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Effects of dietary fat, nitrate, and 3-nitrooxypropanol and their combinations on methane emission, feed intake, and milk production in dairy cows

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ABSTRACT

The objective of the present study was to investigate the effect of individual and combined use of dietary fat, nitrate, and 3-nitrooxypropanol (3-NOP) on dairy cows' enteric methane (CH_4) emission and production performance. Twenty-four primiparous and 24 multiparous Danish Holstein cows (111 ± 44.6 d in milk; mean ± standard deviation) were included in an incomplete 8 × 8 Latin square design with six 21-d periods. Dietary treatments were organized in a 2 × 2 × 2 factorial arrangement aiming for 2 levels of FAT (30 or 63 g of crude fat/kg of dry matter [DM]; LF or HF, respectively), 2 levels of NITRATE (0 or 10 g of nitrate/kg of DM; UREA or NIT, respectively), and 2 levels of 3-NOP (0 or 80 mg/kg DM; BLANK or NOP, respectively). Treatments were included in ad libitum-fed partial mixed rations in bins that automatically measured feed intake and eating behavior. Additional concentrate was offered as bait in GreenFeed units used for measurement of gas emission. For total DM intake (DMI), a FAT × NITRATE interaction showed that DMI, across parities and levels of 3-NOP, was unaffected by separate fat supplementation, but reduced by nitrate with 4.6% and synergistically decreased (significant 2-way interaction) with 13.0% when fat and nitrate were combined. Additionally, 3-NOP decreased DMI by 13.4% and the combination of 3-NOP with fat and nitrate decreased DMI in an additive way (no significant 3-way interaction). The decreasing effects on DMI were more pronounced in multiparous cows than in primiparous cows. For treatments with largest reductions in DMI, eating behavior was altered toward more frequent, but smaller meals, a slower eating rate and increased attempts to visit unassigned feed bins. Energy-corrected milk (ECM) yield increased by 6.3% with fat supplementation, whereas ECM yield did not differ among diets including nitrate (FAT × NITRATE interaction). Cows supplemented with 3-NOP had 9.0% lower ECM yield than cows fed no 3-NOP. Based on three 2-way interactions including FAT, NITRATE, and 3-NOP, the combined use of the additives resulted in antagonistic effects on CH_4 reduction. A 6% to 7% reduction in CH_4 yield (CH_4/kg of DMI) could be ascribed to the effect of fat, a 12% to 13% reduction could be ascribed to the effect of nitrate and an 18% to 23% reduction could be ascribed to the effect of 3-NOP. Hence, no combinations of additives resulted in CH_4 yield-reductions that were greater than what was obtained by separate supplementation of the most potent additive within the combination. The CH_4 yield reduction potential of additives was similar between parities. Increased apparent total-tract digestibility of organic matter (OM) in cows fed combinations including nitrate or 3-NOP was a result of a NITRATE × 3-NOP interaction. Apparent total-tract digestibility of OM was also increased by fat supplementation. These increases reflected observed decreases in DMI. In conclusion, combined use of fat, nitrate, and 3-NOP in all combinations did not result in CH_4 reductions that were greater than separate supplementation of the most potent additive within the combination (3-NOP > nitrate > fat). Additionally, separate supplementation of some additives and combined use of all additives reduced DMI.

Key words: feed additive, eating behavior, combined effect, 3-NOP, methanogen inhibitor

INTRODUCTION

Globally, mitigation of enteric CH_4 is of high priority because it has high effect on global warming and a short life span compared with other greenhouse gases from the dairy cattle sector. Strategies to reduce enteric emission include, among others, changes in diet formulation and the use of feed additives such as fat, nitrate, and 3-nitrooxypropanol (**3-NOP**).

Dietary fats have a moderate potential for CH_4 reduction (Beauchemin et al., 2020), and the potential of CH_4 reduction has been evaluated in different reviews

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and meta-analyses. Across these, fats have a potential of achieving a 10% to 15% decline in CH_4 emissions under practical conditions, depending on fat source and inclusion level (Grainger and Beauchemin, 2011; Patra, 2013; Knapp et al., 2014; Arndt et al., 2022). Nitrate, a known sink for H_2, has been evaluated as CH_4 mitigation additive in a review by van Gastelen et al. (2019) and a meta-analysis by Feng et al. (2020). These authors claimed a CH_4 yield (g CH_4/kg DMI) reduction potential of nitrate of 22% and 16% in dairy cows, respectively. The inhibitor 3-NOP has in several studies been supplemented to dairy cows resulting in reductions in CH_4 emissions (Lopes et al., 2016; van Gastelen et al., 2020; Melgar et al., 2021) as well. A meta-analysis by Kebreab et al. (2023) found a CH_4 yield reduction potential in dairy cows of 31%, and any variation in the efficacy of 3-NOP across studies to some extent could be explained by dose of 3-NOP and diet composition (Dijkstra et al., 2018; Melgar et al., 2020c; van Gastelen et al., 2022). For dietary fat inclusion, the meta-analysis by Patra (2013), showed that increasing concentration of fat in diets for cattle decreased feed intake, but also increased milk yield (Patra, 2013). For nitrate and 3-NOP, a review from van Gastelen et al. (2019), showed that feed intake and production performance were generally not affected by dietary inclusion of these additives, however more recent available literature have in some cases indicated reductions in DMI and milk production from cows supplemented with 3-NOP (Kjeldsen et al., 2022; van Gastelen et al., 2022).

Fats, nitrate, and 3-NOP have different and unique modes of action for CH_4 reduction. Fats replace fermentable OM in cows' diets with nonfermentable OM, resulting in less available substrate (H_2) for methanogenesis. This is quantitatively the most important effect of fat for CH_4 reduction. Furthermore, inclusion of fatty acids, depending on chain length and degree of saturation, inhibit the populations of protozoa and methanogens in the rumen (Giger-Reverdin et al., 2003) and in addition, biohydrogenation of unsaturated fatty acids in the rumen redirects H_2 away from methanogenesis. However, Jenkins et al. (2008) suggested that the amount of H_2 used for biohydrogenation is relatively small. Nitrate is a much more effective H_2-sink, redirecting H_2 into reduction of nitrate (NO_3^-) to nitrite (NO_2^-) and further to ammonium (NH_4^+). Both reducing reactions are naturally occurring in the rumen being energetically favorable for microorganisms (Ungerfeld and Kohn, 2006). Another suggested mode of action of nitrate is the direct toxic effect of the intermediate nitrite (NO_2^-) on the methanogens (Liu et al., 2017). This secondary effect has been supported in studies observing elevated net H_2 emissions from nitrate-supplemented cows (Olijhoek et al., 2016). The chemical inhibitor 3-NOP has a very specific mode of action, due to its blocking of methyl coenzyme M-reductase, a key enzyme in the methanogenesis (Duin et al., 2016).

Despite that dietary fats, alternative H_2-sinks such as nitrate, and chemical inhibitors such as 3-NOP have separately been widely investigated and are suggested as potential dietary interventions for CH_4 reduction by several reviews (Beauchemin et al., 2020; Fouts et al., 2022), their combined effects have only been investigated to a limited extent. Due to their diverse modes of action, it appears possible that combinations of these potent additives can achieve even greater CH_4 reductions, than what can be achieved by separate supplementation. The suggested mode of action for such combination of additives assumes that fat is being energy source for the animal while reducing fermentable matter in the rumen, whereas nitrate, in the rumen, can limit the H_2 availability for the methanogens, while 3-NOP directly inhibits the methanogenesis. Few in vivo studies have combined additives and the results of combining fat and nitrate indicated a synergistic effect on CH_4 reduction in a study by Villar et al. (2020), where the effect of the combined treatment was greater than the individual effects. However, an additive effect of nitrate and fat and 3-NOP and fat was found by Guyader et al. (2015) and Zhang et al. (2021), respectively, meaning that the combined effect equaled the sum of the 2 individual effects. To date, no studies have investigated the CH_4 reduction potential and the response in dairy cows' performance, when combinations of fat, nitrate, and 3-NOP are fed to dairy cows, which therefore was the aim of the present study. Due to the well-documented effect on CH_4 reduction when supplemented separately and considering the additives' unique modes of action, we hypothesized that the separate CH_4 mitigating effects of fat, nitrate, and 3-NOP would be additive when combined, and that supplementing combinations of additives would not potentially compromise dairy cows' feed intake and milk production.

