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Enteric methane emission, milk production, and composition of dairy cows fed 3-nitrooxypropanol

A. Melgar,[1] C. F. A. Lage,[1,2] K. Nedelkov,[1,3] S. E. Räisänen,[1] H. Stefenoni,[1] M. E. Fetter,[1] X. Chen,[1,4] J. Oh,[1]* S. Duval,[5] M. Kindermann,[6] N. D. Walker,[6] and A. N. Hristov[1]†
[1]Department of Animal Science, The Pennsylvania State University, University Park 16802
[2]Department of Animal Science, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil 31270-901
[3]Department of Animal Husbandry, Faculty of Veterinary Medicine, Trakia University, Stara Zagora 6000, Bulgaria
[4]School of Computing, University of Ulster, Co. Antrim, Northern Ireland, BT37 0QB, United Kingdom
[5]Research Centre for Animal Nutrition and Health, DSM Nutritional Products, Saint Louis Cedex 68305, France
[6]Department of Animal Nutrition, DSM Nutritional Products, Wurmisweg 576, 4303 Kaiseraugst, Switzerland

ABSTRACT

This study examined the effect of 3-nitrooxypropanol (3-NOP), an investigational substance, on enteric methane emission, milk production, and composition in Holstein dairy cows. Following a 3-wk covariate period, 48 multi- and primiparous cows averaging (± standard deviation) 118 ± 28 d in milk, 43.4 ± 8 kg/d milk yield, and 594 ± 57 kg of body weight were blocked based on days in milk, milk yield, and enteric methane emission and randomly assigned to 1 of 2 treatment groups: (1) control, no 3-NOP, and (2) 3-NOP applied at 60 mg/kg feed dry matter. Inclusion of 3-NOP was through the total mixed ration and fed for 15 consecutive weeks. Cows were housed in a freestall barn equipped with a Calan Broadbent Feeding System (American Calan Inc., Northwood, NH) for monitoring individual dry matter intake and fed ad libitum once daily. Enteric gaseous emissions (methane, carbon dioxide, and hydrogen) were measured using 3 GreenFeed (C-Lock Inc., Rapid City, SD) units. Dry matter intake, cow body weight, and body weight change were not affected by 3-NOP. Compared with the control group, 3-NOP applied at 60 mg/kg feed dry matter decreased daily methane emission, emission yield, and emission intensity by 26, 27, and 29%, respectively. Enteric emission of carbon dioxide was not affected, and hydrogen emission was increased 6-fold by 3-NOP. Administration of 3-NOP had no effect on milk and energy-corrected milk yields and feed efficiency, increased milk fat and milk urea nitrogen concentrations, and increased milk fat yield but had no other effects on milk components. Concentration of C6:0 and C8:0 and the sum of saturated fatty acids in milk fat were increased by 3-NOP. Total *trans* fatty acids and the sum of polyunsaturated fatty acids were decreased by 3-NOP. In this experiment, 3-NOP decreased enteric methane daily emission, yield, and intensity without affecting dry matter intake and milk yield, but increased milk fat in high-producing dairy cows.

Key words: 3-nitrooxypropanol, enteric methane, milk fat, dairy cattle

INTRODUCTION

The global livestock industries are facing challenges related to enteric methane (CH_4) emission. Enteric CH_4 contributes significantly to anthropogenic greenhouse gas emissions worldwide (Gerber et al., 2013), and enteric CH_4 represents an energy loss of 2 to 12% of the total feed energy consumed by ruminants (Johnson and Johnson, 1995). A synthetic compound, 3-nitrooxypropanol (**3-NOP**), which is a structural analog to methyl-coenzyme M, was identified as a potential enteric CH_4 inhibitor by Duval and Kindermann (2012). The 3-NOP molecule is a specific inhibitor of methyl-coenzyme M reductase (**MCR**), the enzyme involved in the biological synthesis and anaerobic oxidation of CH_4 in methanogenic archaea (Ermler et al., 1997). Thus, 3-NOP inhibits the reduction of CO_2 by dissolved H_2 to form CH_4 by targeting the active site of MCR in the terminal step of methanogenesis in rumen archaea (Duin et al., 2016).

Several experiments have examined the effects of 3-NOP on enteric CH_4 production and lactational performance in dairy cows. From the studies of Haisan et al. (2014), Reynolds et al. (2014), and Hristov et al. (2015) to the most recent (Van Wesemael et al., 2019; Melgar et al., 2020a,c), the reported antimethanogenic

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*Current address: Cargill Animal Nutrition, Seongnam, South Korea 13630.
†Corresponding author: anh13@psu.edu

effects of 3-NOP have been consistent. The extent of enteric CH_4 mitigation by 3-NOP in cattle appears to be affected by the inclusion level and administration technique of 3-NOP and the diet composition (Dijkstra et al., 2018). Mixing 3-NOP in the TMR allows its continual consumption throughout the day and has been shown to be effective to decrease daily enteric CH_4 emission, emission yield, and emission intensity (Hristov et al., 2015; Vyas et al., 2018b; Melgar et al., 2020a). As demonstrated in an analysis by Hristov and Melgar (2019), even if included in the TMR, the mitigation effect of 3-NOP varies due to variation in DMI and therefore 3-NOP intake during a 24-h feeding cycle.

