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Long-term effects of 3-nitrooxypropanol on methane emission and milk production characteristics in Holstein-Friesian dairy cows

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ABSTRACT

The objective was to determine the long-term effect of 3-nitrooxypropanol (3-NOP) on CH_4 emission and milk production characteristics from dairy cows receiving 3-NOP in their diet for a full year, covering all lactation stages of the dairy cows. Sixty-four late-lactation Holstein-Friesian cows (34% primiparous) were blocked in pairs, based on expected calving date, parity, and daily milk yield. The experiment started with an adaptation period of 1 wk followed by a covariate period of 3 wk in which all cows received the same basal diet and baseline measurements were performed. Directly after, cows within a block were randomly allocated to 1 of 2 dietary treatments: a diet containing on average 69.8 mg 3-NOP/kg DM (total ration level, corrected for intake of nonsupplemented GreenFeed bait) and a diet containing a placebo. Forage composition as well as forage-to-concentrate ratio altered with lactation stage (i.e., dry period and early, mid, and late lactation). Diets were provided as a total mixed ration, and additional bait was fed in GreenFeed units (C-Lock Inc.), which were used for emission measurements. Supplementation of 3-NOP did not affect total DMI, BW, or BCS, but resulted in a 6.5% increase in the yields of energy-corrected milk and fat- and protein-corrected milk (FPCM). Furthermore, milk fat and protein as well as feed efficiency were increased upon 3-NOP supplementation. Overall, a reduction of 21%, 20%, and 27% was achieved for CH_4 production (g/d), yield (g/kg DMI), and intensity (g/kg FPCM), respectively, upon 3-NOP supplementation. The CH_4 mitigation potential of 3-NOP was affected by the lactation stage dependent diet to which 3-NOP was supplemented. On average, a 16%, 20%, 16%, and 26% reduction in CH_4 yield (g/kg DMI) was achieved upon 3-NOP supplementation for the dry period, and early, mid, and late-lactation diets, respectively. The CH_4 mitigation potential of 3-NOP was affected by the length of 3-NOP supplementation within a lactation stage dependent diet and by variation in diet composition within a lactation stage dependent diet as a result of changes in grass and corn silage silos. In conclusion, 3-NOP reduced CH_4 emission from cows receiving 3-NOP for a year, with a positive effect on production characteristics. The CH_4 mitigation potential of 3-NOP was influenced by diet type, diet composition, and nutrition value, and the efficacy of 3-NOP appeared to decline over time but not continuously. Associated with changes in diet composition, increased efficacy of 3-NOP was observed at the start of the trial, at the start of a new lactation, and, importantly, at the end of the trial. These results suggest that diet composition has a large effect on the efficacy of 3-NOP, perhaps even larger than the week of supplementation after first introduction of 3-NOP. More studies are needed to clarify the long-term effects of 3-NOP on CH_4 emission and to further investigate what influence variation in diet composition may have on the mitigation potential of 3-NOP.

Key words: dairy cow, enteric methane production, feed additive, methanogen inhibitor

INTRODUCTION

Methane emission accounts for 44% of the total GHG emission from dairy production, with enteric fermentation contributing more than 92% (Opio et al., 2013). Due to its considerable contribution, enteric CH_4 production has been targeted to reduce GHG emission from the dairy sector. Several enteric CH_4 mitigation strategies have been proposed, including changes in animal breeding and management, and dietary strategies (Hristov et al., 2013a,b). The proposed dietary strategies to mitigate enteric CH_4 production include improvement of forage quality, reformulation of diets (i.e., different forage types), supplementing lipids, and rumen manipulation by using feed additives. One such feed additive, 3-nitrooxy-

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propanol (**3-NOP**; marketed as Bovaer, DSM-Firmenich AG, Kaiseraugst, Switzerland), has been reported to reduce enteric CH_4 emission by over 30% on average in dairy cattle based on 14 experiments, comprised of 48 treatment means (Kebreab et al., 2023). Arndt et al. (2022) concluded that 3-NOP was the most effective CH_4-mitigating feed additive within the category of rumen manipulation caused by feeding CH_4 inhibitors. By molecular docking of 3-NOP at the site of methylcoenzyme M binding, 3-NOP specifically targets the nickel enzyme methylcoenzyme M reductase, which catalyzes the last step in the CH_4-forming pathway of rumen archaea (Duin et al., 2016).

Several short-term studies with dairy cattle, where 3-NOP was fed 4 wk or less (e.g., Haisan et al., 2014, 2017; van Gastelen et al., 2022), reported that 3-NOP effectively reduced CH_4 emission. However, long-term studies are required to evaluate whether the CH_4-mitigating effect of 3-NOP is persistent and whether potential adaptation of the rumen microbiota to 3-NOP occurs. The CH_4 mitigating effect of 3-NOP in dairy cattle was persistent in the studies of Melgar et al. (2020a) and van Gastelen et al. (2020) from calving until the end of the study at 105 or 115 DIM, respectively. Later in lactation, the CH_4 mitigation effect of 3-NOP persisted for 12 wk (Hristov et al., 2015) and 15 wk (Melgar et al., 2021). In the study of Schilde et al. (2021) dairy cows received 3-NOP in their diet for 4 wk antepartum until 17 wk postpartum. In this study, the inhibitory effect of 3-NOP remained persistent only for 3-NOP in the high-concentrate diet, whereas CH_4 emission with the low-concentrate diet containing 3-NOP gradually increased to the level of the control group (Schilde et al., 2021). The authors suggested that differences in feed intake may have played an important role for the observed loss in persistency. Analysis of the feed (low-concentrate diet) in the study by Schilde et al. (2021) also showed that the actual 3-NOP dose was well below target (i.e., 48 vs. 60 mg/kg DM), which may have been a factor when considering the differences in response to 3-NOP supplementation. In their review, Hristov et al. (2022) re-examined CH_4 emission in 2 earlier studies of 15-wk duration. They reported a linear decrease (significant in only 1 study) in the CH_4 yield mitigation effect of 3-NOP over time and suggested that full or multiple lactation studies are required to further investigate possible change in efficacy of 3-NOP over time. Overall, none of the studies published to date have fed 3-NOP to dairy cows for a full year, covering all lactation stages of the dairy cows. Hence, the objective of this study was to determine the effect of 3-NOP on CH_4 emission and milk production characteristics from dairy cows receiving 3-NOP in their diet for a full year, covering all lactation stages, including the dry period. We hypothesized that 3-NOP supplementation would persistently decrease CH_4 emission throughout the year without affecting DMI or milk production.

MATERIALS AND METHODS

Experimental Design

The experiment was conducted from October 2020 to October 2021 at the research facilities of Wageningen Livestock Research (Dairy Campus, Leeuwarden, the Netherlands), under the Dutch law on Animal Experiments in accordance with European Union Directive 2010/63, and approved by the Animal Welfare Body of Wageningen Research (Lelystad, the Netherlands). The study followed a randomized complete block design with 2 dietary treatments and 64 lactating Holstein-Friesian dairy cows (244 ± 54 DIM at start of the trial; mean ± SD). Of the 64 cows, 22 were first-lactation cows, 13 cows were in their second lactation, and 29 were in their third lactation or higher. The cows were blocked in pairs based on parity, expected calving date, and fat- and protein-corrected milk (**FPCM**) yield, resulting in 32 blocks of 2 cows. Within each block, cows were randomly assigned to 1 of the 2 dietary treatments. The experiment consisted of 2 phases. First, a pre-trial phase consisting of an adaptation period of 1 wk in which the cows were adapted to the basal diets and a covariate period of 3 wk in which baseline measurements took place. Second, a trial phase consisting of 48 wk in which the cows received 1 of the 2 dietary treatments. The use of 3-NOP in animal feed was preapproved by the Veterinary Drugs Directorate Division (Utrecht, the Netherlands), with no withdrawal period implemented, and the milk was not discarded.

Diets and Dietary Treatments

The experiment started with a pre-trial phase, during which all cows received the same basal diet, consisting of 39% perennial ryegrass silage, 22% corn silage, 10% barley meal, 6% soybean meal, and 23% concentrate on DM basis. This diet contained 174 g of CP/kg DM, 172 g of starch/kg DM, and 352 g of NDF/kg DM. After the pre-trial phase, the trial phase started, and cows were assigned to an experimental diet. The chemical composition of the individual feed ingredients is presented in Table 1. The ingredient and chemical compositions of the experimental diets are presented in Table 2. For all diets, the chop length of grass silage was ~15 mm and that of corn silage ~7 mm. To each of these diets, water was added to target a DM content of 370 g/kg during lactation and a DM content of 400 g/kg during the dry period. The concentrate was in meal form and produced by Agrifirm (Apeldoorn, the Netherlands; Table 1). All

Table 1. Average chemical composition (in g/kg DM, unless otherwise stated) of the individual ration components for the complete study

Item	Grass silage	Corn silage	Wheat straw	Concentrate[1]	Barley meal	Soybean meal	CTL premix[2]	3-NOP premix[3]	GreenFeed bait[4]
DM (g/kg product)	438	358	924	892	864	874	898	897	872
OM	882	958	928	911	975	928	912	910	927
CP	204	77	39	165	127	521	162	163	145
Crude fat	36	31	11	48	32	27	43	43	37
Gross energy (MJ/kg DM)	18.9	18.8	18.2	18.2	18.6	19.6	18.2	18.2	18.0
NDF	443	346	784	348	160	123	369	372	370
ADF	255	197	489	207	50	64	201	202	229
ADL	13	8	48	50	6	0	50	49	19
Starch	ND[5]	363	21	181	580	17	196	201	118
Sugar	76	ND	11	65	30	117	65	65	105
NE_L[6] (MJ/kg DM)	6.3	6.9	2.6	7.4	7.7	7.8	7.0	7.0	7.0
DVE[7]	60	54	10	107	90	264	109	109	86
OEB[8]	61	−44	−41	1	−27	169	−22	−22	−6

[1] Ingredient composition (g/kg DM): palm kernel flakes = 340, wheat semolina = 149, citrus pulp = 148, corn = 116, wheat = 77, rumen-protected rapeseed meal (Mervobest, NuScience) = 41, sugar beet pulp = 26, barley = 20, NaCl = 18, $CaCO_3$ = 18, citrocol = 10, vinasse = 9, corn gluten meal = 7, rapeseed meal = 7, MgO = 5, urea = 5, sunflower kernel flakes = 2, and trace mineral and vitamin mix = 2.