MATERIALS AND METHODS

The experiment was conducted from September 2020 to January 2021 and complied with the guidelines set out by the Danish Ministry of Food, Agriculture and Fisheries, The Danish Veterinary and Food Administration under act no. 474 (May 15, 2014) and executive order no. 2028 (December 14, 2020), and under consideration of the ARRIVE guidelines (Percie du Sert et al., 2020).

Animals, Experimental Design, and Housing

Forty-eight Danish Holstein cows were enrolled in an incomplete 8 × 8 Latin square (**LS**) design with 6 periods of 21 d each (126 d in total) at the experimental facilities of Aarhus University, AU Viborg–Research Centre Foulum, Denmark. In each period, the first 14 d were used for adaptation and the last 7 d were used as sampling period. Cows were assigned to 6 blocks of 8 cows each, according to parity (24 primiparous, 24 multiparous) and DIM. Primiparous cows were 114 ± 45.7 (mean ± SD) DIM, BW of 595 ± 45.8 kg, and milk production of 27.3 ± 5.53 kg and multiparous cows (13 second parity, 9 third parity, and 2 fourth parity) were 109 ± 44.3 DIM, BW of 675 ± 61.9 kg, and milk production of 39.3 ± 5.76 kg at the beginning of the experiment. Latin squares were first designed as balanced 8 × 8 LS, whereafter the last 2 periods were discarded, thus cows only received 6 of 8 dietary treatments during the experiment. Six different LS were subsequently used to balance out first order carry-over effects between treatments, so that possible carry-over effects were as balanced between treatments and changes in periods as possible. Blocks of cows were then randomly assigned to a given LS and within LS, cows were at the start of the experiment randomly assigned to receive one of 8 dietary treatments.

Cows were grouped into 2 pens according to parity (primiparous or multiparous) with 24 cows in each pen. Cows were loose-housed in pens with concrete floor and cubicles with mattresses and sawdust as bedding material. Throughout the trial, each cow had access to its own individual feeding trough to receive the assigned experimental diet. All cows in each pen had access to 4 water troughs. The water troughs were refilled with approximately 36 L between visits. Individual feed intake, water intake and feeding/drinking behavior was registered automatically with the Insentec RIC system (Insentec, Marknesse, the Netherlands). Cows were milked twice daily at 0530 and 1630 h in a 2 × 12 parallel milking parlor (SAC A/S, S. A. Christensen and Co.). Milk yield was recorded automatically at every milking as was BW of individual cows when they exited the milking parlor. Lights were on between 0500 h and 2300 h and reduced during nighttime.

Diets and Feeding

Dietary treatments were organized in a 2 × 2 × 2 factorial arrangement aiming for 2 levels of fat (**FAT**: 30 or 63 g of crude fat/kg of DM; **LF** or **HF**, respectively), 2 levels of dietary nitrate (Silvair; Cargill Inc., the Netherlands; $5Ca[NO_3]_2{\cdot}NH_4NO_3{\cdot}10H_2O$ **NITRATE**: 0 or 10 g of nitrate/kg of DM; **UREA** or **NIT**, respectively), and 2 levels of 3-NOP (Bovaer; DSM Nutritional Products, Switzerland; **3-NOP**: 0 or 80 mg/kg DM; **BLANK** or **NOP**, respectively). The fat source was whole cracked rapeseeds, cracked on a Skiold roller (Sæby, Denmark) with roller distance <0.15 mm. Rapeseed was included in the HF diet by balancing the amount of kilograms of nonfat originating from rapeseed products (rapeseed meal and cracked rapeseed) between the 2 levels. Because the nitrate source contained nitrogen, the diets were made iso-nitrogenous with inclusion of urea in the diets without nitrate (UREA). Cows were adapted to nitrate by inclusion of 50% of the intended dose on the first day of exposure to NIT diets. The 3-NOP was included in a carrier substance (silicon dioxide + 1,2 propanediol) including 10.5% of the active compound of 3-NOP. In the diets without 3-NOP, 100% carrier compound was added as a placebo. Premixes with minerals, vitamins and sodium sulfate were composited including either 3-NOP or merely carrier substance before added to the individual partial mixed ration (**PMR**). Forage constituted 53% of the total PMR DM and forage consisted of 48% grass-clover silage and 52% corn silage on a DM basis. Concentrates constituted 47% of total PMR DM and was composed of NaOH treated wheat, rapeseed meal, dried beet pulp, soybean meal, and whole cracked rapeseed (for HF only). Diets were formulated by using the Nordic Feed Evaluation System (NorFor; Volden, 2011). Chemical composition of individual feed ingredients is presented in Table 1. Partial mixed rations were fed daily at 1030 and 2030 h for ad libitum intake aiming for 7% residues but minimum 3 kg (refilled at 0515 h if needed). Residues were removed daily before feeding at 1030 h. Additional concentrate (commercially produced at DLG A.m.b.a., Denmark; see composition in Table 1) was offered as feed bait in the GreenFeed system (maximum allowance of 1.3 kg of DM per day). Planned ingredient composition and analyzed chemical composition of the 8 diets are presented in Table 2.

Samplings

Feed ingredients were sampled on d 20 of each experimental period, whereas each PMR was sampled on d 20 and 21 of each experimental period. A subsample of each sample was used for DM determination. The remaining samples of each PMR were pooled across days, within period. Subsamples of forage samples and PMR representing each period were pooled for period 1 and 2, 3 and 4, and 5 and 6, respectively. Samples of concentrates (soybean meal, rapeseed cake, whole rapeseeds, wheat, and dried beet pulp) were pooled by ingredient across all periods, whereas GreenFeed bait was pooled to represent each of 3 deliveries of the

Table 1. Chemical composition (mean ± SD; g/kg DM unless otherwise stated) of feed ingredients

Item	Grass-clover silage (primary growth)[1]	Grass-clover silage (secondary growth)[1]	Corn silage[1]	Soybean meal, dehulled	Rapeseed meal	Beet pulp	Whole rapeseed, cracked	Wheat, NaOH treated	GreenFeed bait[2]
n	3	3	3	1	1	1	1	1	3
DM, g/kg	405 ± 11.4	348 ± 28.0	327 ± 2.2	913 ± 1.3[3]	896 ± 0.6[3]	914 ± 0.2[3]	936 ± 0.3[3]	830 ± 0.7[3]	903 ± 1.77
Ash	74.0 ± 1.68	83.8 ± 2.37	27.6 ± 0.54	70.1	79.2	93.8	40.4	57.0	67.8 ± 4.90
CP	137 ± 4.25	127 ± 6.33	84 ± 1.90	508	387	80	178	111	211 ± 1.91
Crude fat	29.0 ± 3.61	28.3 ± 1.53	26.7 ± 1.53	25.0	43.0	14.0	484	21.0	44.3 ± 6.81
Starch	NA[4]	NA	356 ± 4.95	NA	NA	NA	NA	634	126 ± 2.86
NDF	321 ± 9.49	473 ± 10.1	336 ± 18.0	NA	NA	NA	NA	NA	275 ± 11.5
iNDF[5]	32.5 ± 1.85	74.9 ± 3.36	61.1 ± 2.43	NA	NA	NA	NA	NA	54.8 ± 1.64
OMD,[6] %	82.4 ± 0.48	70.8 ± 0.09	76.6 ± 0.57	91.6	78.4	86.3	82.8	90.8	83.8 ± 1.04
NO_3	0.04 ± 0.006	0.05 ± 0.017	0.23 ± 0.060	NA	NA	NA	NA	NA	NA

[1]Fermentation characteristics for primary growth grass-clover silage, secondary growth grass-clover silage, and corn silage, respectively: pH = 4.31 ± 0.07, 4.04 ± 0.07, and 3.66 ± 0.05; L-lactate (g/kg DM) = 28.5 ± 0.87, 34.0 ± 4.55, and 38.4 ± 1.45 (L-lactate constitute about half of total lactate; Johansen et al., 2020); acetic acid (g/kg DM) = 11.4 ± 0.66, 19.6 ± 3.29, and 17.7 ± 0.53; propionic acid (g/kg DM) = 0.24 ± 0.031, 0.46 ± 0.321, and 0.29 ± 0.032; butyric acid (g/kg DM) = 0.12, 0.09, and 0.15; and NH_4-N of total N (%) = 5.4 ± 0.28, 4.9 ± 0.26, and 6.8 ± 0.49.