Short-term preference studies involving dietary supplementation with 3-NOP have shown that 3-NOP is not unpalatable and therefore does not affect DMI in beef (Lee et al., 2019) or dairy cattle (Melgar et al., 2020b), and it had no effect on milk yield in long-term production studies (Hristov et al., 2015; Van Wesemael et al., 2019; Melgar et al., 2020a). However, there have been inconsistent reports of effects of 3-NOP on milk components in dairy cattle. Reynolds et al. (2014) observed an increase in milk protein and casein. A tendency to increase true milk protein was reported by Hristov et al. (2015). Milk fat concentration increased (Lopes et al., 2016; Melgar et al., 2020c) and milk fat yield increased (Melgar et al., 2020c) or tended to increase (Lopes et al., 2016) with 3-NOP supplementation. In contrast, Reynolds et al. (2014) reported no effects of 3-NOP on milk fat concentration or milk fat yield. Furthermore, Reynolds et al. (2014) reported no effect of 3-NOP on milk fatty acid (**FA**) profile, whereas in other studies an increase was observed in the sum of SFA (Hristov et al., 2015; Melgar et al., 2020c) and in de novo synthetized short-chain milk FA (Hristov et al., 2015; Melgar et al., 2020a,c), and a decrease in *trans* FA was also observed by Melgar et al. (2020c). Some studies reported an increase in MUN (Melgar et al., 2020a,c); others, however, found no effect of 3-NOP on MUN (Reynolds et al., 2014; Lopes et al., 2016; Haisan et al., 2017).

The effect of 3-NOP on enteric CH_4 emission has been consistent but further long-term studies are required to conclusively determine its effects on milk production and composition in dairy cows, which would be critically important for future adoption of this CH_4 mitigation practice. Therefore, the objective of this study was to investigate the effect of 3-NOP on enteric CH_4 emission and milk production and composition in high-producing dairy cows. Our hypothesis was that, similar to previous research, 3-NOP will decrease CH_4 emission and emission yield and intensity, would not affect milk production, and may increase milk fat concentration and yield in dairy 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.

Animals and Experimental Design

The study initially involved 48 multi- (30) and primiparous (18) Holstein cows averaging (±SD) 118 ± 28 DIM, 43.4 ± 8 kg/d milk yield, and 594 ± 57 kg of BW at the beginning of the experiment. Four blocks of cows (i.e., a total of 8 cows) were removed at various stages of the experiment for the following reasons: one cow from one block was removed due to limping, one cow from a second block due to distortion on her right frontal leg, one cow from a third block because she never visited GreenFeed, and one cow from a fourth block due to very low (77%) DMI. The blocking pairs of the removed cows were also removed from the study. Thus, data for 40 cows were used in the statistical analysis. Cows were housed in a freestall barn at The Pennsylvania State University's Dairy Teaching and Research Center (University Park), equipped with a Calan Broadbent Feeding System (American Calan Inc., Northwood, NH) for individual monitoring of DMI and 3 units of GreenFeed system (C-Lock Inc., Rapid City, SD) for continuous measurements of enteric CH_4, CO_2, and H_2 emissions.

The experimental design was a randomized complete block design with a 3-wk covariate period at the beginning of the study and a 15-wk experimental period. Following the covariate period, cows were blocked in 20 blocks of 2 based on their lactation number, DIM, current milk production (or genetic potential for milk, if primiparous), and enteric CH_4 emission. Cows within block were randomly assigned to 1 of 2 experimental treatments: (1) control, placebo (no 3-NOP), and (2) 3-NOP applied at 60 mg/kg feed DM.

Cows had free access to drinking water and feeding was ad libitum (aiming at 10% refusals) once a day at approximately 0800 h. Cows were fed a basal TMR (Table 1) for 21 d during the covariate period. Diet was formulated to meet or exceed the NE_L and MP requirements of a mid-lactation cow (BW = 616 kg, milk yield = 38 kg/d, milk fat = 3.50%, milk protein = 3.10%, and DMI = 25.9 kg) according to NRC (2001). The composition of the TMR was the same during both covariate and experimental periods. The basal TMR was prepared using a stationary mixer (Electra-Mix, model 1062, I. H Rissler, Mohnton, PA) 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 3-NOP or placebo supplement (DSM Nutritional Products, Basel, Switzerland), prepared twice a week, kept at 4°C in sealed containers with no head space, and mixed daily with the TMR before feeding. The active supplement contained 10.9% 3-NOP on a carrier of SiO_2 and propylene glycol; the placebo supplement contained SiO_2 and propylene glycol only. Separate feed mixers (Rissler Mobile TMR Mixer Model 1050; I. H. Rissler) were used to mix the control and 3-NOP TMR.