[2] Ingredient composition (g/kg DM): palm kernel flakes = 334, sugar beet pulp = 167, wheat semolina = 147, corn = 112, wheat = 88, rumen-protected rapeseed meal (Mervobest, Nuscience) = 34, barley = 29, sunflower seed meal = 24, NaCl = 18, $CaCO_3$ = 16, trace mineral and vitamin mix = 8, urea = 5, and placebo supplement = 17.

[3] Ingredient composition (g/kg DM): palm kernel flakes = 334, sugar beet pulp = 167, wheat semolina = 147, corn = 112, wheat = 88, rumen-protected rapeseed meal (Mervobest, Nuscience) = 34, barley = 29, sunflower seed meal = 24, NaCl = 18, $CaCO_3$ = 16, trace mineral and vitamin mix = 8, urea = 5, and 3-NOP supplement = 17.

[4] Ingredient composition (g/kg DM): sugar beet pulp = 300, soybean meal = 221, corn gluten meal = 87, wheat = 72, alfalfa = 70, sunflower meal = 70, molasses = 50, barley = 50, corn = 49, rapeseed meal = 21, NaCl = 10.

[5] Not determined.

[6] van Es (1978).

[7] Intestinal digestible protein (van Duinkerken et al., 2011).

[8] Rumen-degradable protein balance (van Duinkerken et al., 2011).

cows started with the late-lactation (**LL**) diet. The cows were dried off 6 wk before their expected calving date but were switched to the dry period (**DP**) diet during their last week of lactation to ensure a reduction in milk yield. The cows were switched to the early-lactation (**EL**) diet upon the first signs of calving and continued receiving this EL diet until they were at least 100 DIM. After 100 DIM, as soon as the FPCM yield of the cows became less than 36.0 kg/d, they were switched to the mid-lactation (**ML**) diet. All dietary changes were executed per block of animals, meaning that both cows within a block had to fulfill the criteria. On average, cows were 271 ± 53 DIM when starting the LL diet, 351 ± 52 DIM when changing from LL to DP, and 134 ± 20 DIM when changing from EL to ML.

All diets (i.e., LL, DP, EL, and ML) were supplemented with a placebo (i.e., silicon dioxide + 1,2 propanediol; control [**CTL**]) or with 3-NOP (i.e., 10% 3-NOP on silicon dioxide + 1,2-propanediol) with a target inclusion level of 80 mg 3-NOP/kg DM. To achieve the 3-NOP inclusion level, a part of the concentrate was replaced by a treatment premix containing either the placebo or 3-NOP (Tables 1 and 2). These premixes were in meal form and produced by Research Diet Services (RDS B.V., Wijk bij Duurstede, the Netherlands). Also, to achieve this target 3-NOP inclusion level, we corrected for a pre-experiment estimated intake of 1.1 kg DM/cow per day of nonsupplemented bait pellets in the GreenFeed units (C-Lock Inc., Rapid City, SD) and an estimated total DMI (i.e., GreenFeed bait and partial mixed ration [**PMR**]) of 22.5 kg DM/d. Hence, the formulated 3-NOP inclusion level in the PMR was slightly greater (i.e., 84 instead of 80 mg 3-NOP/kg DM) to achieve the target inclusion level of 80 mg 3-NOP/kg DM in the total diet (i.e., PMR and GreenFeed bait).

Feeding and Housing

The cows were fed the PMR via an automated feeding system, consisting of the Trioliet feed mixing robot (Triomatic HP 2 300, Trioliet, Oldenzaal, the Netherlands) for mixing the diets, and Insentec feed bin (**FB**; RIC system, Hokofarm Group B.V., Marknesse, the Netherlands) to measure feed intake. The cows had access to every FB that had the allocated diet. The FB were calibrated weekly using a standard weight. The Trioliet mixed the diets 4 times daily in equal portions. Measures to avoid cross-contamination were taken by first mixing the placebo-containing diets (CTL), followed by mixing the 3-NOP diet (3-NOP), followed by a rinsing diet (which was not fed to the cows in the experiment). This was repeated 4 times daily. The operator of the system

Table 2. The calculated average ingredient and chemical composition (g/kg DM, unless otherwise stated) of the diets, supplemented with placebo (CTL) or 3-nitrooxypropanol (3-NOP), based on the composition of the individual ration components that were included during the period that the diets were fed (i.e., lactation stage-dependent)[1]

Item	Late lactation	Dry period	Early lactation	Mid lactation
Ingredient composition				
Grass silage	531	266	389	456
Corn silage	228	185	166	196
Wheat straw	0	362	0	0
Concentrate	63	8	285	172
Barley meal	51	47	67	86
Soybean meal	82	87	47	44
Treatment premix[2]	45	45	46	46
Chemical composition				
DM (g/kg product)	368	401	370	370
OM	918	925	910	907
CP	179	139	182	187
Crude fat	34	25	39	38
Gross energy (MJ/kg DM)	18.9	18.7	18.6	18.6
NDF	357	502	368	372
ADF	197	298	212	213
ADL	12	25	24	20
Starch	133	115	163	163
Sugar	92	45	44	38
NE_L[3] (MJ/kg DM)	6.9	5.4	6.8	6.7
DVE[4]	83	62	85	80
OEB[5]	27	4	23	27

[1] The GreenFeed bait is not included in the calculation of the chemical composition of the diets.

[2] CTL premix for CTL diet and 3-NOP premix for 3-NOP diet.

[3] van Es (1978).

[4] Intestinal digestible protein (van Duinkerken et al., 2011).

[5] Rumen-degradable protein balance (van Duinkerken et al., 2011).

determined on a weekly basis (based on the DM content of each individual ration component from the previous week; determined daily) how much of each of these components was required to prepare the diets. The dietary components were taken automatically from their bunker (i.e., forages; an accuracy of ± 2 kg product), silo (i.e., concentrate, barley meal, and soybean meal; an accuracy of ± 2 kg product), or mini-silo (i.e., premixes; an accuracy of ± 2 g of product), weighed, and transported into the Trioliet feed mixing robot. The premix (i.e., containing placebo or 3-NOP) was added to the Trioliet in 2 equal portions while mixing was ongoing to achieve a greater homogeneity of placebo or 3-NOP in the complete ration. After the last dietary component was added to the Trioliet, the diets were mixed for an additional 10 min and subsequently transported to the FB. The diets were offered at 10% excess to allow ad libitum feed intake.

All cows were housed as 1 group in a freestall barn, with 64 lying cubicles, 32 FB for feeding, and 3 GreenFeed units for emission measurements. Due to variation in the expected calving date, both lactating and dry cows were simultaneously present at a certain period in the experiment. During this period, we separated the cows by placing fences in the barn to create 2 areas: one specifically for the lactating cows and one specifically for the dry cows. These fences were flexible, meaning that we were able to adjust the size of both areas according to the number of lactating and dry cows to ensure sufficient lying cubicles, GreenFeed units, and FB for the number of cows present in each area. During this period, we also added 2 additional GreenFeed units to the barn to ensure no overstocking (maximum of 20 animals per GreenFeed unit). We chose to separate the dry cows from the lactating cows for multiple reasons: (1) we did not want the dry cows to go to the milking carousel twice daily with the lactating animals, and (2) the difference in the concentrate fraction in the PMR was rather large and we wanted to prevent potential stealing behavior. Upon the first signs of calving, cows were moved to a transition barn with deep litter straw bedding, where they had access to FB to continue receiving the diets to which they were assigned, but there were no GreenFeed units for gas emission measurements. As a result, out of the 64 cows, we have no GreenFeed measurements for 19 cows around calving, which summed up to 62 d before calving (i.e., on average 3.3 d/cow) and 31 d after calving (i.e., on average 1.6 d/cow). After calving, when the cows were considered healthy and recovered, the cows moved back to the freestall barn with the other lactating cows. The lying cubicles in the barn for lactating cows and the dry cows were covered with commercially available rubber cattle mats with wood shavings as bedding and were cleaned daily. All cows were exposed to light from 0500 to 2300 h and had free access to clean drinking water throughout the trial, irrespective of barn. The lactating cows were milked twice daily at ~0500 and 1500 h during the entire experiment.

Measurements and Sample Collection

The FB were equipped with an automated identification system (monitor ID system based on transponders within the collar of the cows) to enable access. For each visit of a cow to a FB, the start and end times of the visit as well as the start and end weights of the FB were recorded to determine daily feed intake. Cows were milked in a milking carrousel suited for 40 cows with automatic cow identification, milk recording, and milk cluster removal (AutoRotor PerFormer, Gea Farm Technologies, Leeuwarden, the Netherlands). Body weight (Gea weighing scale; Gea Farm Technologies Nederland B.V., Deventer, the Netherlands) and BCS (DeLaval BCS camera, DeLaval B.V., Steenwijk, the Netherlands) of each cow were recorded twice daily as they exited the milking carrousel.

Samples of individual ration components and GreenFeed bait were taken once weekly and stored at −20°C pending analysis. Once weekly, during 1 morning and 1 evening milking event, a milk sample (10 mL) was collected in a tube containing sodium azide (5 µL) for preservation, stored no longer than 1 d at 4°C, and subsequently analyzed for composition.