[2]Ingredient composition (g/kg DM): dried sugar beet pulp = 297, dried distillers grain = 250, wheat bran = 125, rapeseed cake = 65, wheat = 50, malt sprouts = 50, sunflower meal, decorticated = 46, rye = 41, barley = 35, limestone = 14, cane molasses = 7, palm fat = 5, vitamin/micromineral premix = 3, NaCl = 2.

[3]DM concentration is based on n = 6.

[4]NA: not analyzed.

[5]iNDF: indigestible NDF.

[6]OMD: in vivo OM digestibility in % calculated for grass-clover silage as 4.10 + 0.959 × in vitro OM digestibility, for corn silage as 6.73 + 0.950 × in vitro OM digestibility, and for concentrates as 5.38 + 0.867 × enzymatic in vitro OM digestibility (Åkerlind et al., 2011).

Table 2. Ingredient composition (g/kg DM) and analyzed chemical composition (mean ± SD; g/kg DM unless otherwise stated) of the 8 partial mixed ration (PMR) diets[1]

	LF				HF			
	UREA		NIT		UREA		NIT	
Item	BLANK	NOP	BLANK	NOP	BLANK	NOP	BLANK	NOP
Dietary composition, g/kg DM								
Corn silage	283	283	283	283	271	271	271	271
Grass-clover silage (primary)	135	135	135	135	130	130	130	130
Grass-clover silage (secondary)	121	121	121	121	116	116	116	116
Wheat, NaOH treated	186	186	186	186	178	178	178	178
Soybean meal, dehulled	61	61	61	61	58	58	58	58
Dried beet pulp, rolled	81	81	81	81	78	78	78	78
Rapeseed meal	117	117	117	117	71	71	71	71
Whole rapeseed, cracked	—	—	—	—	82	82	82	82
SilvAir (NO_3 source)[2]	—	—	13.9	13.9	—	—	13.9	13.9
Urea[3]	7.0	7.0	—	—	7.0	7.0	—	—
Calcium carbonate	6.7	6.7	—	—	6.7	6.7	—	—
Bovaer (with 3-NOP)	—	0.80	—	0.80	—	0.80	—	0.80
Bovaer placebo (without 3-NOP)	0.80	—	0.80	—	0.80	—	0.80	—
Mineral mix[4]	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4
Vitamins[5]	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87
Sodium sulfate	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87
Chemical composition, g/kg DM								
DM, g/kg	405 ± 4.3	407 ± 3.6	409 ± 3.9	409 ± 4.7	420 ± 2.3	415 ± 3.7	422 ± 5.6	419 ± 3.1
Ash	70.8 ± 2.43	72.4 ± 1.51	72.1 ± 0.82	72.2 ± 0.98	70.2 ± 0.58	68.3 ± 1.53	69.7 ± 1.76	69.0 ± 1.33
CP	182 ± 4.4	180 ± 6.9	180 ± 2.2	178 ± 2.3	173 ± 3.5	170 ± 1.1	173 ± 1.5	174 ± 1.4
Crude fat	27.7 ± 0.58	26.3 ± 0.58	27.7 ± 1.53	30.0 ± 3.46	66.0 ± 2.65	60.3 ± 5.69	65.7 ± 1.53	67.0 ± 2.65
Starch	215 ± 18.0	211 ± 4.2	208 ± 3.3	207 ± 19.0	195 ± 7.9	207 ± 5.8	197 ± 12.4	197 ± 7.0
NDF	292 ± 11.8	287 ± 8.7	291 ± 18.2	287 ± 10.2	285 ± 16.1	281 ± 6.1	289 ± 14.8	280 ± 5.1
iNDF[6]	52.4 ± 3.72	49.7 ± 0.98	54.4 ± 1.21	52.2 ± 2.18	52.1 ± 2.93	48.4 ± 2.03	51.1 ± 1.53	49.8 ± 1.08
Calculated energy and protein value								
NEL_{20}[7] MJ/kg of DM	6.52	6.52	6.55	6.55	6.96	6.96	6.99	6.99
AAT_{20}[8]	97	97	98	98	93	93	94	94
PBV_{20}[9]	31	31	31	31	30	30	29	29

[1]The PMR diets were 2 × 2 × 2 factorially arranged aiming for 2 levels of fat (FAT: 30 or 63 g of crude fat/kg DM; LF or HF, respectively), 2 levels of dietary nitrate (NITRATE: 0 or 10 g of nitrate/kg DM; UREA or NIT, respectively), and 2 levels of 3-nitrooxypropanol (3-NOP: 0 or 80 mg/kg DM; BLANK or NOP, respectively). GreenFeed bait is not included in the analyzed chemical composition, but included in the calculated energy and protein value with 1.1 kg of DM per day.

[2]Calcium ammonium nitrate; $5Ca(NO_3)_2{\cdot}NH_4NO_3{\cdot}10H_2O$; 75% NO_3 in DM (Cargill Inc., the Netherlands).

[3]Vilofoss Urea, ingredient composition (g/kg DM): urea = 800, sodium sulfate = 135, calcium carbonate = 65.

[4]Vilofoss Komix Type 3, analyzed or declared macro mineral composition (g/kg DM): Ca = 147, Mg = 141, Na = 116, S = 1. Added vitamins and microminerals (per kg of DM): vitamin A = 600,000.10 IU, vitamin D_3 = 190,000.10 IU, vitamin E = 4,000 IU, Mn = 4,000 mg, Cu = 1,500 mg, Zn = 4,500 mg, I = 225, Co = 25 mg, Se = 50 mg.

[5]Vilofoss Suplex ADE, analyzed or declared macromineral composition (g/kg DM): Ca = 139, Mg = 91, Na = 95. Added vitamins and microminerals (per kg of DM): vitamin A = 900,000 IU, vitamin D_3 = 200,000 IU, vitamin E = 2,000 IU, Se = 50 mg.

[6]iNDF: indigestible NDF.

[7]Net energy for lactation at 20 kg of DMI per day, calculated according to NorFor (Åkerlind and Volden, 2011).

[8]Amino acids absorbed in the small intestine at 20 kg of DMI per day, calculated according to NorFor (Åkerlind and Volden, 2011).

[9]Protein balance in the rumen at 20 kg of DMI per day, calculated according to NorFor (Åkerlind and Volden, 2011).

GreenFeed concentrate. All samples were stored at −20°C until pooling and later chemical analysis. Milk samples were taken over the last 3 d (6 consecutive milkings) in the last week of each experimental period. These were stored at 5°C with bronopol until analysis of milk composition. Samples of feces (500 mL) of each cow were collected on d 20 (1300 h) and 21 (0700 h) in the 3 last periods (4–6). Individual fecal samples from d 20 were stored at 5°C until they were pooled with the sample from d 21. Feces consistency of the pooled samples (n = 144) was evaluated by visual scoring on a 5-point scale (1–5; 0.5 point intervals, 1 is loose and 5 is firm). On d 20 in the 4 last periods (3–6), a blood sample was taken from the tail vein, 3 h after returning from morning milking. The samples were taken in sodium heparin Vacutainers and stored on ice until analysis on whole blood.