Sampling and Measurements

Diet and Feed Ingredient Analyses. 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 were determined weekly and used to calculate DMI from the as-fed TMR intake. Samples of the forages were collected once weekly, concentrate feeds every 2 wk, 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) and submitted to Cumberland Valley Analytical Services (Waynesboro, PA) for wet chemistry analyses using methods described in CVAS (2018). Specifically, the methods were 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). Concentrations of RDP, RUP, NE_L, and NFC and NE_L balance were estimated based on NRC (2001) using averaged DMI, milk yield, milk composition, and BW of the cows during the experiment.

During experimental wk 1, 7, and 14, samples of control and 3-NOP TMR, including the basal TMR without added premixes, were collected, kept frozen at −20°C, and submitted for analysis of 3-NOP concentration (DSM Nutritional Products, Global R&D Analytics, Kaiseraugst, Switzerland).

Enteric Gas Emissions. During the covariate and experimental periods, 3 GreenFeed units were permanently available for individual cows to visit, and during visits, enteric gas emissions were measured. A pelletized bait feed (Stocker Grower 14, Purina Animal Nutrition LLC, Shoreview, MN) was available at each cow visit and the weight of pellets dispensed was recorded and

Table 1. Ingredient and chemical composition of the basal diet fed to dairy cows in the experiment

Item	Diet[1]
Feed ingredient, % of diet DM	
Corn silage[2]	39.8
Alfalfa haylage[3]	16.5
Grass hay[4]	4.0
Corn grain, ground[5]	11.7
Soybean seeds, roasted[6]	8.4
Canola meal[7]	8.1
SoyPlus[8]	5.0
Molasses[9]	4.6
Mineral and vitamin premix[10]	1.9
Composition,[11] % of DM (or as indicated)	
CP[11]	16.4
RDP[12]	9.7
RUP[12]	6.6
NDF[11]	31.4
ADF[11]	21.2
Starch	22.5
Ether extract	4.9
NE_L,[11] Mcal/kg	1.57
Average NE_L balance,[13] Mcal/d	4.2
Average MP balance,[13] g/d	198
NFC[12]	43.3
Ash[11]	6.51
Ca[11]	0.80
P[11]	0.38

[1]Average basal diet composition.

[2]Corn silage was 35.9% DM and contained (DM basis) 6.6% CP, 39.5% NDF, and 31.5% starch.

[3]Haylage was 33.9% DM and contained (DM basis) 21.7% CP and 41.3% NDF.

[4]Grass hay was 87.1% DM and contains (DM basis) 11.5% CP and 68.5% NDF.

[5]Corn grain was 89.3% DM and contained (DM basis) 6.7% CP.

[6]Soybean seeds, roasted, were 95.4% DM and contained (DM basis) 34.9% CP.

[7]Canola meal was 90.2% DM and contained (DM basis) 39.6% CP.

[8]SoyPlus (West Central Cooperative, Ralston, IA) contained (DM basis) 46.6% CP.

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

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

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

[12]Estimated based on NRC (2001).

[13]Estimated based on NRC (2001) using actual DMI, milk yield, milk composition, and BW of the cows throughout the experiment.

included in the daily DMI estimation. Cows were identified with a unique radio-frequency identification ear tag and were adapted to using the GreenFeed before the beginning of the experiment. GreenFeed units were calibrated following the manufacturer's recommendations (http://greenfeed.c-lockinc.com). GreenFeed is equipped with a head position sensor and gas emission data are rejected when the cow's head position criteria are not met. Each cow was allowed a maximum of 6 visits in 24 h, with a 4-h interval between visits, and not more than 12 feed drops of approximately 35 g each per visit. A total of 21,343 GreenFeed visits (an average of 5 visits/cow per day) were collected and processed from the study. Weekly average DMI and milk and ECM yields were used to estimate weekly averages of CH_4 and CO_2 yields (i.e., g/kg DMI) and intensity (i.e., g/kg milk or ECM).

Milk Production and Composition. Cows were milked twice daily at 0600 and 1800 h and milk production of the cows was recorded daily at each milking. Milk samples were collected from 2 consecutive milkings (p.m. on Wednesdays and a.m. on Thursdays) weekly during the entire experiment. One aliquot of each milk sample was placed in tubes with a preservative (2-bromo-2-nitropropane-1,3-diol) and samples submitted to Dairy One Cooperative Inc. (Ithaca, NY) for analysis of milk fat, true protein, lactose, SCC, TS, and MUN using Milkoscan models 6000, FT+, and Fossomatic models 5000 of FC (Foss Electric A/S, Hillerød, Denmark). The individual cow average daily milk yields and milk compositions during each study week were used to calculate yields of milk fat, true protein, and lactose. Energy-corrected milk was calculated according to Sjaunja et al. (1990): ECM (kg/d) = kg of milk × [(38.3 × % fat × 10 + 24.2 × % true protein × 10 + 16.54 × % lactose × 10 + 20.7) ÷ 3,140]. Another milk sample was collected and placed in tubes without preservative and stored at −20°C until composited as follows: one sample for the covariate period and wk 4 to 6, 7 to 9, 10 to 12, and 13 to 15, and analyzed for milk FA composition as described in Rico and Harvatine (2013) and casein (Barbano and Sherbon, 1984). Cows receiving 3-NOP were milked after the control cows, and milk from 3-NOP cows was discarded for the duration of the study and for an additional 7 d upon completion of the study.