Gas Emissions

Emissions of CH_4, H_2, and CO_2 were measured on an individual cow level for the duration of the trial by using 5 GreenFeed units (Zimmerman et al., 2011). Irrespective of treatment or lactation stage, 3 GreenFeed units could be visited by every cow during lactation, and 2 GreenFeed units could be visited by every cow during the DP. The GreenFeed units measured airflow as well as CH_4, H_2, and CO_2 concentrations in both the environment and exhaled air when a cow visited the system. Each GreenFeed unit was equipped with a head position sensor, and gas emission data were rejected when the head position criteria were not met. Each individual cow could visit the GreenFeed units every 3 h (with a maximum of 8 visits per day), and data collection was dependent on the cows' voluntary visits to the GreenFeed units. A concentrate was offered as bait (Table 1) for enticement and to encourage the cows to maintain a suitable head position for accurate measurements. This GreenFeed bait was provided from a hopper above the GreenFeed hood using a computer-controlled rotating cup dispenser, with a maximum of 9 so-called cup drops per visit, 1 cup drop per 25 s, and 35.1 ± 1.14 g of feed per cup drop. Emissions of CH_4, H_2, and CO_2 were calculated by subtracting the recorded background gas concentration from the gas concentrations recorded during the animal's time in the GreenFeed. This difference in concentration was subsequently multiplied by the airflow to come to an emission rate in grams per day, using the ideal gas law and correcting for temperature and pressure. See van Gastelen et al. (2022) for more details regarding the procedures applied for the GreenFeed units in the present study. The average CO_2 recovery was 99.1% (for individual GreenFeed units between 97.7% and 99.9%).

Chemical Analysis

Feed samples were thawed at room temperature, pooled (i.e., per 2 wk for grass silage and corn silage, and per 2 mo for all other ingredients and the GreenFeed bait), freeze-dried until constant weight (silages only), and ground to pass a 1-mm screen by using a cross beater mill for both silages (Peppink 100AN, Peppink, Olst, the Netherlands) and an ultra-centrifugal mill for all other ration components (Retsch ZM200, Retsch GmbH, Haan, Germany). The samples were subsequently analyzed by using wet chemistry for DM, ash, N, starch (except for grass silage), reducing sugars (i.e., all carbohydrates with reducing properties and soluble in 40% ethanol, except for corn silage), crude fat, NDF, ADF, and ADL as described by Abrahamse et al. (2008). Gross energy (**GE**) was determined using bomb calorimetry (ISO 9831; International Organization for Standardization, 1998), and CP was calculated as N × 6.25, where N was determined using the Kjeldahl method (ISO 5983; International Organization for Standardization, 2005). The N concentrations in both silages were determined in fresh material according to Klop et al. (2016).

Samples of the CTL and 3-NOP premixes were collected on a weekly basis, and samples of the CTL and 3-NOP PMR were collected once for every lactation stage–dependent diet. These samples were frozen at −20°C and, at the end of the study, transported to DSM Nutritional Products (Kaiseraugst, Switzerland) on dry ice for analysis of 3-NOP content, as described by van Gastelen et al. (2020). Milk samples were analyzed by Qlip (Qlip B.V., Zutphen, the Netherlands). Milk fat, protein, lactose, and urea content were determined by mid-infrared spectroscopy using MilkoScan FT 6000 equipment (Foss, Hillerod, Denmark), and SCC was determined using Fossomatic instruments (Foss, Hillerod, Denmark), both with the manufacturer-supplied basic calibration models according to manufacturer's recommended procedures (Foss, Hillerod, Denmark; ISO 9622; International Organization for Standardization, 2013).

Calculations

Based on the measured 3-NOP content of the premixes, combined with the ration composition as mixed by the feed mixing robot, we calculated the 3-NOP dose in the PMR on a weekly basis. Based on the DMI of both PMR and GreenFeed bait, on an individual animal level and daily basis, we calculated the actual 3-NOP dose relative to the total DMI (i.e., PMR + GreenFeed bait). Milk composition was corrected for differences in milk yield between milking events on the same day, and the weighed milk composition on a daily basis was used for data analysis. The FPCM yield was subsequently calculated according to the equation FPCM yield (kg/d) = (0.337 + 0.116 × fat % + 0.06 × CP %) × milk yield (kg/d; CVB, 2016). We calculated ECM yield according to the equation ECM yield (kg/d) = [milk yield (kg/d) × 0.327] + [fat yield (kg/d) × 12.95] + [CP yield (kg/d) × 7.25] (Tyrrell and Reid, 1965). Body weight change was calculated per cow per week as the difference between average BW in the current week and average BW in the previous week.

Dry matter intake was calculated daily for GreenFeed bait and PMR and summed for total daily DMI. Feed efficiency was calculated per week by dividing the FPCM or ECM with the total DMI. Production of CH_4, H_2, and CO_2 was expressed in g/d. Yields of CH_4, H_2, and CO_2 were calculated per week by dividing the production of CH_4, H_2, and CO_2 by the total DMI, and intensity of CH_4, H_2, and CO_2 was calculated per week by dividing the production of CH_4, H_2, and CO_2 by FPCM yield. The ratio of CH_4 to CO_2 was calculated by dividing CH_4 production (g/d) by CO_2 production (g/d). The relative reduction of CH_4 yield (%) was calculated per week as

$$\text{Relative reduction} = \frac{\text{CH}_4\ \text{yield}_{\text{CTL}} - \text{CH}_4\ \text{yield}_{\text{3NOP}}}{\text{CH}_4\ \text{yield}_{\text{CTL}}} \times 100\%.$$

Statistical Analysis

Data Exclusion. The final data set included 62 cows. Due to continuous spilling of large amounts of feed, we could not obtain reliable feed intake measures for 1 cow. Another cow was determined to be nonpregnant. Both cows were excluded from the trial. In the course of the complete experiment, another 8 animals (i.e., 4 animals receiving the CTL diet, and 4 animals receiving the 3-NOP diet) were removed from the experiment because of health issues unrelated to the dietary treatments or experimental conditions. These cows' data were retained in the data set up to the moment where the first sign of health issues was observed. For these 8 cows, the number of weeks in the study after the covariate period was on average 25 ± 2.8.

In total, including the adaptation period, covariate period, and 48-wk trial phase, the data set consisted of 21,776 cow-days. From this complete data set, days on which cows had a diet change (e.g., from EL to ML diet, in total 478 d) and days on which cows had health issues were deleted. Additionally, all data of the final week of lactation of all cows were deleted from the data set because the cows already received the DP diet to ensure a reduction in milk yield before being dried off. This week is representative neither of late lactation, because of the DP diet and large drop in milk yield, nor of the dry period, because the cows were still lactating. Moreover, the reduction in milk yield and the change in diet type and DMI were expected to affect CH_4 production, yield, and intensity. Furthermore, 1,405 cow-days were removed from the data set because on those days the FB recorded feed intake from other FB than the FB to which the cows were assigned (i.e., consumption of the wrong diet; stealing behavior). When stealing behavior was recorded, cows consumed on average 0.35 ± 0.04 kg DM/d from the wrong diet. Only the day at which consumption of the wrong diet was recorded was removed from the data set, because the small quantity was expected to have limited consequences for gas emissions or the effect of 3-NOP. This together resulted in a data set of 18,629 cow-days. All parameters related to feed intake, lactation performance, and gaseous exchange were averaged per cow per week. This resulted in 2,992 cow-weeks, including the adaptation period and the covariate period. The data set consisted of both experimental weeks (**ExpWk** = counting started after the covariate period; wk 1–48) and weeks that a cow had received a certain diet (**DietWk** = wk within a diet). As the first week of the DP was excluded from the data set because the cows were still lactating, the first DietWk in DP was 2.

The minimum number of GreenFeed visits for reliable measurements in our data set was evaluated in a subset of 10 blocks (20 cows) including only cow-weeks with at least 30 GreenFeed visits (736 cow-weeks). For these cow-weeks, we made 100 replicates of 14 random GreenFeed visits per cow per week and calculated the absolute error (|mean CH_4 of a replicate − mean CH_4 using all visits|), the absolute error as a fraction of the mean, and the standard deviation for each replicate. The number of 14 GreenFeed visits was based on visual evaluation, where the error of considering fewer visits than 35 flattened from 14 or more visits onward (data not shown). In our data set, using 14 GreenFeed visits per cow per week resulted in an overall mean absolute error of 15.3 g/d, a mean error of 4.1% of the absolute error, and a mean standard deviation of the absolute error of 11.3, compared with using all GreenFeed visits. These errors were arbitrary and considered to be acceptable. Using this threshold of 14 observations per cow-week resulted in deletion of 187 cow-weeks, spread over 59 cows (on average 3 wk per cow, ranging from 1 to 14 wk), all diets, and both treatments. We additionally used a threshold of 10 animals per treatment group within a diet (i.e., LL, DP, EL, and ML) within an ExpWk and 10 animals per treatment group within a DietWk. A minimum of 10 animals was based on a power calculation resulting in a power of 0.62 for 10 cows per week, 0.52 for 8 cows per week, and 0.39 for 6 cows per week. When fewer than 10 animals per treatment group were observed, we deleted those particular cow-weeks from the data set, resulting in a data set of 2,347 cow-weeks. These deleted cow-weeks due to fewer than 10 animals per treatment group involved cows that had relatively early or late calving in the study and therefore started or ended a diet relatively early or late. As a result of this, combined with the lack of a GreenFeed around the time of calving and the recovery time required after calving before moving back to the freestall barn with GreenFeed, the first week of EL was excluded for all cows because of insufficient GreenFeed visits (i.e., minimum of 14), and EL started with DietWk 2. Finally, the adaptation period (wk 1) was deleted from the data set and the data of the covariate period (wk 2–4) served as baseline measurement (covariate) in the statistical model. Hence, this resulted in a final data set of 2,108 cow-weeks during the 48-wk trial phase.

Data Analysis. Statistical analyses were performed by using SAS 9.4 (SAS Institute Inc., Cary, NC). Normality of the residuals was assessed by visual evaluation, and non-normal data were transformed to the natural logarithm to approximate a normal distribution. Values are presented as covariate-adjusted least squares means (LSM) ± pooled standard error of the mean. All P-values of pairwise comparisons of LSM were corrected with a Tukey-Kramer adjustment. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. The covariate period (wk 2–4) was used to calculate a covariate mean for all variables, which was added in the statistical models as baseline measurement.