Analytical Procedures

Subsamples of PMR representing each period were analyzed at an external laboratory for concentration of 3-NOP (DSM Nutritional Products, Kaiseraugst, Switzerland). Dry matter concentration of feed ingredients and PMR samples were determined by drying at 60°C for 48 h (Åkerlind et al., 2011). The DM concentration of fecal samples was determined by drying for 72 h at 60°C. For chemical analyses, samples were freeze-dried and subsequently ground (Ultra Centrifugal Mill ZM 200, Verder Scientific, Hann, Germany) by using a 1.0 mm screen. Subsamples for starch determination were grinded by using a 0.5 mm screen. Ash concentration was determined in duplicates by combustion at 525°C for 6 h. In feed samples, total nitrogen (N) concentration was determined according to the Dumas method (Hansen, 1989) by using a Vario MAX CN apparatus (Elementar Analysensysteme GmbH, Hanau, Germany). Concentration of CP was calculated as N × 6.25. Crude fat (**CF**) concentration was analyzed in feed samples after hydrolysis with hydrochloric acid (HCl) by using a Hydrotherm HT6 apparatus (C. Gerhardt GmbH and Co. KG) followed by Soxhlet extraction with petroleum ether using a Soxtherm SOX 416 apparatus (C. Gerhardt GmbH and Co. KG; Eurofins Steins Laboratories, Vejen, Denmark). Starch was enzymatically determined in feed samples by using a heat-stable α-amylase and amyloglucosidase, whereafter starch concentration was measured as liberated glucose (YSI 2900D Biochemistry Analyzer, YSI Inc.; Kristensen et al., 2007). In feed ingredients, PMR and fecal samples, NDF concentration was determined according to Mertens (2002) by using heat-stable amylase and sodium sulfite following the ANKOM procedure (A2000; ANKOM, 2022). Results were reported as ash-free NDF and iNDF. Indigestible NDF was determined as residual NDF, where samples were incubated in duplicates in F57 ANKOM bags in the rumen of 2 out of 3 used rumen-cannulated nonlactating cows for 288 h (12 d; Åkerlind et al., 2011), before NDF analysis. Samples from the same period were incubated in the same 2 cows. The nonlactating cows were fed a standard ration at maintenance level as described by Brask et al. (2013a). After rumen incubation, the bags were rinsed with cold-tap water followed by rinsing in a domestic washing machine for 2 × 5 min at 25°C.

In vitro digestibility of OM in silages was determined according to Tilley and Terry (1963) by anaerobic incubation in rumen fluid for 48 h. The rumen fluid was collected from 3 nonlactating rumen-cannulated cows (fed as described for iNDF analysis) and filtered through 2 layers of cheesecloth before incubation. After rumen fluid incubation, the sample was incubated in a solution of HCl and pepsin for additional 48 h. Enzymatic OM digestibility of concentrates was determined by dissolving the sample in HCl and pepsin following incubation with cellulolytic enzymes (Álvarez et al., 2020). In vivo OM digestibility was then calculated for grass-clover silage as 4.10 + 0.959 × in vitro OM digestibility, for corn silage as 6.73 + 0.950 × in vitro OM digestibility, and for concentrates as 5.38 + 0.867 × enzymatic in vitro OM digestibility (Åkerlind et al., 2011).

To analyze fermentation products and nitrate concentration in silages, water extracts were prepared by blending of 100 g of fresh silage with 1,000 mL of deionized water. The blends were subsequently centrifuged and pH, VFA, L-lactate, and ammonium (NH_4) concentrations were measured in the metaphosphoric acid stabilized supernatant. Concentrations of VFA were measured by using gas chromatography (Trace 1310, Thermo Scientific). The gas chromatograph used split/splitless injector at 225°C and a flame ionization detector at 250°C with a 30 m × 0.53 mm × 1 μm HP-FFAP column using helium as carrier gas (0.3405 atm). Glucose and L-lactate was analyzed using membrane-immobilized substrate-specific oxidases (YSI 2900D Biochemistry Analyzer; YSI Inc.), whereas NH_4 was determined in phosphate buffered samples by using a Cobas Mira Plus (Roche Diagnostics Systems) with a Randox Ammonia Kit-AM1015. Nitrate was determined by using spectrophotometry as outlined by Best (1976).

Total hemoglobin (**tHb**), methemoglobin (**MetHb**), and hematocrit (**Hct**) in whole blood samples were analyzed immediately after sampling by oximetry by using an acid base laboratory analyzer (Radiometer ABL90 Flex). Concentration of milk protein, fat, and lactose monohydrate were analyzed at an external laboratory (Eurofins Steins Laboratorium, Vejen, Denmark) using

a Milkoscan 7 RM infrared analyzer (Foss, Hillerød, Denmark).

Gas Measurements

Measurements of CH_4, CO_2, and H_2 were obtained from individual animals by using the GreenFeed system (C-Lock Inc., Rapid City, SD). Four GreenFeed units were used in the experiment; 2 units in each pen with 24 cows. Thus, each cow had access to 2 GreenFeed units. Concentrations of CH_4, CO_2, and H_2 in exhaust air were measured by nondispersive near-infrared sensors (**NDIR**) and the individually measured concentrations were corrected for gas concentrations in ambient air and temperature and pressure according to the ideal gas law. Gas concentrations were subsequently converted to a daily flux of emission (g/d) by multiplying with the measured airflow rate. The NDIR sensors were calibrated daily by using a zero-gas mixture serving as baseline (N_2 with 20% O_2) and a span-gas mixture (N_2 with 10 ppm H_2, 500 ppm CH_4, 5,000 ppm CO_2, and 21% O_2) provided by C-Lock Inc. Accuracy of measurements were monitored before and after the experiment and before each sampling period by releasing a gravimetrically determined amount of CO_2 into the system. Average CO_2 recovery for all units was 99.8% ± 1.83% (mean ± SD). If recovery tests were repeatedly biased, the airflow coefficient used for calculations of flux was adjusted by C-Lock Inc. Procedure for calibrations and CO_2 recoveries are presented in a slightly modified form by Hristov et al. (2015a). Cows were allowed a maximum of 5 daily visits with an interval between visits of minimum 4 h and a maximum of 6 feed drops per visit (of 44.4 ± 3.30 g [mean ± SD] of each drop; see Table 1 for nutrient composition), fed with an interval of 40 s between drops. Certain criteria regarding head proximity and visit duration were set by C-Lock Inc., which filtered out unreliable gas measurements. This resulted in 30 ± 7.2 (mean ± SD) approved visits per animal per sampling week (d 15 to 21 of each period), subsequently to be included in the statistical analysis. Individual gas emission observations were averaged over the last 7 d of each period.

Calculations

In PMR samples, NEL_{20}, AA absorbed in the small intestine (AAT_{20}), and protein balance in the rumen (PBV_{20}) at 20 kg DMI/d, were calculated according to NorFor (Åkerlind and Volden, 2011).

Feed intake of PMR and GreenFeed concentrate was averaged over the last 7 d in each period (sampling week). Intake of DM was calculated based on feed intake in the sampling week and an averaged DM concentration measured over 2 of the 7 last days in each period.

Intake behavior (feed and water) was averaged over the last 7 d in each period. For eating and drinking behavior data, a new meal, or a new drinking bout was initiated if the time of termination of one visit to initiation of another visit exceeded 30 min for feed and 4 min for water (Howie et al., 2009). Total eating and drinking duration corresponded to the time the gate to the respective bin was open, whereas meal duration corresponded to the time from initiation to termination of a meal given the criteria above. Efficient eating time was calculated as the share of total eating duration out of total meal duration.

Individual BW was averaged per day and BW change over the experimental periods was calculated by linear regression by using day in period as fixed effect.

Milk yield data were averaged over the last 7 d in each period and milk composition data were obtained from yield-weighted averages of fat, protein and lactose monohydrate concentration in milk samples taken over the last 3 d in each period. Energy-corrected milk yield (3.14 MJ/kg) was calculated by using the formula ECM = milk yield (kg) × [(38.3 × fat (g/kg) + 24.2 × protein (g/kg) + 15.71 × lactose (g/kg) + 20.7)/3,140] (Sjaunja et al., 1991).