Body Weight and Body Weight Change. Cow BW was recorded twice daily upon exiting the milking parlor using an AfiFarm 3.04E scale system (S.A.E. Afikim, Rehovot, Israel). Body weight change was calculated in grams per day as the difference between average BW during the last week of treatment period (i.e., wk 15) and the average BW during the third week of the covariate period and divided by 105 (days on study).

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. Dry matter intake and enteric gas emission data were processed for outliers' identification based on an absolute studentized residual value >3 (PROC REG of SAS), and along with milk yield and milk composition were averaged per 3-wk periods (i.e., 200 averages for the experiment). The outlier analysis identified one cow that was removed, along with her blocking pair, from the study due to her low DMI. In total, 4 blocks of cows were removed from the study and the analysis was performed with 40 cows (24 multiparous and 16 primiparous). The model used was as follows:

$$Y_{ijk} = \mu + B_i + \tau_j + B\tau_{ij} + W_k + \tau W_{jk} + e_{ijk},$$

where Y_{ijk} is the dependent variable, μ is the overall mean, B_i is the block, τ_j is treatment (control vs. 3-NOP), $B\tau_{ij}$ is the block × treatment interaction, W_k is the study week period (1 to 5; a study week period was the average of 3 experimental weeks), and τW_{jk} is the treatment × study week interaction, with the error term e_{ijk} assumed to be normally distributed with mean = 0 and constant variance. Block and block × treatment were random effects and all others were fixed. Data were analyzed as repeated measures using AR(1) covariance structure with study week period being the repeated term. All data are presented as covariate-adjusted least squares means. Statistical differences were considered significant at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

In this experiment, high-producing dairy cows fed 3-NOP administered via the TMR at 60 mg/kg feed DM decreased ($P \leq 0.001$) daily enteric CH_4 emission by 26%, increased ($P \leq 0.001$) 6-fold H_2 emission, and did not affect CO_2 emission (Table 2). Compared with control, the reduction of CH_4 emission yield and intensity by 3-NOP was 27 and 29%, respectively ($P \leq 0.001$). These results are consistent with observations in long-term experiments with dairy cattle where the decrease in daily emission of enteric CH_4 by 3-NOP was from approximately 26 to 30% (Hristov et al., 2015; Van

Table 2. Effect of the methane inhibitor 3-nitrooxypropanol (3-NOP) on gas emissions in dairy cows

Item	Treatment[1]		SEM[2]	*P*-value[3]
	Control	3-NOP		
CH_4				
CH_4, g/d	411	301	6.10	<0.001
CH_4 per DMI, g/kg	16.4	11.9	0.25	<0.001
CH_4 per milk yield, g/kg	11.3	8.2	0.37	<0.001
CH_4 per ECM,[4] g/kg	11.6	8.2	0.29	<0.001
CO_2				
CO_2, g/d	13,360	13,167	122.9	0.28
CO_2 per DMI, g/kg	531	519	6.91	0.19
CO_2 per milk yield, g/kg	363	359	12.0	0.80
H_2, g/d	0.4	2.6	0.12	<0.001

[1]Treatments were control (basal diet) and 3-NOP (basal diet plus 60 mg of 3-NOP/kg of feed DM). Data are covariate-adjusted LSM.

[2]Largest SEM published in table; n = 200 (n represents number of observations used in the statistical analysis). Data are from 40 cows.

[3]Main effect of treatment.

[4]ECM (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).

Wesemael et al., 2019; Melgar et al., 2020a). All these studies incorporated 3-NOP into the TMR at target inclusion rates from 40 to 80 mg/kg feed DM, allowing continual consumption of the inhibitor throughout the day.

The mitigation effect of 3-NOP persisted over the 15 wk of treatment (Figure 1) and was consistent with reports from long-term studies with dairy cows by Hristov et al. (2015), Van Wesemael et al. (2019), and Melgar et al. (2020a). Analysis of the diurnal variability in the relationship between DMI and enteric CH_4 emission of data collected in the current experiment revealed that CH_4 emission and DMI were the lowest 2 h before feeding and highest within 6 h after feeding (Hristov and Melgar, 2019). The maximum mitigation effect of 3-NOP (45% decrease) was observed immediately after feeding, persisted within 10 h after feeding (an average of 35% decrease), decreased beyond 20 h after feeding (average of 13% decrease), and was nonexistent 2 h before feeding, which is consistent with the concept that 3-NOP must continuously enter the rumen to be an efficient mitigant.