A repeated measurements model (PROC MIXED) was used to test the effects of treatment, diet, DietWk, their interactions, and the covariate mean on dependent variables related to feed intake, lactation characteristics, and emissions:

$$\begin{aligned} y_{ijkl} = \mu + \mathrm{TRT}_i + \mathrm{Diet}_j + \mathrm{DietWk}_k + (\mathrm{TRT} \times \mathrm{Diet})_{ij} \\ + (\mathrm{TRT} \times \mathrm{DietWk})_{ik} + (\mathrm{Diet} \times \mathrm{DietWk})_{jk} \\ + (\mathrm{TRT} \times \mathrm{Diet} \times \mathrm{DietWk})_{ijk} + \mathrm{Cov}_l + \varepsilon_{ijkl}, \end{aligned}$$

where y_{ijkl} represents the dependent variables; μ represents the mean; TRT_i represents the treatment (i = CTL or 3-NOP); Diet_j represents the diet (j = LL, DP, EL, or ML, with the exception of traits involving milk, which have only LL, EL, and ML); DietWk_k represents the week within a diet (k = LL: 1, 2, . . . , 12; DP: 2, 3, . . . , 7; EL: 2, 3, . . . , 20; ML: 1, 2, . . . , 11); $(\mathrm{TRT} \times \mathrm{Diet})_{ij}$ represents the interaction between treatment and diet; $(\mathrm{TRT} \times \mathrm{DietWk})_{ik}$ represents the interaction between treatment and DietWk; $(\mathrm{Diet} \times \mathrm{DietWk})_{jk}$ represents the interaction between diet and DietWk; $(\mathrm{TRT} \times \mathrm{Diet} \times \mathrm{DietWk})_{ijk}$ represents the interaction between treatment, diet, and DietWk; Cov_l represents the covariate; and ε_{ijkl} represents the random error term from a normal distribution. The model included a repeated measurement effect of ExpWk with cow as the repeated subject and a random effect of block. As blocks were based on parity, parity was not included as a fixed effect in the models. The covariance structures first-order autoregressive, compound symmetry, unstructured, and variance components were considered, and first-order autoregressive covariance structure provided the best fit with the lowest overall Akaike's information criterion values. It should be noted that diet is confounded with effects of lactation stage, and effects of diet may not be solely related to diet composition changes but also to lactation stage changes. A Pearson correlation (PROC CORR) was used to test, for each diet separately, the relation between the relative reduction of CH_4 yield and actual 3-NOP dose (i.e., taking into account individual PMR DMI as well as DMI of nonsupplemented GreenFeed bait) as well as between the relative reduction of CH_4 yield and the dietary content of the following nutrients on a weekly basis: OM, ADF, ADL, NDF, CP, fat, starch, sugar, GE, and NE_L.

RESULTS

The objective of this study was to determine the effect of 3-NOP on CH_4 emission and production characteristics from cows receiving 3-NOP in their diet for 1 full year. Hence, despite the complex experimental design and statistical model, we will focus specifically on the treatment effect and all interactions with treatment when discussing the results. The 3-NOP dose in the PMR samples of the CTL diet was 0 mg/kg DM. Based on the 3-NOP dose of the premix samples collected on a weekly basis, the calculated average 3-NOP dose in the PMR was 75.9 ± 3.59 mg/kg DM for LL, 76.6 ± 3.06 mg/kg DM for DP, 75.6 ± 3.64 mg/kg DM for EL, and 74.0 ± 3.67 mg/kg DM for ML. Taking into account both DMI of the PMR and that of the nonsupplemented GreenFeed bait on an individual cow level, the overall inclusion levels of 3-NOP for total daily DMI (i.e., the actual 3-NOP dose) were on average 69.8 ± 0.17 mg/kg DM, 70.2 ± 0.43 mg/kg DM, 70.9 ± 0.17 mg/kg DM, and 69.2 ± 0.48 mg/kg DM for LL, DP, EL, and ML diets, respectively. Intake of 3-NOP was on average 1.44 ± 0.028 g/d, 1.15 ± 0.020 g/d, 1.70 ± 0.035 g/d, and 1.64 ± 0.034 g/d for LL, DP, EL, and ML diets, respectively.

Effect of 3-NOP on Gas Emissions

For all units of CH_4 emission, the production and yield of H_2, and the ratio of CH_4 to CO_2, a treatment × diet × DietWk interaction ($P \leq 0.05$) was observed (Table 3). The interactions for CH_4 and H_2 yield are visualized in Figure 1 and resulted from a difference in the effect of 3-NOP between the diets as well as within the diets over time. For all diets and all weeks within that diet, cows receiving 3-NOP had a lower CH_4 emission and CH_4-to-CO_2 ratio compared with cows receiving CTL ($P < 0.01$ for all Tukey adjusted pairwise comparisons). On average, a 26.0% ± 0.48%, 16.1% ± 0.05%, 19.7% ± 1.02%, and 15.5% ± 0.43% reduction in CH_4 yield was achieved by 3-NOP compared with CTL for the LL, DP, EL, and ML diets, respectively (see also Figure 2). Similarly, for all diets and all weeks within diet, cows receiving 3-NOP had a greater H_2 emission compared with cows receiving CTL ($P < 0.01$ for all Tukey-adjusted pairwise comparisons). On average, 235% ± 0.2%, 276% ± 3.5%, 251% ± 5.4%, and 179% ± 4.0% increases in H_2 yield were observed for 3-NOP compared with CTL for the LL, DP, EL, and ML diets, respectively. The realized relative reduction in CH_4 yield upon 3-NOP supplementation was correlated with actual 3-NOP dose (mg/kg DM), diet composition (g/kg DM), and GE and NE_L contents (MJ/kg DM), with the correlations being diet specific (Table 4; correlation determined using weekly averages). For the LL diet, the relative reduction in CH_4 yield was positively correlated with OM, sugar, GE, and NE_L contents, and negatively correlated with ADF, ADL, fat, and starch contents. For the DP diet, the relative reduction in CH_4 yield was positively correlated with 3-NOP, ADF, ADL, and NDF contents, and was negatively correlated with NE_L content. For the EL diet, the relative reduction in CH_4 yield was positively correlated with OM, sugar, GE, and NE_L contents, and negatively correlated with ADL, CP, and fat contents. Finally, for the ML diet, the relative reduction in CH_4 yield was positively correlated with 3-NOP, sugar, and NE_L contents, and negatively correlated with ADF, ADL, NDF, CP, and starch contents. The weekly variation in diet composition of the PMR, underlying these correlations, is shown in Figure 3.

A treatment × diet interaction ($P = 0.01$) was observed for H_2 intensity (Table 3). Over all lactation diets, cows receiving 3-NOP had a greater H_2 intensity compared with cows receiving CTL (i.e., 0.05 vs. 0.14 g/kg FPCM; 2.8-fold increase), but the increase in H_2 intensity with 3-NOP compared with CTL was different between the diets (i.e., LL: 0.07 vs. 0.22 g/kg FPCM [3.1-fold increase]; EL: 0.03 vs. 0.11 g/kg FPCM [3.7-fold increase]; ML: 0.05 vs. 0.12 g/kg FPCM [2.4-fold increase]; $P < 0.01$ for every diet). No interactions between treatment and other factors or effect of treatment were observed for the number of GreenFeed visits or for the production and intensity of CO_2, whereas CO_2 yield was higher ($P = 0.04$) for 3-NOP compared with CTL, and approximately double in size compared with the decrease in CH_4 yield.

Effect of 3-NOP on Feed Intake

No treatment × diet × DietWk interaction, treatment × diet interaction, or treatment × DietWk interaction was observed for any of the feed intake variables (Table 5). Cows receiving 3-NOP tended ($P = 0.10$) to have a greater DMI of GreenFeed bait compared with the cows receiving CTL, although this difference remained small (70 g of DM/d; 0.3% of total DMI). In addition, cows receiving 3-NOP had a 6.4% greater ($P = 0.02$) feed efficiency for both FPCM and ECM compared with cows receiving CTL. Treatment did not affect DMI of PMR or total DMI.

Effect of 3-NOP on Lactation Performance and Body Measures

A treatment × diet × DietWk interaction was observed for milk lactose content ($P = 0.01$) and milk fat content ($P = 0.10$; tendency only; Table 6). For wk 1, 2, and 3 within the LL diet, cows receiving 3-NOP had a greater milk lactose content ($P < 0.05$ for all comparisons), and for wk 4 within the LL diet cows receiving 3-NOP tended to have a greater milk lactose content ($P = 0.06$) compared with cows receiving CTL. For all other weeks within that diet, and for other diets, milk lactose content did not differ between treatments. For wk 1 to 10 within the LL diet, cows receiving 3-NOP had a greater milk fat content ($P < 0.02$), and for wk 11 within the LL diet cows receiving 3-NOP tended to have a greater milk fat content ($P = 0.08$) compared with cows receiving CTL. For wk 2 to 7 within the EL diet, cows receiving 3-NOP had a lower milk fat content ($P < 0.05$ for all comparisons), and for wk 8 within the EL diet cows receiving 3-NOP tended to have a lower milk fat content ($P = 0.07$) compared with cows receiving CTL. For other weeks within those diets, and for other diets, milk fat content did not differ between treatments. A tendency for a treatment × diet interaction was observed for milk protein content ($P = 0.06$), and a treatment × DietWk interaction was observed for milk urea content ($P = 0.05$). Overall, in the first weeks of a diet, cows receiving 3-NOP had a greater milk urea content compared with cows receiving CTL. In the later weeks of a diet, the milk urea content did not differ between 3-NOP and CTL. Overall, cows receiving

Table 3. Gas emissions of dairy cows receiving a diet supplemented with a placebo (CTL) or with 3-nitrooxypropanol (3-NOP) for 1 yr (LSM ± pooled SEM)[1]