For calculation of apparent total-tract digestibility of DM, OM, and NDF, concentration of iNDF in PMR was averaged for LF diets whereafter this value was subsequently corrected by a factor of 0.965 to represent the diluting effect of fat in HF diets. This procedure eliminated any possible analytical bias of iNDF determination. Concentrations of iNDF in diets, GreenFeed bait, and feces were used to calculate apparent total-tract digestibility.

Statistical Analysis

Feed intake, eating behavior, milk production, gas emission, BW change, and digestibility data were included in the statistical analysis conducted in R 4.0.2 (R Core Team, 2020). The effect of treatment on the response variables were analyzed by using the following model fitted with REML and the lmer function from the lme4 and lmeTest packages (Bates et al., 2015):

$$Y_{ijklpc} = \mu + A_i + B_j + C_k + D_l + AB_{ij} + AC_{ik} + AD_{il} + BC_{jk} + BD_{jl} + CD_{kl} + ABC_{ijk} + ABD_{ijl} + BCD_{jkl} + ACD_{ikl} + ABCD_{ijkl} + P_p + \delta_c + \varepsilon_{ijklpc},$$

where Y_{ijklpc} is the dependent response variable; μ is the overall mean; A is the fixed effect of FAT (i = LF, HF);

B is the fixed effect of NITRATE (j = UREA, NIT); C is the fixed effect of 3-NOP (k = BLANK, NOP); D is the fixed effect of parity (l = primiparous, multiparous); AB_{ij}, AC_{ik}, AD_{il}, BC_{jk}, BD_{jl} and CD_{kl} and ABC_{ijk}, ABD_{ijl}, BCD_{jkl}, and ACD_{ikl} and $ABCD_{ijkl}$ are the possible 2-, 3-, and 4-way interactions between FAT, NITRATE, 3-NOP, and parity, respectively; P is the fixed effect of period (p = 1 to 6); δ is the random effect of cow (c = 1 to 48); and ε_{ijklpc} is the random residual error assumed to be normally distributed and independent with constant variance. Ten observations were discarded from the statistical analysis due to animal related issues or technical problems with equipment. Hence, 278 observations were included in the statistical analysis. Estimated marginal means (**EMM**) and standard error of means were reported by using the emmeans package (Lenth, 2020) and *P*-values were computed by using a type 2 ANOVA. Statistical significances were declared at $P \leq 0.05$. Tendencies were declared when *P*-values $0.05 < P \leq 0.10$. When an interaction was significant, EMM comparisons were based on Tukey's multiple comparison test and pairwise comparisons. In the tables, EMM for the 4-way interactions were presented, whereas EMM for the main effects or interactions across factors were presented in the text. Normality of residuals and homogeneity of variance were tested by using qqplots and residual plots, respectively. In case of heterogeneous variance, data were log-transformed (natural log; ln) to obtain homogeneity of variance. For such data (eating behavior, feed bin visit attempts and drinking behavior), the EMM from the model without log-transformation were presented in the tables along with the *P*-values associated with the log-transformed data.

RESULTS

Diet Composition

Analyzed chemical compositions of experimental diets are reported in Table 2. Crude fat concentration ranged from 26 to 30 g/kg DM in LF diets and 60 to 67 g/kg DM in HF diets. Concentration of ash, CP, starch, NDF, and iNDF was slightly lower in HF diets compared with LF diets due to a dilution caused by fat addition in HF diets. The addition of fat increased calculated energy concentration (NEL_{20}) of HF diets compared with LF diets (Table 2). Average 3-NOP recovery in NOP diets was 95% ± 9.4% (mean ± SD) of target concentration (84 mg 3-NOP/kg of DM). This resulted in an actual dose of 76 ± 2.4 mg 3-NOP/kg of DM (mean ± SD) when corrected for intake of GreenFeed bait.

Feed Intake and Eating Behavior

Dry matter intake of PMR was affected by dietary treatments (Table 3) and total DMI of PMR varied across treatments from 15.9 to 20.2 kg/d for primiparous cows and from 16.2 to 24.3 kg/d for multiparous cows. A significant FAT × NITRATE interaction and a tendency for a FAT × 3-NOP interaction showed that PMR DMI was unaffected by fat addition alone, however when fat was combined with nitrate ($P_{FAT \times NITRATE} < 0.01$), or 3-NOP ($P_{FAT \times 3\text{-NOP}} = 0.08$) DMI of PMR was more reduced than when nitrate or 3-NOP were fed alone. Cows fed HF, NIT, or NOP had greater DMI from GreenFeed bait (all $P < 0.01$), than cows fed LF, UREA, or BLANK, respectively. Though the FAT × NITRATE interaction ($P < 0.01$) remained significant for total DMI and showed, across parities, that total DMI was similar in LF-UREA and HF-UREA diets (21.4 vs. 21.3 kg; $P = 0.96$), reduced by 4.6% in LF-NIT diets compared with LF-UREA diets (20.4 vs. 21.4 kg; $P = 0.01$), and even more reduced (−13.0%) in HF-NIT diets compared with LF-UREA diets (18.7 vs. 21.4 kg; $P < 0.01$). Total DMI was lower in NOP diets compared with BLANK diets (19.0 vs. 21.9 kg; −13.4%; $P < 0.01$). Additionally, FAT also interacted with parity ($P_{Parity \times FAT} = 0.02$), as multiparous cows on HF reduced DMI compared with multiparous cows on LF, whereas primiparous cows were unaffected. The effects of NITRATE and 3-NOP were present in both parities, but more pronounced in multiparous cows, compared with primiparous cows ($P_{Parity \times NITRATE} = 0.03$, $P_{Parity \times 3\text{-NOP}} < 0.01$). Effects on DMI were likewise reflected in total intake of OM, CP, NDF, ST, and CF. A FAT × NITRATE interaction ($P < 0.01$) was similarly observed for BW change during the experimental periods, where cows on LF-UREA, HF-UREA, and LF-NIT had similar BW change (479, 142, and 363 g/d; all $P > 0.10$, but cows on HF-NIT had significantly lower BW change than LF-UREA (−697 vs. 479 g/d; $P < 0.01$). Additionally, across parities, cows on NOP had lower BW change than cows on BLANK (−320 vs. 463 g/d; $P < 0.01$).

For total eating duration (min/d) a FAT × NITRATE interaction ($P = 0.03$) was observed and for total meal duration (min/d) a NITRATE × 3-NOP interaction ($P = 0.02$) was observed (Table 4). Both interactions showed that eating and meal duration was unaffected by NIT alone, but decreased even more when NIT was combined with HF or NOP, respectively, than when HF or NOP was fed separately. Relating total eating duration to meal duration (efficient eating time), a NITRATE × 3-NOP interaction ($P = 0.04$) showed that efficient eating time was greater for cows

Table 3. Intake and BW change of dairy cows fed the 8 partial mixed ration (PMR) diets[1]