Hydrogen and CO_2 are the main energy substrate of rumen methanogenesis (Wolin, 1981). It is expected that inhibition of rumen methanogenesis causes accumulation of H_2 in the rumen (Trei et al., 1972). It has been observed that H_2 accumulates when methanogenesis is inhibited by 3-NOP (Hristov et al., 2015; Melgar et al., 2020a); therefore, the 3-NOP molecule is a specific inhibitor of MCR isoform II, which enables archaea to utilize H_2 at higher concentrations (McAllister et al., 2015; Tapio et al., 2017). Hydrogen emission remained 6-fold greater for 3-NOP cows over the 15 wk of treatment compared with the control group (Figure 2). Because methanogenesis is one of the main H_2 sinks in rumen fermentation, the increase in H_2 emission can be directly associated with the inhibition of methanogenesis by 3-NOP. Increased concentration of formate and ethanol, other potential H_2 sinks, in ruminal fluid was also observed under 3-NOP supplementation (Melgar et al., 2020a). Similar increases in H_2 emission were observed in both early-lactation cows (Melgar et al., 2020a) and mid-to-late-lactation cows (Hristov et al., 2015) fed 3-NOP, as well as other inhibitors of enteric CH_4 (Trei et al., 1972; Demeyer and Van Nevel, 1975; Russell and Martin, 1984). Increased H_2 emission is a reflection of accumulation of dissolved H_2 in ruminal fluid (Melgar et al., 2020a), although the relationship is relatively weak (Wang et al., 2016). If 4 mol of H_2 is required to yield 1 mol of CH_4, the 110 g/d, or 6.9 mol/d, average reduction (411 vs. 301 g/d) in CH_4 emission with 3-NOP in the current experiment would represent 27.2 mol/d H_2 not being used for CH_4 synthesis. The increase in gaseous H_2 emission of about 1 mol/d (3-NOP cows) is clearly only a small fraction of the H_2 not used for CH_4 synthesis in 3-NOP cows. We have observed and discussed this discrepancy in our previous studies (Hristov et al., 2015; Melgar et al., 2020a); apart from CH_4, other potential sinks for H_2 (not measured in the current study but increased concentrations were reported by Melgar et al., 2020a) are some rumen VFA (specifically propionate, but also

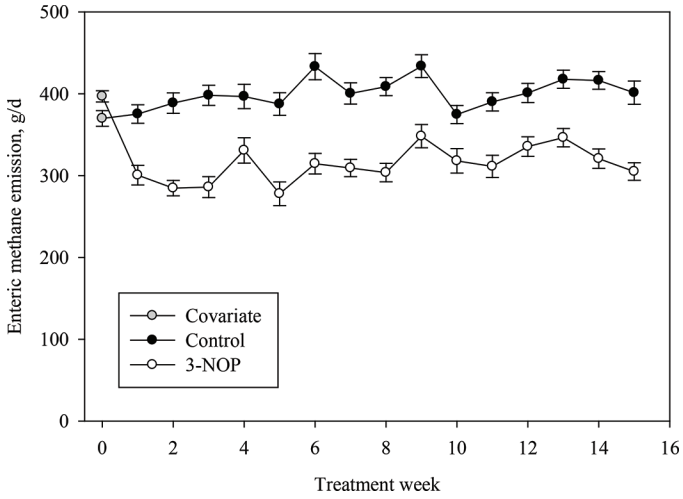

Figure 1. Effect of the methane inhibitor 3-nitrooxypropanol (3-NOP) on enteric methane emission in lactating dairy cows over the course of the experiment. Treatments were control (basal diet) and 3-NOP (basal diet plus 60 mg of 3-NOP/kg of feed DM). Data are covariate-adjusted LSM, and error bars represent SEM; n = 20 [number of independent data points (i.e., 20 cows) used for each mean value]. Main effect of treatment ($P < 0.001$); effect of week, $P < 0.001$; treatment × week interaction, $P = 0.07$.

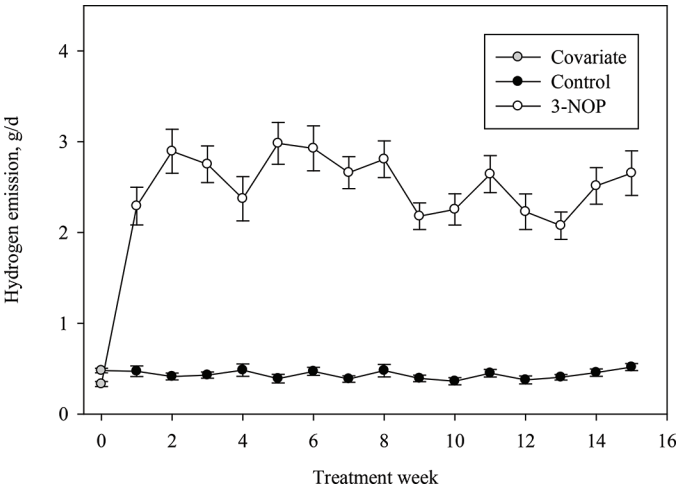

Figure 2. Effect of the methane inhibitor 3-nitrooxypropanol (3-NOP) on hydrogen emission in lactating dairy cows over the course of the experiment. Treatments were control (basal diet) and 3-NOP (basal diet plus 60 mg of 3-NOP/kg of feed DM). Data are covariate-adjusted LSM, and error bars represent SEM; n = 20 [number of independent data points (i.e., 20 cows) used for each mean value]. Main effect of treatment ($P < 0.001$); effect of week, $P = 0.18$; treatment × week interaction, $P = 0.39$.

butyrate and valerate), microbial mass (microbial long-chain FA), formate, ethanol, and above all the dissolved H_2 pool in the rumen (Melgar et al., 2020a).