	Treatment			*P*-value						
Item	CTL	3-NOP	SEM	Trt[2]	Diet[3]	DietWk[4]	Trt × diet	Trt × DietWk	Diet × DietWk	Trt × diet × DietWk
GreenFeed visits[5]	34.4	35.7	0.87	0.52	<0.01	<0.01	0.51	0.14	<0.01	0.36
CH_4 emission										
Production (g/d)	425	336	7.0	<0.01	<0.01	<0.01	<0.01	0.92	<0.01	<0.01
Yield (g/kg DMI)	20.3	16.2	0.29	<0.01	<0.01	<0.01	0.01	0.45	<0.01	<0.01
Intensity[6] (g/kg FPCM[7])	13.9	10.2	0.40	<0.01	<0.01	<0.01	0.08	0.62	<0.01	0.03
H_2 emission										
Production (g/d)	1.44	4.74	0.138	<0.01	<0.01	0.05	<0.01	0.43	<0.01	0.02
Yield (g/kg DMI)	0.07	0.23	0.006	<0.01	<0.01	<0.01	<0.01	0.17	<0.01	<0.01
Intensity[6] (g/kg FPCM)	0.05	0.14	0.005	<0.01	<0.01	<0.01	0.01	0.72	<0.01	0.48
CO_2 emission										
Production (g/d)	13,311	13,306	107.3	0.99	<0.01	<0.01	0.43	0.98	<0.01	0.41
Yield (g/kg DMI)	642	650	6.6	0.04	<0.01	<0.01	0.83	0.46	<0.01	0.20
Intensity[6] (g/kg FPCM)	439	414	12.3	0.12	<0.01	<0.01	0.71	0.62	<0.01	0.95
CH_4 to CO_2 ratio	0.032	0.025	0.0005	<0.01	<0.01	0.98	0.01	0.52	<0.01	0.01

[1] In all models, the average of the variable in the covariate period (wk 2, 3, and 4) was included and always had a significance of $P < 0.01$.

[2] Trt = treatment (3-NOP or CTL).

[3] Diet = late lactation, dry period, early lactation, or mid lactation.

[4] DietWk = week within a diet.

[5] Successful GreenFeed visits with gas emission measurements per week.

[6] Non-normal data were transformed to the natural logarithm for analysis, and back-transformed.

[7] FPCM = fat- and protein-corrected milk yield.

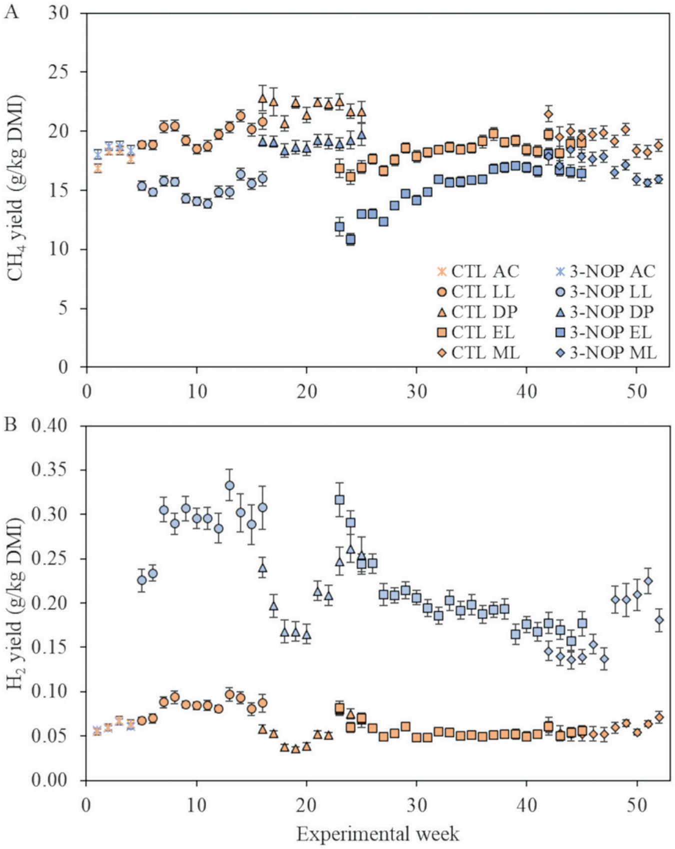

Figure 1. CH_4 yield (panel A) and H_2 yield (panel B) for the complete experimental period per diet (AC = adaptation + covariate period, LL = late lactation, DP = dry period, EL = early lactation, or ML = mid lactation) of cows receiving a diet supplemented with a placebo (CTL) or with 3-nitrooxypropanol (3-NOP; mean ± SEM).

3-NOP had greater ($P \leq 0.05$) FPCM, ECM, fat, and protein yields, and milk SCC compared with cows receiving CTL (Table 6). Supplementation of 3-NOP did not affect BW, BW change, or BCS.

DISCUSSION

CH_4 Emission

In agreement with results of the meta-analysis of Kebreab et al. (2023), 3-NOP supplementation reduced CH_4 emission relative to CTL in the present study, where an overall reduction of 21%, 20%, and 27% was achieved for the duration of the study for CH_4 production, yield, and intensity, respectively. The CH_4 mitigation potential of 3-NOP was, however, affected by the diet to which 3-NOP was supplemented as well as by the week of 3-NOP supplementation within a diet. The lactation diets used in the present study (i.e., LL, EL, and ML) were representative of current practice in the Dutch dairy sector. The amount of concentrate, for example, depended on lactation stage, being 44.5%, 34.8%, and 24.1% (on a DM basis) for the EL, ML, and LL diets, respectively. Contrary to Dutch practice, though, the DP diet contained more concentrate (18.7%) because of the method of 3-NOP supplementation and diet formulation to meet the energy and protein requirements for maintenance and pregnancy. The forage composition also changed accordingly with stage of lactation, containing 70% grass silage and 30% corn silage for all lactation diets, and containing 33% grass silage, 23% corn silage, and 44% straw for the DP diet (all on a DM basis). These shifts in dietary in-

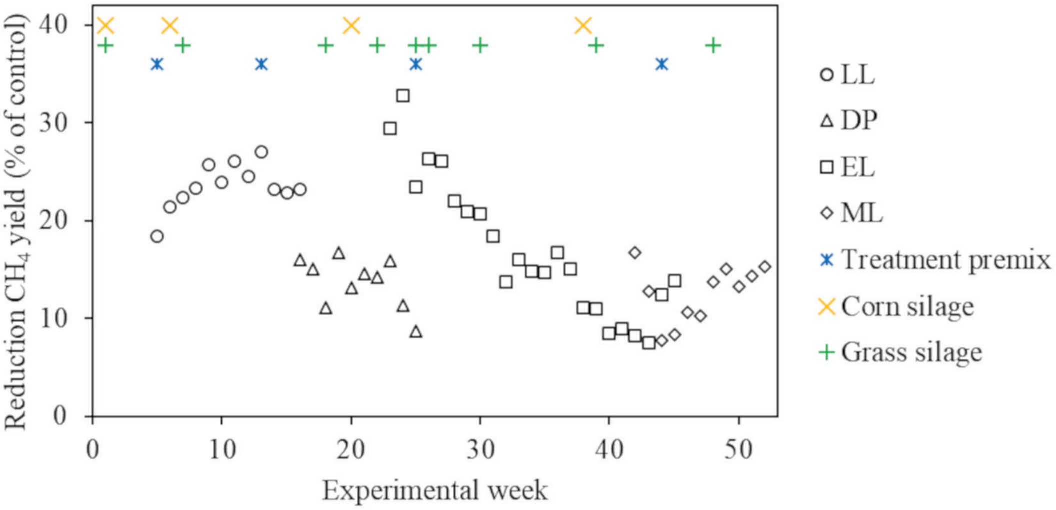

Figure 2. Relative reduction of CH_4 yield ([CTL − 3-NOP]/CTL × 100; %) for the complete experimental period, per diet (LL = late lactation, DP = dry period, EL = early lactation, or ML = mid lactation). Changes in grass silage silo, corn silage silo, and treatment premix batch are illustrated in time. Diets were supplemented with placebo (CTL) or with 3-nitrooxypropanol (3-NOP).

Table 4. Pearson correlations between the relative reduction of CH_4 yield ([CTL − 3-NOP]/CTL × 100; %)[1] and diet composition (in g/kg DM, unless otherwise stated) on a weekly basis[2]

Diet[3]	No. weeks within a diet (DietWk)	Correlation with relative reduction in CH_4 yield	Actual 3-NOP dose (mg/kg DM)	OM	ADF	ADL	NDF	CP	Fat	Starch	Sugar	GE (MJ/kg DM)	NE_L (MJ/kg DM)
LL	12	r	NS[4]	0.58	−0.71	−0.90	NS	NS	−0.60	−0.80	0.90	0.55	0.73
		P-value	NS	0.05	<0.01	<0.01	NS	NS	0.04	<0.01	<0.01	0.07	<0.01
DP	6	r	0.79	NS	0.96	0.80	0.89	NS	NS	NS	NS	NS	−0.89
		P-value	0.06	NS	<0.01	0.06	0.02	NS	NS	NS	NS	NS	0.02
EL	19	r	NS	0.86	NS	−0.90	NS	−0.97	−0.59	NS	0.97	0.77	0.98
		P-value	NS	<0.01	NS	<0.01	NS	<0.01	<0.01	NS	<0.01	<0.01	<0.01
ML	11	r	0.67	NS	−0.71	−0.98	−0.96	−0.97	NS	−0.94	0.97	NS	0.96
		P-value	0.03	NS	0.01	<0.01	<0.01	<0.01	NS	<0.01	<0.01	NS	<0.01

[1] Diet supplemented with a placebo (CTL) or with 3-nitrooxypropanol (3-NOP).

[2] Correlations are shown when $P < 0.10$.

[3] Diet: LL = late lactation, DP = dry period, EL = early lactation, ML = mid lactation.

[4] No significant ($P > 0.10$) correlation.

gredient composition across diets resulted in differences in chemical composition (e.g., dietary NDF content), and subsequently in variability in the mitigating effect of 3-NOP. According to the meta-analysis of Kebreab et al. (2023), the reduction efficiency for a given 3-NOP dose is dependent on diet composition, including NDF content.