	LF				HF												
	UREA		NIT		UREA		NIT			*P*-value							
Item	BLANK	NOP	BLANK	NOP	BLANK	NOP	BLANK	NOP	SEM[2]	Parity	FAT	NITRATE	3-NOP	FAT × NITRATE	FAT × 3-NOP	NITRATE × 3-NOP	FAT × NITRATE × 3-NOP
Observations, n																	
Primiparous	18	18	18	17	18	17	18	18									
Multiparous	17	18	18	18	18	16	14	17									
Intake, kg/d																	
PMR DM																	
Primiparous	19.6	17.4	18.9	17.3	20.2	17.8	17.4	15.9	0.61	<0.01	<0.01*	<0.01*	<0.01*	<0.01	0.08	0.26	0.96
Multiparous	24.3	20.8	22.4	19.2	24.1	19.1	21.0	16.2									
GreenFeed bait DM																	
Primiparous	0.99	0.99	1.03	1.04	0.98	1.06	1.08	1.07	0.042	<0.01	<0.01	<0.01	<0.01*	0.46	0.22	0.15	0.48
Multiparous	0.78	0.91	0.89	0.95	0.80	0.96	0.92	1.04									
DM																	
Primiparous	20.6	18.3	19.9	18.4	21.2	18.9	18.5	17.0	0.60	<0.01	<0.01*	<0.01*	<0.01*	<0.01	0.09	0.30	0.99
Multiparous	25.0	21.7	23.3	20.2	24.9	20.1	21.9	17.3									
OM																	
Primiparous	19.1	17.0	18.5	17.1	19.7	17.6	17.2	15.8	0.56	<0.01	<0.01*	<0.01*	<0.01*	<0.01	0.11	0.30	0.94
Multiparous	23.3	20.1	21.6	18.7	23.1	18.7	20.4	16.1									
CP																	
Primiparous	3.78	3.34	3.62	3.31	3.72	3.26	3.23	2.99	0.108	<0.01	<0.01*	<0.01*	<0.01*	<0.01	0.31	0.14	0.59
Multiparous	4.58	3.94	4.24	3.62	4.35	3.47	3.82	3.05									
NDF																	
Primiparous	6.00	5.24	5.78	5.25	6.02	5.30	5.30	4.76	0.176	<0.01	<0.01*	<0.01	<0.01*	<0.01	0.16	0.38	0.67
Multiparous	7.30	6.21	6.77	5.79	7.06	5.66	6.32	4.85									
Starch																	
Primiparous	4.33	3.79	4.04	3.75	4.06	3.83	3.59	3.27	0.131	<0.01	<0.01*	<0.01*	<0.01*	<0.01	0.78	0.77	0.06
Multiparous	5.31	4.50	4.75	4.11	4.79	4.10	4.26	3.32									
Crude fat																	
Primiparous	0.59	0.49	0.56	0.58	1.37	1.11	1.20	1.11	0.032	<0.01	<0.01*	<0.01	<0.01*	<0.01	<0.01*	0.01	0.17
Multiparous	0.70	0.58	0.66	0.61	1.62	1.19	1.42	1.14									
BW change, g/d																	
Primiparous	823	106	804	161	660	−36.4	−16.8	−376	244.9	<0.01	<0.01	<0.01	<0.01	<0.01	0.23	0.56	0.50
Multiparous	618	370	710	−222	565	−622	−457	−1,937									

[1]The PMR diets were 2 × 2 × 2 factorially arranged aiming for 2 levels of fat (FAT: 30 or 63 g of crude fat/kg DM; LF or HF, respectively), 2 levels of dietary nitrate (NITRATE: 0 or 10 g of nitrate/kg DM; UREA or NIT, respectively), and 2 levels of 3-nitrooxypropanol (3-NOP: 0 or 80 mg/kg DM; BLANK or NOP, respectively).

[2]Standard error of estimated marginal mean. The lowest value within the variable is reported as SEM.

*Indicates significant interaction ($P < 0.05$) to parity group (parity; primiparous, multiparous).

on UREA-NOP diets than for cows on UREA-BLANK diets (55.9% vs. 48.0%; $P < 0.01$), but not when NOP was combined with NIT or when NIT was fed alone. Eating rate (kg DM/min) was significantly greater in HF cows compared with LF cows (0.126 vs. 0.121 kg DM/min; $P_{FAT} < 0.01$). In contrast, eating rate was lower for cows on NOP compared with BLANK (0.122 vs. 0.126 kg DM/min; $P_{3\text{-NOP}} < 0.01$) and tended to be lower for cows on NIT compared with UREA (0.123 vs. 0.125 kg DM/min; $P_{NITRATE} = 0.06$).

Number of visits in the assigned feed bin (Table 4) was lower in cows on NOP compared with cows on BLANK (15.4 vs. 16.6; $P < 0.01$), whereas number of attempts to visit an unassigned feed bin as well as percent of visits in unassigned feed bins of total daily visit attempts increased additively by FAT, NITRATE, and 3-NOP (all $P < 0.05$). Daily number of meals was greater for cows on NOP compared with BLANK (8.43 vs. 8.10 meals/d; $P < 0.01$; Table 4), whereas meal size was lower for these cows (2.2 vs. 2.7 kg DM/meal; $P < 0.01$). A similar pattern for number and size of meals was observed, when NIT and HF were fed in combination ($P_{FAT \times NITRATE}$ <0.03 and <0.01, respectively), whereas we observed no significant separate effect of HF or NIT.

Milk Production

For milk and ECM yield (Table 5) a FAT × NITRATE interaction ($P \leq 0.01$) was observed. Cows on HF-UREA had 8.6% and 6.3% greater milk and ECM yield, respectively, than cows on LF-UREA (31.7 vs. 29.2 kg/d and 32.1 vs. 30.2 kg, respectively; $P < 0.01$), whereas for cows on LF-NIT or HF-NIT, we observed no difference in milk and ECM yield, compared with cows on a LF-UREA diet. Cows on NOP had 11.7% and 9.0% lower milk and ECM yield compared with BLANK (28.2 vs. 31.5 kg/d and 29.3 vs. 31.9 kg/d, respectively; $P < 0.01$), and the differences were more pronounced in multiparous (31.2 vs. 35.8 kg/d and 31.9 vs. 35.7 kg/d, respectively; $P < 0.01$) than in primiparous cows (25.3 vs. 27.3 kg/d and 29.3 vs. 31.9 kg/d, respectively; $P < 0.01$; $P_{Parity \times 3\text{-NOP}} < 0.01$).

Milk fat concentration was lower for HF cows compared with LF cows (40.2 vs. 41.5 g/kg; $P < 0.01$), greater in NIT than in UREA cows (41.2 vs. 40.4 g/kg; $P < 0.01$), and greater in NOP than in BLANK cows (42.0 vs. 39.7 g/kg; $P < 0.01$). However, milk protein concentration was unaffected by 3-NOP ($P = 0.69$), lower in HF cows than LF cows (36.5 vs. 38.5 g/kg; $P < 0.01$) and lower in NIT than UREA cows (37.1 vs. 37.9 g/kg; $P < 0.01$). For milk lactose concentration a FAT × NITRATE interaction ($P < 0.01$) showed that lactose concentration was greater in HF-UREA cows compared with LF-UREA (49.9 vs. 48.8 g/kg; $P < 0.01$), however, we observed no difference when fed LF-NIT or HF-NIT diets, compared with LF-UREA diets. Lactose concentration was lower in cows fed NOP compared with BLANK (48.9 vs. 49.3 g/kg; $P < 0.01$). A FAT × 3-NOP interaction ($P < 0.01$) for milk urea concentration, showed that urea concentration was lower in HF-BLANK than in LF-BLANK cows (3.64 vs. 5.05 mmol/L; $P < 0.01$). Although milk urea concentration was similar in LF-NOP cows compared with LF-UREA cows, urea concentration was lower for HF-NOP compared with LF-NOP cows (4.00 vs. 5.10 mmol/L; $P < 0.01$). Although milk fat concentration decreased by fat addition, the greater milk yield in HF cows than in LF cows resulted in greater fat yield (kg/d) in HF cows, but only when fat was not combined with nitrate ($P_{FAT \times NITRATE} < 0.01$). Similarly, the observed effects on milk yield affected total protein and lactose yield accordingly. Milk N efficiency increased additively by FAT, NITRATE, and 3-NOP with 2.8, 1.1, and 1.2 percentage points (all $P < 0.01$), respectively, and was generally greater in multiparous cows than in primiparous cows (31.1% vs. 28.7%; $P_{Parity} = 0.01$).

