haisan et al. (2014), Romero-Pérez et al. (2015), and Lopes et al. (2016) in studies with beef or dairy cattle fed 3-NOP.

In the current study, increasing 3-NOP dose decreased linearly ($P = 0.002$) the sum of *trans* fatty acids, particularly *trans*-18:1 isomers, in milk fat. Based on milk fatty acid composition in the current experiment, it appeared that rumen biohydrogenation was following the normal pathways (Bauman and Griinari, 2003). The increased ($P = 0.03$) concentration of SFA and the tendency for decreased ($P = 0.07$) MUFA (and to a lesser extent PUFA, $P = 0.12$) in milk fat observed

Table 3. Dose-response effects of 3-nitrooxypropanol (3-NOP) on milk composition, BW, and BW change in lactating dairy cows

Item	Treatment ¹								P-value ³		
	CON	40NOP	60NOP	80NOP	100NOP	150NOP	200NOP	SEM ²	C vs. Trt	L	Q
Milk fat, %	3.20	3.86	3.94	3.97	3.70	4.04	4.16	0.229	0.001	0.005	0.18
Yield, kg/d	1.37	1.54	1.83	1.67	1.60	1.64	1.95	0.131	0.01	0.004	0.74
Milk true protein, %	3.03	3.08	3.06	2.99	3.07	3.03	3.08	0.046	0.65	0.67	0.63
Yield, kg/d	1.31	1.25	1.38	1.28	1.37	1.21	1.42	0.056	0.88	0.37	0.36
Milk lactose, %	4.84	4.83	4.82	4.84	4.83	4.88	4.77	0.041	0.85	0.57	0.38
Yield, kg/d	2.10	1.98	2.18	2.03	2.13	2.00	2.22	0.087	0.90	0.41	0.37
MUN, mg/dL	8.75	9.69	9.06	10.2	9.04	10.6	11.2	0.67	0.08	0.005	0.69
SCC ⁴ × 10 ³ cells/mL	65.5	105.7	76.5	80.9	37.1	77.1	101.6	27.71	0.39	0.89	0.82
BW, kg	638	635	630	639	635	627	632	6.3	0.41	0.28	0.87
BW change, kg	-4.11	-6.11	-11.7	-3.39	-4.69	-11.5	-8.66	6.116	0.52	0.46	0.92

¹Treatments were control (CON) and 3-NOP mg/kg feed DM (40, 60, 80, 100, 150, and 200 for 40NOP, 60NOP, 80NOP, 100NOP, 150NOP, and 200NOP, respectively). Measured concentration of 3-NOP in TMR were 0, 37, 55, 73, 92, 137, and 183 mg/kg feed DM, for each dose level, respectively. Data are covariate-adjusted LSM.

²Largest SEM published in table; n = 49 (n represents number of observations used in the statistical analysis).

³Contrasts: C vs. Trt, control vs. all 3-NOP treatments; L, linear effect of 3-NOP dose; Q, quadratic effect of 3-NOP dose.

⁴Statistical analysis was performed on log-transformed data.

⁵Average BW during the last week of treatment period.

⁶Average BW during the last week of treatment period – average BW during the last week of covariate period.

in the current study and also reported by Hristov et al. (2015a) suggest that rumen biohydrogenation may have provided an additional, although minor (Nagaraja et al., 1997), sink for H₂ in cows fed 3-NOP. Although data are not conclusive, our results suggest that rumen biohydrogenation may be enhanced by 3-NOP. According to Lourenço et al. (2010), biohydrogenation is not necessarily stimulated by availability of H₂ itself but may have been affected through other H₂-utilization processes.

Compared with control, sum of C16 fatty acids remained unchanged by 3-NOP, whereas concentration of the sum of preformed (>C16) fatty acids in milk fat tended to decrease ($P = 0.08$) with 3-NOP inclusion. The sum of odd-and branched-chain fatty acids tended ($P = 0.08$) to generally increase with 3-NOP dose. Changes in the concentrations of *trans* fatty acids can be explained by changes in microbial processes in the rumen that induce a shift in the molar proportions of VFA toward decreasing acetate and increasing propionate (Van Soest, 1963; Bauman and Grinari, 2003). A decrease in the molar proportion of acetate is commonly observed in 3-NOP-fed cows (Reynolds et al., 2014; Haisan et al., 2014; Lopes et al., 2016) and can be related to H₂ accumulation due to the inhibition of CH₄ synthesis (van Lingen et al., 2016). Melgar et al. (2020) showed that inclusion of 3-NOP decreased rumen acetate molar proportion and did not affect propionate molar proportion, but increased butyrate and valerate molar proportions of total VFA in ruminal fluid.