Across subsequent weeks within diet, the variation in diet composition within each diet appears associated with the considerable variability in the observed CH_4 mitigation potential of 3-NOP. Due to the length of duration of the trial, we had to feed 9 different silos of grass silage and 4 different silos of corn silage (Figure 2), with silos having different chemical composition and NE_L. This resulted in substantial variation in diet composition of the PMR over time (Figure 3). Within lactation stage, this resulted in high correlations between the relative reduction of CH_4 yield and the different chemical fractions and NE_L of the total diet. Organic matter digestibility is a measure for ration quality, but this was not available for all ration components in the present study, and, when it was available, it was only estimated using near-infrared spectroscopy. Hence, we considered NE_L (van Es, 1978) as a measure for ration quality. There were differences between the diets with respect to the correlations found, but overall (when significant) the directions of the relations were similar (negative or positive), with the exception of the DP diet. The DP diet strongly contrasts with the lactation diets by its high proportion of straw but also due to the lower DMI that was realized. Under such conditions the correlations between the relative reduction of CH_4 yield and the dietary chemical fractions and NE_L for the DP diet across weeks are apparently opposite to those established for the LL, EL, and ML diets. This would imply that the effect of these factors on the relative reduction of CH_4 by 3-NOP was opposite to that in the lactation diets. As shown in Figure 3, the variation in the fibrous fraction of the diet (i.e., ADL, ADF, and NDF) is substantial over the duration of the complete experiment, but also within each lactation stage-dependent diet. This may have contributed to the positive relationship found between the fibrous fraction of the diet and the relative reduction of CH_4 yield for the DP diet, which is contrary to the results of meta-analyses of Dijkstra et al. (2018) and Kebreab et al. (2023), and warrants further investigation. However, the general negative relationships found between the relative reduction of CH_4 yield and the fibrous fraction of the diet for the lactation diets are in agreement with the meta-analyses of Dijkstra et al. (2018) and Kebreab et al. (2023). The negative relation found between the relative reduction of CH_4 yield and dietary starch content for both LL (starch ranging between 112 and 168 g/kg DM) and ML (starch ranging between 164 and 175 g/kg DM) as well as the lack of

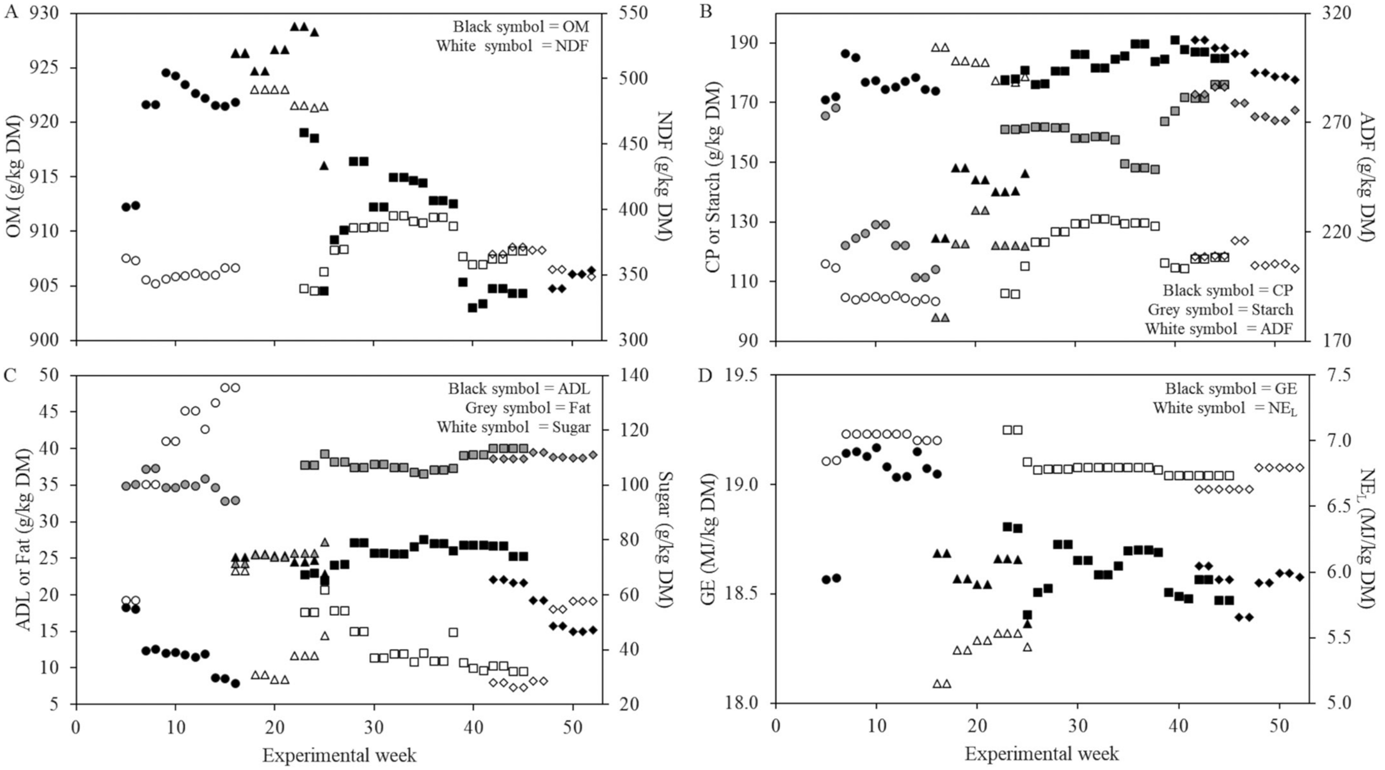

Figure 3. Weekly variation in diet composition of the partial mixed ration for the complete experimental period per diet, averaged over the diet supplemented with a placebo or with 3-nitrooxypropanol (circles = late lactation, triangles = dry period, squares = early lactation, and diamonds = mid lactation). Panel A shows OM and NDF (g/kg DM); panel B shows CP, starch, and ADF (g/kg DM); panel C shows ADL, fat, and sugar (g/kg DM); and panel D shows gross energy (GE) and NE_L (MJ/kg DM).

relation for EL (starch ranging between 148 and 176 g/kg DM) are contrary to what one would expect based on both Dijkstra et al. (2018) and Kebreab et al. (2023), and at present cannot be explained. For each lactation diet, NE_L was positively related to the relative reduction of CH_4 yield. The variation in NE_L was substantial over the complete trial, ranging from 5.2 MJ/kg DM for the DP diet to 7.1 MJ/kg DM for the EL diet; also within the EL diet, NE_L varied greatly (i.e., between 6.7 and 7.1 MJ/kg DM). A higher ration quality is generally associated with

Table 5. Feed intake and efficiency of dairy cows receiving a diet supplemented with a placebo (CTL) or with 3-nitrooxypropanol (3-NOP) for 1 yr (LSM ± pooled SEM)[1]

	Treatment			*P*-value						
Item	CTL	3-NOP	SEM	Trt[2]	Diet[3]	DietWk[4]	Trt × Diet	Trt × DietWk	Diet × DietWk	Trt × Diet × DietWk
DMI PMR[5] (kg/d)	19.9	19.7	0.27	0.25	<0.01	0.39	0.34	0.64	<0.01	0.12
DMI GreenFeed bait (kg/d)	1.33	1.40	0.027	0.10	0.15	0.01	0.45	0.72	0.01	0.31
DMI total (kg/d)	21.2	21.1	0.27	0.36	<0.01	0.30	0.38	0.65	<0.01	0.15
Feed efficiency (kg FPCM[6]/kg DMI total)	1.44	1.53	0.031	0.02	<0.01	<0.01	0.68	0.77	<0.01	0.90
Feed efficiency (kg ECM/kg DMI total)	1.57	1.67	0.034	0.02	<0.01	<0.01	0.71	0.76	<0.01	0.92

[1] In all models, the average of the variable in the covariate period (wk 2, 3, and 4) was included and always had a significance of $P < 0.01$.

[2] Trt = treatment (3-NOP or CTL).

[3] Diets = late lactation, dry period, early lactation, or mid lactation.

[4] DietWk = week within a diet.

[5] PMR = partially mixed ration.

[6] FPCM = fat- and protein-corrected milk.

Table 6. Body measures and lactation characteristics of dairy cows receiving a diet supplemented with a placebo (CTL) or with 3-nitrooxypropanol (3-NOP) for 1 yr (LSM ± pooled SEM)[1]

	Treatment			*P*-value						
Item	CTL	3-NOP	SEM	Trt[2]	Diet[3]	DietWk[4]	Trt × Diet	Trt × DietWk	Diet × DietWk	Trt × Diet × DietWk
BW (kg)	710	717	5.6	0.14	0.88	<0.01	0.36	0.64	<0.01	0.71
BW change (kg/wk)	1.8	2.1	1.05	0.92	0.03	0.55	0.86	0.97	0.82	0.93
BCS	3.3	3.4	0.04	0.16	<0.01	<0.01	0.23	0.26	<0.01	0.67
Lactation characteristics										
Milk yield (kg/d)	29.3	30.6	0.93	0.21	<0.01	<0.01	0.16	0.89	<0.01	0.80
FPCM[5] (kg/d)	32.3	34.4	0.81	0.03	<0.01	<0.01	0.54	0.84	0.01	0.62
ECM (kg/d)	35.2	37.5	0.88	0.03	<0.01	<0.01	0.53	0.84	0.01	0.59
Fat content (%)	4.82	4.91	0.077	0.65	<0.01	0.40	<0.01	0.55	<0.01	0.10
Protein content (%)	3.73	3.80	0.042	0.24	<0.01	<0.01	0.06	0.83	<0.01	0.45
Lactose content (%)	4.39	4.40	0.018	0.07	<0.01	0.81	0.01	0.28	<0.01	0.01
Fat yield (kg/d)	1.38	1.49	0.036	0.01	<0.01	<0.01	0.34	0.82	0.01	0.31
Protein yield (kg/d)	1.07	1.13	0.026	0.03	<0.01	<0.01	0.41	0.81	<0.01	0.68
Lactose yield (kg/d)	1.29	1.35	0.043	0.17	<0.01	<0.01	0.53	0.98	<0.01	0.69
Urea content (mg/dL)	21.5	21.7	0.63	0.01	<0.01	<0.01	0.40	0.05	<0.01	0.54
SCC × 1,000 cells[6]	92	104	17.8	0.05	0.30	0.28	0.58	0.18	0.71	0.86

[1] In all models, the average of the variable in the covariate period (wk 2, 3, and 4) was included and always had a significance of $P < 0.01$ except for BW change ($P = 0.24$).