Gas Emissions

Averaged across parities, CH_4 production (g/d), yield (g/kg DMI), and intensity (g/kg ECM) varied from 217 to 380 g/d, 12.0 to 16.7 g/kg DMI, and 7.7 to 12.3 g/kg ECM, respectively, between treatments (Table 6). For CH_4 yield, two 3-way interactions (FAT × 3-NOP × parity, $P = 0.03$ and NITRATE × 3-NOP × parity, $P = 0.03$) were found and were a result of the more pronounced decreasing effect of combinations of fat and 3-NOP, and nitrate and 3-NOP on DMI in multiparous compared with primiparous cows. Additionally, three 2-way interactions (FAT × NITRATE, FAT × 3-NOP, and NITRATE × 3-NOP; all $P < 0.01$) were found. Figure 1 shows the CH_4 yield of the 3 interactions. The FAT × NITRATE interaction (Figure 1a) showed that cows on HF-UREA, LF-NIT, and HF-NIT had a 7.1%, 12.1%, and 8.2% lower CH_4 yield than cows on LF-UREA, respectively (13.5, 12.7, and 13.3 vs. 14.5 g/kg DMI; $P \leq 0.01$), whereas we observed no significant difference in the CH_4 mitigating effect between the 3 diets including HF or NIT. The FAT × 3-NOP interaction (Figure 1b) revealed that CH_4 yield was reduced by 6.0% for cows on HF-BLANK compared with cows on LF-BLANK (14.3 vs. 15.2 g/kg DMI; $P = 0.04$), and reduced by 21.0% and 18.0% for cows on LF-NOP and HF-NOP, respectively (12.0 and 12.5 vs. 15.2 g/kg DMI; $P < 0.01$), though we observed no difference between the 2 latter diets ($P = 0.57$). Similarly, the NITRATE × 3-NOP interaction (Figure 1c) resulted

Table 4. Eating behavior and visit attempts to feed bins of dairy cows fed the 8 partial mixed ration (PMR) diets[1]

	LF				HF					*P*-value							
	UREA		NIT		UREA		NIT										
Item	BLANK	NOP	BLANK	NOP	BLANK	NOP	BLANK	NOP	SEM[2]	Parity	FAT	NITRATE	3-NOP	FAT × NITRATE	FAT × 3-NOP	NITRATE × 3-NOP	FAT × NITRATE × 3-NOP
Observations, n																	
Primiparous	18	18	18	17	18	17	18	18									
Multiparous	17	18	18	18	18	16	14	17									
Eating behavior																	
Eating duration,[3] min/d																	
Primiparous	181	162	174	167	174	151	155	144	8.1	0.63	<0.01	<0.01	<0.01	0.03	0.47	0.22	0.51
Multiparous	190	166	180	163	180	159	167	140									
Meal duration,[4] min/d[5]																	
Primiparous	433	328	414	374	434	315	360	359	50.2	0.56	<0.01	0.55	<0.01	0.58	0.95	0.02	0.95
Multiparous	516	369	497	426	443	330	452	367									
Efficient eating time, %																	
Primiparous	47.7	56.7	49.8	54.9	46.7	56.2	51.0	51.4	0.35	0.76	0.88	0.31	<0.01	0.26	0.78	0.04	0.70
Multiparous	47.9	53.0	50.1	50.7	49.7	57.7	45.7	50.7									
Meals,[4] number/d																	
Primiparous	8.1	8.1	8.0	8.2	7.9	8.8	8.5	9.1	0.26	0.70	<0.01	0.22	<0.01	0.03	0.12	0.30	0.75
Multiparous	8.0	8.5	8.0	8.1	8.0	8.4	8.3	8.4									
Meal size, kg DM/meal																	
Primiparous	2.49	2.20	2.41	2.19	2.60	2.13	2.13	1.80	0.114	<0.01	<0.01	<0.01	<0.01*	<0.01	0.10	0.05	0.69
Multiparous	3.19	2.54	2.91	2.47	3.17	2.37	2.61	2.07									
Eating rate,[6] kg DM/min[5]																	
Primiparous	0.113	0.112	0.114	0.111	0.120	0.122	0.118	0.117	0.007	0.06	<0.01	0.06	<0.01	0.83	0.88	0.60	0.29
Multiparous	0.134	0.131	0.133	0.123	0.141	0.127	0.134	0.131									
Visit attempts, number/d																	
Assigned bin[5]																	
Primiparous	16.2	13.7	15.4	14.8	16.6	15.1	15.2	16.7	1.55	0.85	0.81	0.94	<0.01	0.33	0.36	0.06	0.98
Multiparous	18.2	15.9	17.2	16.3	16.7	14.6	17.1	16.0									
Unassigned bin[5]																	
Primiparous	2.7	4.0	3.5	4.6	4.0	5.2	5.7	7.6	1.12	0.64	<0.01	<0.01	<0.01	0.52	0.17	0.98	0.23
Multiparous	2.7	3.3	3.7	3.6	3.8	4.4	4.7	8.2									
Unassigned bin, % of total[5]																	
Primiparous	13.4	19.4	16.8	22.3	18.5	22.1	21.3	26.0	2.64	0.57	<0.01	0.02	<0.01	0.83	0.36	0.64	0.40
Multiparous	13.5	16.1	17.4	17.7	13.7	20.7	18.8	26.9									

[1]The PMR diets were 2 × 2 × 2 factorially arranged aiming for 2 levels of fat (FAT: 30 or 63 g of crude fat/kg DM; LF or HF, respectively), 2 levels of dietary nitrate (NITRATE: 0 or 10 g of nitrate/kg DM; UREA or NIT, respectively), and 2 levels of 3-nitrooxypropanol (3-NOP: 0 or 80 mg/kg DM; BLANK or NOP, respectively).

[2]Standard error of estimated marginal mean. The lowest value within the variable is reported as SEM.

[3]Duration of time where gate to Insentec bin was open and cow's head was placed inside the bin.

[4]Meal initiation was defined as visits started after 30 min from the last visit.

[5]Means and SEM are from model without log-transformation, but *P*-values are from log-transformed model.

[6]Eaten DM per minute of total eating time.

*Indicates significant interaction ($P < 0.05$) to parity group (Parity; primiparous, multiparous).

Table 5. Milk production parameters of dairy cows fed the 8 partial mixed ration (PMR) diets[1]

	LF				HF					*P*-value							
	UREA		NIT		UREA		NIT										
Item	BLANK	NOP	BLANK	NOP	BLANK	NOP	BLANK	NOP	SEM[2]	Parity	FAT	NITRATE	3-NOP	FAT × NITRATE	FAT × 3-NOP	NITRATE × 3-NOP	FAT × NITRATE × 3-NOP
Observations, n																	
Primiparous	18	18	17	17	18	17	18	17									
Multiparous	17	18	18	18	18	16	14	17									
Yield, kg/d																	
Milk																	
Primiparous	26.5	24.3	26.3	24.4	29.2	26.5	27.1	26.0	1.12	<0.01	<0.01	<0.01	<0.01*	0.01	0.29	0.68	0.60
Multiparous	34.7	31.1	34.4	30.3	38.0	33.0	35.9	30.4									
ECM																	
Primiparous	27.9	25.9	27.8	26.3	29.8	27.8	27.3	27.0	0.10	<0.01	<0.01	<0.01	<0.01*	<0.01	0.70	0.52	0.90
Multiparous	35.1	31.8	34.7	31.5	37.4	33.4	35.6	30.9									
Fat																	
Primiparous	1.08	1.02	1.09	1.06	1.13	1.09	1.05	1.07	0.041	<0.01	<0.01	0.23	<0.01*	<0.01	0.58	0.56	0.75
Multiparous	1.33	1.23	1.34	1.26	1.40	1.33	1.37	1.25									
Protein																	
Primiparous	1.03	0.94	1.01	0.94	1.09	0.99	0.99	0.95	0.038	<0.01	0.64	<0.01	<0.01*	0.01	0.25	0.54	0.76
Multiparous	1.32	1.18	1.28	1.13	1.37	1.17	1.28	1.05									
Lactose																	
Primiparous	1.32	1.20	1.30	1.20	1.48	1.34	1.36	1.30	0.057	<0.01	<0.01	<0.01	<0.01*	<0.01	0.23	0.55	0.46
Multiparous	1.68	1.49	1.66	1.44	1.89	1.61	1.75	1.47									
Milk composition, g/kg																	
Fat																	
Primiparous	41.6	43.6	41.9	43.9	39.1	42.1	40.0	41.9	1.17	0.20	<0.01*	<0.01	<0.01	0.70	0.24	0.87	0.13
Multiparous	38.9	39.9	39.5	42.4	37.4	40.6	38.8	41.4									
Protein																	
Primiparous	39.3	39.3	38.7	38.8	37.7	37.9	36.8	36.8	0.42	0.07	<0.01	<0.01	0.69	0.53	0.24	0.83	0.65
Multiparous	38.2	38.3	37.4	37.7	36.3	35.8	35.7	35.1									
Lactose																	
Primiparous	49.7	49.2	49.4	49.1	50.7	50.4	50.1	49.7	0.28	<0.01	<0.01	<0.01	<0.01	<0.01	0.63	0.60	0.52
Multiparous	48.3	48.0	48.2	47.8	49.6	48.8	48.6	48.2									
Urea, mmol/L																	
Primiparous	5.17	5.19	5.15	5.14	3.75	4.02	3.85	4.07	0.131	0.19	<0.01	0.79	<0.01	0.56	<0.01	0.62	0.60
Multiparous	4.94	4.92	4.93	5.14	3.56	4.00	3.43	3.90									
Milk N/dietary N, %																	
Primiparous	26.8	27.7	27.6	27.9	28.7	30.0	30.0	31.2	0.78	0.01	<0.01	<0.01	<0.01	0.36	0.20	0.47	0.79
Multiparous	28.3	29.4	29.5	30.6	30.8	33.1	32.8	34.1									