The lack of a significant effect of 3-NOP on CO_2 emission yield in cattle has been consistent among studies (Hristov et al., 2015; Van Wesemael et al., 2019; Melgar et al., 2020a,c). About 1/4 to 1/3 of CO_2 produced during OM fermentation by rumen microorganisms is absorbed into blood and removed through the lungs by respiration (Kinsman et al., 1995). Only a small fraction of CO_2 emission measured with GreenFeed is from rumen fermentation. A study by Caetano et al. (2018) reported that from 6 to 20% of the total CO_2 output per day in cattle was composed of rumen-derived CO_2.

The decrease in enteric CH_4 emission with 3-NOP was not associated with decreased DMI and milk yield of the cows in the current experiment (Table 3). Overall, long-term studies have shown no negative effects of feeding 3-NOP on DMI and lactation performance of dairy cattle (Hristov et al., 2015; Van Wesemael et al., 2019; Melgar et al., 2020a). A study by Melgar et al. (2020c) suggested that within a wide range of 3-NOP inclusion (30 to 120 mg/kg feed DM), 3-NOP appeared not to affect DMI in dairy cows. Similarly, Kim et al. (2019) observed no effect of feeding 3-NOP at a rate of 100 mg/kg on DMI in beef cattle.

Although it has been suggested that inhibition of methanogenesis may provide extra ME that can be used for production purposes (Blaxter and Czerkawski, 1966), a meta-analysis by Kim et al. (2020) showed that 3-NOP inclusion in dairy diets did not result in increased milk yield in both short-term (Haisan et al., 2014, 2017; Reynolds et al., 2014; Lopes et al., 2016) and long-term (Hristov et al., 2015; Van Wesemael et al., 2019; Melgar et al., 2020a) experiments. In the current experiment, it appears the extra ME energy from inhibited methanogenesis was redirected toward milk fat synthesis.

The similar DMI and milk yield between 3-NOP and control cows resulted in lack of effect of treatment on milk and ECM feed efficiency (Table 3). Feed efficiency was also not affected by 3-NOP in mid-to-late-lactation dairy cows (Hristov et al., 2015; Van Wesemael et al., 2019). In contrast, Melgar et al. (2020a) observed about 6% improvement in feed efficiency by 3-NOP in early-lactation cows, but the effect was explained by the lower DMI compared with the control cows.

Inclusion of 3-NOP in the TMR did not affect overall milk composition of the cows in the current experiment, except it increased ($P \leq 0.05$) milk fat concentration by 6.5%, milk fat yield by 5%, and MUN by about 4% (Table 3). Similarly, milk fat concentration and yield were linearly increased by 3-NOP in a dose-response study by Melgar et al. (2020b). In contrast, Hristov et al. (2015) and Melgar et al. (2020a) did not observe changes in milk fat concentration and yield with 3-NOP supplementation up to 80 mg/kg feed DM. However, all these studies reported effects of 3-NOP on milk FA composition, which is consistent with the results from the current study (Table 4). Concentration of the short-chain FA 6:0 and 8:0 increased ($P = 0.03$), and 10:0 tended to increase ($P = 0.08$). Total *trans* FA were lowered ($P = 0.01$) and sum of SFA was increased ($P = 0.05$) by 3-NOP. Sum of MUFA tended ($P = 0.09$) to decrease and sum of PUFA decreased ($P = 0.03$) with 3-NOP. In contrast, Reynolds et al. (2014) observed no effect on milk FA profile when 3-NOP was dosed directly into the rumen twice daily rather than continuously fed via a TMR, which may explain why these authors reported only a 7% decrease in enteric CH_4.

Researchers have reported increased butyrate and decreased acetate molar proportions with 3-NOP (Lopes et al., 2016; Haisan et al., 2017; Melgar et al., 2020a). Thus, the increase in milk fat concentration and yield observed in the current experiment and another experiment from our laboratory (Melgar et al., 2020c) is likely related to a shift in rumen fermentation toward increased production of butyrate. About 50% of the fat in milk is derived from the uptake of FA by the mammary gland and the other 50% originates from de novo FA synthesis (Bauman and Davis, 1974). The main substrate for FA synthesis in the cow is acetate, but butyrate provides half of the initial 4 carbons (Palmquist

et al., 1969). Thus, short-chain FA in the mammary gland are mainly synthesized from acetate and, to a lesser extent, from butyrate (Shingfield et al., 2008). Melgar et al. (2020a) suggested that under rumen inhibition of methanogenesis by 3-NOP, butyrate and not acetate appears to become the primary substrate for short-chain FA synthesis in the mammary gland.