[2] Trt = treatment (3-NOP or CTL).

[3] Diets = late lactation, dry period, early lactation, or mid lactation.

[4] DietWk = week within a diet.

[5] FPCM = fat- and protein-corrected milk.

[6] Non-normal data were transformed to the natural logarithm for analysis, and back-transformed.

a shorter mean retention time of the feed in the rumen (Moe and Tyrrell, 1979), faster fermentation, and tendency toward increased propionate production (Pacheco et al., 2014; van Lingen et al., 2016). This may result in reduced methanogens in the rumen and subsequently a lower concentration of methylcoenzyme M reductase, the enzyme inhibited by 3-NOP. Hence, adding 3-NOP to a high-quality diet might inhibit methylcoenzyme M reductase with greater efficacy, consequently resulting in a greater CH_4 mitigation, compared with a low-quality diet. Alternatively, with a high-quality diet, the ruminal fermentation conditions may react more strongly to disturbances of H_2 dynamics via inhibition of methanogens by 3-NOP, leading to a stronger shift in fermentation profile, H_2 production, and methanogenesis on top of the effect of 3-NOP. The positive relation between actual 3-NOP dose and the relative reduction of CH_4 yield observed is in line with the meta-analyses of both Dijkstra et al. (2018) and Kebreab et al. (2023) as well as with Hristov et al. (2022). This relation was, however, only present for the DP and ML diet in the current study. This appears to be related to a higher amount of nonsupplemented GreenFeed bait intake and subsequently lower relative actual 3-NOP dose on complete ration level for these diets compared with the LL and EL diets. The GreenFeed bait DMI was 1.31 ± 0.032 kg/d, 1.45 ± 0.027 kg/d, 1.37 ± 0.032 kg/d, and 1.42 ± 0.041 kg/d for LL, DP, EL, and ML, respectively. This equals GreenFeed bait DMI fractions (relative to total DMI) of 6.5% ± 0.21%, 9.0% ± 0.22%, 5.9% ± 0.24%, and 6.0% ± 0.20% for LL, DP, EL, and ML, respectively.

The week of 3-NOP supplementation within a specific diet also affected the CH_4 mitigation potential of 3-NOP in the present study. For all diets and all weeks within each diet, cows receiving 3-NOP had a lower CH_4 emission compared with cows receiving CTL. However, first examination indicated that the CH_4 mitigation potential of 3-NOP was diminishing over time, both within diet (DP and EL particularly) and over the time course of the study, and this was therefore explored further. First to be considered was within-diet effects. Within the EL diet, the mitigation potential of 3-NOP decreased from 29% reduction in CH_4 yield at the start of EL to 8% reduction in CH_4 yield relative to CTL near the end of EL (reduction increased again to 14% in the last 2 wk of EL). A similar although smaller effect was also visible for the DP diet, where the mitigation potential of 3-NOP decreased from 16% reduction at the start of DP in CH_4 yield to 9% reduction in CH_4 yield relative to CTL near the end of DP (Figure 2). Part of this decline over time within diet can be proposed to be linked to the grass silage and corn silage silo changes and subsequently variation in diet composition, as explained earlier and visualized in Figures 2 and 3. In contrast to the EL and DP diets, the mitigation potential of 3-NOP during LL did not decrease, but rather increased from 18% reduction in

CH_4 yield relative to CTL at the start to 23% reduction at the end. Additionally, the mitigation potential of 3-NOP during ML initially decreased from 17% to 8% reduction in CH_4 yield relative to CTL during the first 3 wk, but increased to 15% reduction at the end of ML. Also considered was that over the course of this study, the CH_4 mitigation potential of 3-NOP appeared to diminish over time. From LL to ML, the observed CH_4 yield reductions were 26.0%, 16.1%, 19.7%, and 15.5%. Based on the actual 3-NOP dose and the dietary NDF content for each diet, the prediction equation of Kebreab et al. (2023) estimates reductions of 28.6%, 18.4%, 26.9%, and 26.5% in CH_4 yield. We acknowledge that this prediction equation of Kebreab et al. (2023) was developed specifically for lactating cows; hence, applying the prediction equation to DP may not be fully correct and can only serve as an indication. Comparing the realized CH_4 yield reduction of the present study with the predicted potential (Kebreab et al., 2023), the realized efficacy of 3-NOP supplementation was 91% for LL, 87% for DP, 73% for EL, and 58% for ML (in percentage of expected efficiency). That shows a decline pattern in efficacy, suggesting a lower effect of 3-NOP the longer it is supplemented. Contrary to this, however, the increased CH_4 mitigation potential of 3-NOP at the end of the EL diet as well as for the ML diet immediately following the EL diet, without an increase in dietary 3-NOP dose, does not suggest a decline in CH_4-reducing efficacy or adaptation to 3-NOP in the rumen (e.g., a diminished response of the microbiota and methanogenesis after long-term exposure to 3-NOP). This observed response is confounded by changes in diet composition and its fermentability, and therefore requires further investigation as it does not follow a classic adaptation pattern, where efficacy would continue to decline with time.

Several studies have, however, proposed that 3-NOP supplementation may lose persistency over time, whereas other studies reported persistent effects over time. McGinn et al. (2019) used 2 micrometeorological methods, a concentration ratio method and an inverse dispersion method, to measure CH_4 emission in beef cattle feedlots upon 3-NOP supplementation (i.e., 125 mg/kg DM). Over a 90-d period, the CH_4 reduction upon 3-NOP supplementation declined over time with both CH_4 measurements methods, suggesting some adaptation to 3-NOP in the rumen. It should be noted, though, that the micrometeorological methods used by McGinn et al. (2019) do not distinguish between enteric and manure CH_4 emission. The pens in that study were not cleaned for the duration of the finishing period, and the decrease in efficacy might have been more related to greater proportion of CH_4 emission coming from manure as the study progressed, because 3-NOP is expected to have no effect on manure CH_4 emission, as 3-NOP is completely metabolized in the rumen. The observation of McGinn et al. (2019) was not observed in the longer-term beef cattle study of Romero-Perez et al. (2015; 112 d using respiration chambers). Alemu et al. (2021a) fed low (124.6 mg 3-NOP/kg DM), medium (192.8 mg 3-NOP/kg DM), and high doses (226.8 mg 3-NOP/kg DM) in 3 successive 28-d phases to beef cattle and measured emissions using the GreenFeed system. The authors reported an overall decrease of 21.7% in CH_4 yield upon 3-NOP supplementation, and showed decreases of 20.2%, 25.5%, and 21.5% in CH_4 yield for the low, medium, and high 3-NOP doses. These results suggest a persistent effect of 3-NOP, although it must be mentioned that the 3-NOP dose increased over time (from 124.6 to 226.8 mg 3-NOP/kg DM) whereas the mitigation potential of 3-NOP did not increase, even though a dose-response can be expected (Dijkstra et al., 2018). Furthermore, Alemu et al. (2021b) fed low (100 mg/kg DM), medium (125 mg/kg DM), and high (150 mg/kg DM) doses of 3-NOP to crossbred steers for 112 d. The authors reported that CH_4 yield was decreased by 52%, 76%, and 63% for low, medium, and high 3-NOP doses, respectively, compared with the control. However, they also observed that the low dose decreased in efficacy from 59% in the first and in the second period of 28 d, to 37% in the third period of 28 d, whereas the efficacy of the medium and high 3-NOP doses remained consistent over time, although with considerable variation (e.g., at highest dose, efficacy decreased from 78% in first period to 48% in second period, to increase again to 62% in third period). Despite the considerable variation, it was therefore concluded that a possible rumen adaptation at low levels of supplementation occurred (Alemu et al., 2021b). Schilde et al. (2021) reported a loss in persistency of 3-NOP over time when dairy cows were fed a low-concentrate diet, but not when cows were fed a high-concentrate diet. Interestingly, a steep increase in CO_2 production (+30%) in the last 6 to 8 wk of the 21-wk total period was recorded by Schilde et al. (2021) for the 3-NOP treatments, but not for the control treatments. A change in DMI seems unlikely to explain this steep increase in CO_2 production, especially because Schilde et al. (2021) reported a stable GE intake over time and we assumed an unchanged dietary GE content. Such an increase in CO_2 emission cannot be related to the realized outcome, because in hydrogenotrophic methanogenesis, 1 mol CO_2 is used to form 1 mol CH_4 (McAllister and Newbold, 2008). These results may thus suggest that the observed loss in efficacy of 3-NOP is perhaps confounded by the gas measurements as evidenced by the combined increase in both CH_4 and CO_2 emissions for the low-concentrate diet with 3-NOP. More recently, Hristov et al. (2022) regressed the averaged CH_4 mitigation effect of 3-NOP, expressed as relative reduction in CH_4 yield compared with the control treatment, over the duration of two 15-wk experiments with a 3-NOP dose of 60 mg/kg DM (i.e., Melgar et al., 2020a, 2021). In the case of Melgar et al. (2020a), Hristov et al. (2022) reported that the CH_4 mitigation effect of 3-NOP decreased linearly from 24% (wk 2) to 17% (wk 15). This linear decrease was, however, not significant, and the study of Melgar et al. (2020a) itself reported a nonsignificant treatment × study week interaction, suggesting a persistent effect of 3-NOP over the duration of 15 wk. In the case of Melgar et al. (2021), Hristov et al. (2022) reported that the CH_4 mitigation effect of 3-NOP decreased linearly from 31% (wk 3) to 24% (wk 15). Also Melgar et al. (2021) themselves reported a tendency for a treatment × week interaction for CH_4 production, although they reported the effect of 3-NOP to be persistent. These observations suggest a possible temporary, or inconsistent, effect of 3-NOP over time. In contrast, in the studies of Hristov et al. (2015) and van Gastelen et al. (2020), there was no such change in CH_4 mitigation potential of 3-NOP over time.