We can determine by calculation that the decrease in daily enteric CH₄ emissions (by 96 to 176 g/d of CH₄) would have increased the availability of feed digestible energy from 1.25 to 2.29 Mcal/d. If energy in CH₄ synthesized in the rumen is converted to NE_L with efficiency similar to that of dietary digestible energy (NRC, 1981), and we assume no differences in efficiency of energy use between treatments (and no change in BW), the reduction in emitted CH₄ with 3-NOP would represent, in theory, an additional (over the control) 0.40, 0.44, 0.43, 0.74, 0.72, and 0.71 Mcal/d NE_L for 40NOP, 60NOP, 80NOP, 100NOP, 150NOP, and 200NOP, respectively, that could potentially be used for productive purposes. Previous studies in mid- to late-lactation cows reported from 0.26 (Hristov et al., 2015a) to 0.34 (Melgar et al., 2019) Mcal/cow NE_L per day digestible energy gain as a result of the reduction of emitted enteric CH₄ with the inclusion of 3-NOP at 60 mg/kg feed DM. In an early-lactation study, when maintenance and milk production requirements exceed the available energy from feed intake, Melgar et al. (2020) reported a potential increase in NE_L availability of 0.29 Mcal/d in 3-NOP-fed cows, which could support an additional milk yield of 0.39 kg/d. In the

Table 4. Dose-response effects of 3-nitrooxypropanol (3-NOP) on fatty acid composition of milk fat (g/100 g of total fatty acids) in lactating dairy cows

Item	Treatment ¹							P-value ³			
	CON	40NOP	60NOP	80NOP	100NOP	150NOP	200NOP	SEM ²	C vs. Trt	L	Q
4:0	4.03	4.59	4.77	4.93	4.93	5.13	5.24	0.230	<0.001	<0.001	0.11
6:0	1.90	2.40	2.50	2.51	2.57	2.84	2.68	0.158	<0.001	<0.001	0.02
8:0	0.98	1.29	1.40	1.38	1.43	1.61	1.44	0.114	<0.001	<0.001	0.01
10:0	2.24	2.84	3.18	3.05	3.18	3.63	3.12	0.306	0.003	0.01	0.03
12:0	2.67	3.14	3.53	3.40	3.46	3.92	3.38	0.328	0.02	0.04	0.05
14:0	9.60	9.80	10.24	9.92	9.78	10.01	9.35	0.395	0.56	0.62	0.19
cis-9 14:1	0.98	0.83	0.78	0.85	0.65	0.71	0.68	0.109	0.03	0.03	0.28
15:0	1.09	1.17	1.24	1.02	0.96	1.03	0.86	0.114	0.70	0.05	0.54
16:0	24.6	25.2	23.9	24.1	22.9	24.8	25.2	0.79	0.74	0.67	0.10
cis-9 16:1	1.50	1.22	1.11	1.12	0.81	0.89	1.27	0.152	0.006	0.08	0.001
17:0	0.49	0.49	0.50	0.49	0.46	0.46	0.47	0.022	0.68	0.25	0.79
18:0	11.0	11.6	11.9	12.1	13.6	13.0	11.9	0.852	0.14	0.21	0.08
trans-4 18:1	0.029	0.025	0.026	0.029	0.028	0.026	0.020	0.0031	0.22	0.03	0.22
trans-5 18:1	0.026	0.018	0.023	0.022	0.020	0.019	0.017	0.0026	0.02	0.02	0.70
trans-6,8 18:1	0.69	0.53	0.53	0.52	0.42	0.34	0.40	0.081	0.005	0.002	0.16
trans-9 18:1	0.53	0.44	0.42	0.44	0.36	0.30	0.35	0.057	0.01	0.003	0.21
trans-10 18:1	3.44	2.05	2.05	1.87	0.98	0.52	1.08	0.809	0.02	0.01	0.15
trans-11 18:1	1.40	1.32	1.25	1.17	1.23	1.04	1.42	0.263	0.50	0.77	0.26
trans-12 18:1	0.80	0.63	0.71	0.67	0.69	0.60	0.57	0.059	0.007	0.004	0.61
cis-9 18:1	20.0	18.8	18.5	18.9	19.9	17.8	19.3	0.93	0.26	0.56	0.39
cis-11 18:1	1.11	0.91	0.92	0.86	0.77	0.69	0.73	0.087	0.003	<0.001	0.13
cis-9, cis-12 18:2	3.38	3.17	3.24	3.20	3.27	3.12	3.39	0.148	0.32	0.98	0.18
18:3	0.49	0.46	0.47	0.46	0.48	0.44	0.46	0.019	0.16	0.15	0.60
20:0	0.12	0.12	0.12	0.12	0.15	0.13	0.11	0.008	0.53	0.72	0.04
cis-9, trans-11 CLA	0.73	0.68	0.59	0.53	0.54	0.42	0.63	0.088	0.06	0.08	0.03
cis-12, trans-10 CLA	0.013	0.009	0.008	0.014	0.003	<0.001	0.007	0.0065	0.38	0.26	0.60
Others	6.14	6.31	6.13	6.30	6.47	6.49	5.98	0.233	0.55	0.94	0.14
Total trans fatty acids	6.92	5.02	5.01	4.71	3.73	2.85	3.85	0.871	0.003	0.002	0.06
Σ SFA	54.7	58.0	58.5	58.1	58.4	61.5	58.4	1.85	0.03	0.05	0.11
Σ MUFA	30.5	26.8	26.3	26.5	25.9	22.9	25.9	1.71	0.07	0.01	0.06
Σ PUFA	4.61	4.32	4.31	4.21	4.30	3.98	4.48	0.209	0.12	0.40	0.07
Σ De novo ⁴	22.4	24.9	26.4	26.0	26.0	27.9	25.9	1.21	0.003	0.01	0.02
Σ Mixed ⁴	26.1	26.4	25.0	25.2	23.7	25.7	26.4	0.78	0.37	0.91	0.02
Σ Preformed ⁴	45.1	42.2	42.1	42.3	43.9	39.8	41.7	1.70	0.08	0.10	0.45
Σ OBCFA ⁵	3.03	3.17	3.32	3.12	3.02	3.42	2.91	0.185	0.08	0.84	0.23