Inclusion of 3-NOP in the diet decreased milk total *trans* FA in the current experiment, particularly *trans* 18:1. Increased trans FA in milk may be explained by the shift in ruminal fermentation pathways toward decreased acetate and increased butyrate and valerate concentrations. Similar to previous data from our laboratory (by Melgar et al., 2020a,c) and based on milk FA profile, rumen biohydrogenation in the current experiment appeared to follow normal pathways (Bauman and Griinari, 2003). Other studies have also shown that biohydrogenation pathways were not significantly affected by 3-NOP (Reynolds et al., 2014; Hristov et al., 2015) or bromochloromethane (Abecia et al., 2012). The increased sum of SFA and the decreased sum of PUFA (and to a lesser extent MUFA) observed in the current study and also reported by Hristov et al. (2015) and Melgar et al. (2020c) suggest that biohydrogenation may have provided a minor H_2 sink when rumen methanogenesis was inhibited by 3-NOP.

The lack of effect of 3-NOP on lactose concentration and yield is consistent with results from previous experiments (Hristov et al., 2015; Van Wesemael et al., 2019; Melgar et al., 2020a). In some studies, 3-NOP increased or tended to increase (Reynolds et al., 2014; Hristov et al., 2015) milk protein concentration, but similar to the current experiment, milk protein yield was not affected. Milk casein concentration was reported to be increased by 3-NOP by Reynolds et al. (2014), whereas in the current experiment there was no difference in casein yield between control and 3-NOP-fed cows. No other studies have reported casein data when cows were fed 3-NOP.

The increased MUN concentration in cows receiving 3-NOP is consistent with previous reports from long-term studies with early-lactation (Melgar et al., 2020a) and mid- to late-lactation (Melgar et al., 2020c) dairy cows. In other studies, however, 3-NOP had no effect

Table 3. Effect of the methane inhibitor 3-nitrooxypropanol (3-NOP) on feed DMI, milk yield and components, feed efficiency, and BW in dairy cows

	Treatment[1]			
Item	Control	3-NOP	SEM[2]	*P*-value[3]
DMI, kg	25.4	25.7	0.38	0.54
Milk yield, kg	38.5	38.0	0.95	0.74
Feed efficiency,[4] kg/kg	1.52	1.50	0.038	0.61
Milk fat, %	3.82	4.07	0.073	0.02
Yield, kg/d	1.45	1.52	0.025	0.05
ECM,[5] kg/d	36.8	37.4	0.66	0.54
ECM feed efficiency,[6] kg/kg	1.46	1.46	0.025	0.97
Milk true protein, %	3.11	3.16	0.048	0.53
Yield, kg/d	1.17	1.18	0.022	0.87
Casein, g/d	971	966	44.5	0.92
Lactose, %	4.79	4.77	0.021	0.50
Yield, kg/d	1.85	1.81	0.049	0.60
Milk solids, %	12.7	12.9	0.102	0.11
MUN, mg/dL	11.6	12.1	0.175	0.04
SCC,[7] $\times 10^3$ cells/mL	85.4	53.6	19.22	0.92
Milk NE_L,[8] Mcal/d	27.4	27.9	0.489	0.52
BW, kg	619	619	2.98	0.97
BW change,[9] g/d	480	437	50.9	0.39

[1]Treatments were control (basal diet) and 3-NOP (basal diet plus 60 mg of 3-NOP/kg of feed DM). Data are covariate-adjusted LSM.

[2]Largest SEM published in table; n = 200, except n = 160 for casein and n = 40 for BW change (n represents number of observations used in the statistical analysis). Data are from 40 cows.

[3]Main effect of treatment.

[4]Milk yield ÷ DMI.

[5]ECM (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).

[6]ECM yield ÷ DMI.

[7]Statistical analysis was performed on log-transformed data.

[8]Milk NE_L (Mcal/d) = kg of milk × (0.0929 × % fat + 0.0563 × % true protein + 0.0395 × % lactose), NRC (2001).

[9]BW change: (average BW wk 15 – average BW covariate wk 3) ÷ days on study.

on MUN concentration (Reynolds et al., 2014; Lopes et al., 2016; Haisan et al., 2017). According to Nousiainen et al. (2004) hepatic oxidation of AA is a significant source of MUN. In the present study we did not analyze plasma AA concentrations, but Melgar et al. (2020a) observed a decrease in plasma concentration of some essential and nonessential AA, which, according to the authors, could have contributed to increase MUN in 3-NOP-fed cows. Another possible explanation for the MUN effect of 3-NOP is the observed consistent increase in ruminal butyrate concentration by 3-NOP (Lopes et al., 2016; Guyader et al., 2017; Melgar et al., 2020a). Butyrate is known to stimulate blood flow and, consequently, ammonia absorption (Engelhardt et al., 1978; Rémond et al., 1993), which is supported by the observed decrease in ammonia concentration by 3-NOP (Lopes et al., 2016; Melgar et al., 2020a). Increased absorption of ammonia would likely explain the increase in MUN in the current and previous long-term studies with 3-NOP from our laboratory (Melgar et al., 2020a,c).