H_2 and CO_2 Emissions

Supplementation of 3-NOP resulted in an increase in H_2 emission, which is in agreement with other studies (e.g., Melgar et al., 2020a,b; van Gastelen et al., 2020, 2022). The increase in H_2 emission (production and yield) followed the response in CH_4 emission, being affected by the diet to which 3-NOP was supplemented as well as by the week of 3-NOP supplementation within a diet. For example, the increase in H_2 yield was smallest for the ML diet, for which the reduction in CH_4 yield was also smallest. Additionally, the increase in H_2 yield was diminishing over time for the EL diet, similarly to the CH_4 mitigation potential of 3-NOP. These responses match the changes in diet composition, as was discussed already for CH_4 emission. In general, we measured a smaller increase in H_2 emission than what can be expected based on the calculated amount of unused H_2 due to decreased CH_4 production. This corresponds with previous observations by Hristov et al. (2015) and van Gastelen et al. (2020, 2022), and suggests that the secondary effects of 3-NOP include a redirection of H_2 to alternative H_2 sinks, with a redirection of the ruminal fermentation profile toward propionate fermentation as a H_2 sink being a likely consequence (Haisan et al., 2014; Lopes et al., 2016). However, on certain diets this redirection to propionate production is not always observed, and other metabolic fates of this H_2 then must be presumed (Guyader et al., 2017).

The consistent increase in CO_2 yield upon 3-NOP supplementation for all diets and week within diet, is contrary to Hristov et al. (2015; no difference in CO_2 emission upon 3-NOP supplementation in ML to LL dairy cows) and Melgar et al. (2020a; decreased CO_2 production but unaffected CO_2 yield and intensity upon 3-NOP supplementation in EL dairy cows), but in line with van Gastelen et al. (2022). The observed increase in CO_2 yield upon 3-NOP supplementation might be related to CO_2 not being used any more due to the inhibition of CH_4 formation by 3-NOP. Assuming that in hydrogenotrophic methanogenesis 1 mol CO_2 is used to form 1 mol CH_4 (McAllister and Newbold, 2008), the overall decrease in CH_4 yield (i.e., 4.1 g of CH_4/kg DMI) with 3-NOP compared with CTL could be expected to result in an increase of 11.3 g of CO_2/kg DMI with 3-NOP supplementation. A part of this difference might be explained by the potential shift in ruminal fermentation profile (i.e., increased proportion of ruminal propionate at the expense of acetate; Haisan et al., 2014, 2017). When assuming a VFA production of 70 mol/d and a 1% shift from acetate to propionate (i.e., 0.7 mol), 0.7 mol CO_2 will not be produced by the formation of acetate. This equals 31 g of CO_2 and 1.5 g of CO_2/kg DMI upon 3-NOP supplementation. Considering both CH_4 mitigation and shift in ruminal fermentation profile, an increase of 9.8 g of CO_2/kg DMI with 3-NOP supplementation can be expected. We observed CO_2 yield to increase with 7.8 g/kg DMI, however, which is 80% of the expected 9.8 g/kg DMI. This finding confirms that with 3-NOP part of the energy prevented from emission as CH_4 was retained as absorbed energy (i.e., nutrients) retained in milk and hence not contributing to CO_2 emission.

Feed Intake, Body Measures, and Lactation Performance

Although DMI of the GreenFeed bait tended to be higher for the cows supplemented with 3-NOP, total DMI as well as DMI of the PMR was not affected by 3-NOP supplementation in the present study. This is in agreement with the meta-analysis of Kim et al. (2020), where it was concluded that the DMI of beef cattle tended to decrease when the dose of 3-NOP increased but that the DMI of dairy cattle was unaffected by 3-NOP supplementation. The latter is because none of the individual studies used for that meta-analysis reported a significant change of DMI by 3-NOP. van Gastelen et al. (2020; 51 mg 3-NOP/kg DM), Schilde et al. (2021; ~50 mg 3-NOP/kg DM), and Melgar et al. (2021; 60 mg 3-NOP/kg DM) also reported no effect of 3-NOP supplementation on DMI of dairy cattle. Melgar et al. (2020b) reported that DMI was not different between the 3-NOP and control treatments, but that increasing the 3-NOP dose tended to linearly decrease DMI. Both van Gastelen et al. (2022; 62 and 80 mg/kg DM) and Melgar et al. (2020a; 60 mg/kg DM) reported a decreased DMI upon 3-NOP supplementation. van Gastelen et al. (2022) adopted relatively short measurement periods in a crossover design, and it might be proposed that reductions in DMI occur relatively more easily under such experimental settings. Melgar et al. (2020a) assigned the lower DMI fully to a lower BW. In the present study, neither BW nor BCS were affected by 3-NOP supplementation, which is in agreement with Haisan et al. (2017) and also Hristov et al. (2022), who concluded from their meta-analysis that neither BW nor BW change were affected by 3-NOP supplementation. In contrast, Haisan et al. (2014), Hristov et al. (2015), and van Gastelen et al. (2020) observed a greater BW gain upon 3-NOP supplementation. A difference in BW or BW change might be caused by a shift in the ruminal fermentation profile and partly retention of the prevented energy loss with CH_4 emission. Studies have reported an increased proportion of ruminal propionate at the expense of acetate upon 3-NOP supplementation (e.g., Haisan et al., 2014, 2017). Propionate is classified as glucogenic and may result in increased insulin blood levels reducing mobilization of body reserves (van Knegsel et al., 2007) or increased retention in body reserves. In the present study, however, this did not occur, and the increased absorbed energy actually led to increased milk production.

Milk yield was not significantly affected by 3-NOP supplementation, but was numerically higher for the 3-NOP treatment. Also milk fat and protein content were numerically higher for the 3-NOP treatment. These numerical differences combined resulted in significant increases of FPCM (+2.2 kg/d; +6.5%), ECM (+2.3 kg/d; +6.5%), fat yield (+0.11 kg/d), and protein yield (+0.06 kg/d) upon 3-NOP supplementation, as well as an increased feed efficiency because of the unchanged DMI (relative to both FPCM [+0.09; +6.3%] and ECM [+0.10; +6.4%]). The increased milk fat upon 3-NOP supplementation in the present study is in line with Lopes et al. (2016), Melgar et al. (2020b, 2021), and van Gastelen et al. (2020, 2022; both milk fat content only). The milk fat response likely resulted from a shift in rumen fermentation toward an increased production of short-chain milk fatty acid precursors, such as butyrate (Ellis et al., 2008; Dijkstra et al., 2011). Although not measured in the present study, others observed an increase in ruminal proportions of butyrate upon 3-NOP supplementation (Lopes et al., 2016; Haisan et al., 2017; Melgar et al., 2020a), which may also be an explanation for the results of the present study. Other studies have also reported a shift in the ruminal fermentation profile with 3-NOP resulting in increased proportions of ruminal propionate at the expense of acetate (Haisan et al., 2014, 2017), which may further explain the milk protein response observed in the present study upon 3-NOP supplementation. Propionate is classified as glucogenic. The transfer efficiency of feed N into milk N may increase when glucogenic energy supply to the animal is increased. This is likely because of reduced catabolism of AA for gluconeogenesis, leaving more available for milk protein synthesis (Rulquin et al., 2004; Rius et al., 2010a,b). We also observed an increased milk urea content upon 3-NOP supplementation, which is in agreement with Melgar et al. (2020a,b, 2021) and van Gastelen et al. (2022), but contrary to Lopes et al. (2016), Haisan et al. (2017), and van Gastelen et al. (2020). The increased milk urea observed in the present study (although numerically it must be considered very small with only 1% increase) might be related to an increase in ruminal proportions of butyrate (not measured in the present study, but observed by Lopes et al., 2016, Haisan et al., 2017, and Melgar et al., 2020a). Butyrate is known to stimulate blood flow and, consequently, NH_3 absorption in the rumen (Engelhardt et al., 1978; Rémond et al., 1993). The latter is supported by the observed decrease in ruminal NH_3 concentrations upon 3-NOP supplementation (Lopes et al., 2016; Melgar et al., 2020a). Consequently, the increased absorption of ruminal NH_3 would likely result in increased blood urea or altered dynamics of blood urea, leading to increased milk urea content. An increased feed efficiency was also observed by Melgar et al. (2020a), but for different reasons. In that study, feed intake was decreased by 5% upon 3-NOP supplementation, whereas the cows retained a similar milk production level.

CONCLUSIONS

Supplementation of 3-NOP to the diet of dairy cows for 1 yr, covering all lactation stages and the DP, resulted in a reduction of 21%, 20%, and 27% for CH_4 production, yield, and intensity, respectively. The CH_4 mitigation potential of 3-NOP was influenced by diet type, diet composition, nutrition value, and length of 3-NOP supplementation within a specific diet type (EL diet specifically). Although the efficacy of 3-NOP appeared to decline over time, there was an increased efficacy at the end of the trial. Furthermore, 3-NOP supplementation increased yields of milk fat, milk protein, ECM, and FPCM, and improved feed efficiency.

NOTES

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Nonstandard abbreviations used: 3-NOP = 3-nitrooxypropanol; AC = adaptation; CTL = control; DietWk = week within a diet; DP = dry period; EL = early lactation; ExpWk = experimental week; FB = feed bin; FPCM = fat- and protein-corrected milk; GE = gross energy; LL = late lactation; ML = mid lactation; PMR = partial mixed ration; Trt = treatment.

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