¹Treatments were control (CON), and 3-NOP mg/kg feed DM (40, 60, 80, 100, 150, and 200 for 40NOP, 60NOP, 80NOP, 100NOP, 150NOP, and 200NOP, respectively). Measured concentration of 3-NOP in TMR were 0, 37, 55, 73, 92, 137, and 183 mg/kg feed DM, for each dose level respectively. Data are LSM.

²Largest SEM published in table; n = 49 (n represents number of observations used in the statistical analysis).

³Contrasts: C vs. Trt, control vs. all 3-NOP treatments; L, linear effect of 3-NOP dose; Q, quadratic effect of 3-NOP dose.

⁴De novo fatty acids (<C16) are synthesized by the mammary gland; preformed fatty acids (>C16) originate primarily from extraction from plasma; and mixed fatty acids (C16) originate from both sources.

⁵Odd- and branched-chain fatty acids. Sum of C11:0, *iso* C13:0, *anteiso* C13:0, C13:0, *iso* C14:0, *anteiso* C15:0, C15:0, *iso* C16:0, *anteiso* C17:0, C17:0, and C17:1 *cis*-9.

current experiment, however, the spared CH₄ energy was most likely used for milk fat synthesis and lactose/milk volume were not affected by treatment, and in fact, a trend for decreased milk volume by 3-NOP was observed, which was a result of the trend for decreased DMI (as feed efficiency was not affected by treatment).

CONCLUSIONS

Administration of 3-NOP via the TMR at a target concentration of 0 to 200 mg/kg feed DM quadratically decreased daily enteric CH₄ emissions from 22 to 40%, with an average reduction of 31%. Similar effects were observed for CH₄ emission yield and intensity with average reduction by 26 and 33%, respectively. Maximum mitigation effect of 3-NOP was achieved with 150 mg/kg feed DM, but no statistical difference was observed among the 3 highest doses (100, 150, and 200 mg/kg). Within the 3-NOP doses studied, the average reduction in CH₄ was 26% when expressed as g/kg of feed DM consumed and 33% when expressed in g/kg ECM yield. In the conditions of this short-term experiment, 3-NOP did not affect DMI and milk yield of the cows, but overall, treatment linearly increased milk fat concentration and yield and increased ECM feed efficiency. Concentrations of de novo synthesized fatty acids in milk fat were also increased by 3-NOP, suggesting increased energy availability with 3-NOP, compared with the control. These results suggest that 3-NOP is a promising feed additive for reducing enteric CH₄ emissions, while maintaining lactational performance in dairy cows and potentially increasing milk fat yield.

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