Table 4. Effect of the methane inhibitor 3-nitrooxypropanol (3-NOP) on fatty acid composition of milk fat (g/100 g of total fatty acids) in dairy cows

	Treatment[1]			
Item	Control	3-NOP	SEM[2]	*P*-value[3]
4:0	5.10	5.25	0.091	0.25
6:0	2.68	2.78	0.031	0.03
8:0	1.43	1.50	0.021	0.03
10:0	3.11	3.24	0.051	0.08
12:0	3.50	3.62	0.061	0.15
14:0	11.0	10.9	0.10	0.35
16:0	27.1	27.2	0.3	0.87
18:0	10.3	10.6	0.192	0.21
trans-4 18:1	0.036	0.038	0.0015	0.19
trans-5 18:1	0.024	0.025	0.0006	0.30
trans-6,8 18:1	0.373	0.359	0.0067	0.17
trans-9 18:1	0.279	0.265	0.0052	0.07
trans-10 18:1	0.632	0.497	0.0389	0.02
trans-11 18:1	0.864	0.848	0.0197	0.59
trans-12 18:1	0.424	0.377	0.0136	0.01
cis-9 18:1	20.3	19.9	0.295	0.26
cis-11 18:1	0.984	0.901	0.0168	0.001
cis-12 18:1	0.346	0.316	0.0067	0.003
cis-9,*cis*-12 18:2	2.53	2.42	0.034	0.03
20:0	0.151	0.156	0.0043	0.39
cis-9,*trans*-11 CLA[4]	0.490	0.442	0.0188	0.05
Others	2.79	2.73	0.056	0.22
Total *trans* fatty acids	2.64	2.43	0.056	0.01
ΣSFA	67.6	68.7	0.38	0.05
ΣMUFA	26.9	26.1	0.342	0.09
ΣPUFA	3.69	3.52	0.052	0.03

[1]Treatments were control (basal diet) and 3-NOP (basal diet plus 60 mg of 3-NOP/kg of feed DM). Data are covariate-adjusted LSM.

[2]Largest SEM published in table; n = 160 (n represents number of observations used in the statistical analysis). Data are from 40 cows.

[3]Main effect of treatment.

[4]*cis*-12,*trans*-CLA was <0.003%.

It has been suggested that reduction of CH_4 production in the rumen would provide more ME availability for body tissue gain (Blaxter and Czerkawski, 1966). However, BW gain is not always improved under inhibited methanogenesis in dairy cows (van Zijderveld et al., 2011). Reduction of daily CH_4 emission in the current experiment by 3-NOP did not affect BW or BW change (Table 3). Long-term experiments have reported an increase in BW in mid-lactation cows (Haisan et al., 2014; Hristov et al., 2015), whereas no effect was reported by Melgar et al. (2020a) in early-lactation cows. Similarly, Martinez-Fernandez et al. (2018) observed increased BW in beef steers, but no effect on BW was reported in other studies with beef cattle fed 3-NOP (Romero-Pérez et al., 2014; Vyas et al., 2018a; Kim et al., 2019).

In the current experiment, 3-NOP fed at a rate of 60 mg/kg feed DM decreased daily enteric CH_4 emission by an average of 109 g/d. This decrease would represent 1.42 Mcal/d of feed digestible energy not emitted as CH_4. If energy in CH_4 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 CH_4 with 3-NOP would represent, in theory, an additional (over the control) 0.34 Mcal/d of NE_L that could potentially be used for productive purposes. Thus, if 0.74 Mcal of NE_L is required for the synthesis of 1 kg of milk by 3-NOP cows (NRC, 2001), the reduction in CH_4 emission would allow production of an additional 0.46 kg of milk/d. Previous studies have reported a potential increase in NE_L availability, as a result of the inclusion of 3-NOP in the diet, of 0.29 Mcal/d in early lactation (Melgar et al., 2020a) and 0.26 Mcal/d in mid-to-late-lactation cows (Hristov et al., 2015). Milk yield was not increased by 3-NOP in the current experiment, but fat yield was increased by 70 g/d. Based on NRC (2001), it can be estimated that the NE_L of this extra milk fat is 0.65 Mcal/d; therefore, the potential energy savings from decreased CH_4 emission in the current experiment could directly explain around 52% of the difference in milk fat yield between 3-NOP and the control. Apparently, other mechanisms, such as shifts in rumen VFA, were involved in enhancing milk fat synthesis in 3-NOP-fed cows.

CONCLUSIONS

Results from this long-term experiment are in line with our previous data with 3-NOP in lactating dairy cows. Applied at 60 mg/kg feed DM via the TMR, 3-NOP decreased daily enteric CH_4 emission by 26%, emission yield by 27%, and emission intensity (ECM

basis) by 29%. The inhibitory effect of 3-NOP on methanogenesis persisted over 15 wk of treatment, did not affect overall CO_2 emission, and was accompanied by an increase in H_2 emission. The decrease in CH_4 emission did not affect DMI and milk yield; thus, it did not affect feed efficiency and ECM feed efficiency. Cows receiving 3-NOP, however, had increased milk fat concentration and yield. Similar to previous studies, concentration of de novo synthesized short-chain and sum of SFA in milk was increased by 3-NOP. Overall, this study confirmed the effectiveness of 3-NOP as an enteric CH_4 mitigant. Similar to previous experiments in our laboratory, 3-NOP does not appear to affect feed intake or milk production in dairy cows but increases milk fat concentration and yield; this effect may have important implications in future adoption of this mitigation practice by the dairy industry.

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