[1]The PMR diets were 2 × 2 × 2 factorially arranged aiming for 2 levels of fat (Fat: 30 or 63 g of crude fat/kg DM; LF or HF, respectively), 2 levels of dietary nitrate (NITRATE: 0 or 10 g of nitrate/kg DM; UREA or NIT, respectively), and 2 levels of 3-nitrooxypropanol (3-NOP: 0 or 80 mg/kg DM; BLANK or NOP, respectively).

[2]Standard error of estimated marginal mean. The lowest value within the variable is reported as SEM.

*Indicates significant interaction ($P < 0.05$) to parity group (Parity; primiparous, multiparous).

from that CH_4 yield in cows on NIT-BLANK was 13.3% lower than in cows on UREA-BLANK (13.7 vs. 15.8 g/kg DMI; $P < 0.01$), whereas for cows fed UREA-NOP and NIT-NOP CH_4 yield was reduced by 23.2% and 22.0%, respectively, compared with UREA-BLANK (12.1 and 12.3 vs. 15.8 g/kg DMI; $P < 0.01$), and we observed no difference between the 2 diets including NOP ($P = 0.94$).

For CO_2 yield (g/kg DMI), a FAT × NITRATE interaction was found. Cows on HF-NIT had greater CO_2 yield than cows on LF-UREA (632 vs. 607 g/kg DMI; $P < 0.01$), although we observed no difference comparing HF-UREA and LF-NIT with LF-UREA ($P = 0.37$ and $P = 0.35$, respectively). Yield of CO_2 increased as a response to NOP addition compared with BLANK (638 vs. 590 g/kg DMI; $P < 0.01$).

A NITRATE × 3-NOP interaction was observed for H_2 yield (g/kg DMI) and was a result of a 115% greater H_2 yield in cows on NIT-BLANK compared with UREA-BLANK (0.183 vs. 0.085 g/kg DMI; $P < 0.01$) and a 256% and 240% greater H_2 yield in cows on UREA-NOP and NIT-NOP, respectively, compared with UREA-BLANK (0.301 and 0.288 vs. 0.085 g/kg DMI; $P < 0.01$). However, we observed no difference in H_2 yield between cows on NIT-NOP and cows on UREA-NOP ($P = 0.39$).

Apparent Total-Tract Digestibility

Fecal DM concentration (g/kg) and fecal score increased by FAT, NITRATE, and 3-NOP (all $P < 0.05$; Table 7). A NITRATE × 3-NOP interaction ($P = 0.04$) was observed for digestibility of OM, reflecting that both NIT and NOP increased OM digestibility, although the combination of the 2 did not lead to any further significant increase. Digestibility of OM was similar in cows on NIT-BLANK, UREA-NOP, and NIT-NOP, but all greater than in cows on UREA-BLANK (73.3%, 73.6%, and 74.2% vs. 70.7%, all $P < 0.01$). Additionally, OM digestibility was greater in HF compared with LF cows (73.5% vs. 72.4%; $P = 0.04$). Digestibility of NDF was also greater in HF cows compared with LF cows (58.3% vs. 55.6%; $P < 0.01$), greater in NIT compared with UREA (58.6% vs. 55.4%; $P < 0.01$) and greater in NOP compared with BLANK (57.8% vs. 56.2%; $P = 0.03$). For all digestibility measures, we observed no parity effect.

Blood Values

Total Hb averaged 7.0 ± 0.07 mmol/L and Hct averaged 34.5% ± 0.65%, and we observed no treatment effects (all $P > 0.10$). Methemoglobin tended to be greater in NIT than in UREA supplemented cows (2.41% vs. 2.26% of tHb; $P = 0.06$), but no MetHb values exceeded 3.7% of tHb in NIT supplemented cows.

Supplemental Material

Results on water intake and drinking behavior are reported in Supplemental Table S1 (https://doi.org/10.5281/zenodo.10018284; Maigaard et al., 2023).

DISCUSSION

Feed Intake and Eating Behavior

With nitrate supplementation, reductions in feed intake of around 5% to 8% has previously been reported in some studies with dairy cows fed similar or higher nitrate doses (Veneman et al., 2015; Klop et al., 2016; Meller et al., 2019), but not in all (van Zijderveld et al., 2011; Olijhoek et al., 2016). In the present study, NIT fed separately within the FAT × NITRATE interaction decreased DMI by 4.6%, but 13% when combined with HF. A similar interaction was reported in a study by Villar et al. (2020) supplementing nitrate and rapeseed oil to steers, although there was no negative effect on DMI of nitrate alone. In our study, NOP decreased DMI by 9.5% and 16.8% in primiparous and multiparous cows, respectively (13.4% across parities), when using an actual dose of 76 ± 2.4 mg 3-NOP/kg of DM. Previous literature, applying similar dose of 3-NOP mixed into feed rations for dairy cows, reported smaller (1%–5%), but statistically insignificant decreases in DMI (Hristov et al., 2015b; Van Wesemael et al., 2019). However, more recent studies by Melgar et al. (2020a) and van Gastelen et al. (2022) showed significant 5% and 6% reductions, respectively, by supplementing either 60 or 80 mg of 3-NOP/kg of DM within each study. Haisan et al. (2014) applied even higher doses of 3-NOP to dairy cows (130 mg/kg DM) without significant effects on DMI. Nevertheless, Melgar et al. (2020c) reported a tendency for a linear decrease in DMI of dairy cows by increased dose of 3-NOP (−6% at doses from 100 to 200 mg/kg DM). The discrepancy between the rather substantial negative effect of the applied dose of 3-NOP in the present study compared with others is unknown, but may be related to interactions to regional differences in diet composition (Kebreab et al., 2023) or animal phenotype.

Treatments including NIT or NOP in the present study also altered cows' feeding behavior toward less time spent eating and with shorter meal duration, which is most likely directly related to the lower feed intake. However, for NOP supplemented cows, the meals appeared more efficient, with fewer interruptions of eating events, as indicated by a greater amount of

Table 6. Visits to GreenFeed and gas emissions of dairy cows fed the 8 partial mixed ration (PMR) diets[1]

	LF				HF					*P*-value							
	UREA		NIT		UREA		NIT										
Item	BLANK	NOP	BLANK	NOP	BLANK	NOP	BLANK	NOP	SEM[2]	Parity	FAT	NITRATE	3-NOP	FAT × NITRATE	FAT × 3-NOP	NITRATE × 3-NOP	FAT × NITRATE × 3-NOP
Observations, n																	
Primiparous	18	18	18	17	17	17	18	18									
Multiparous	17	18	18	18	18	16	14	17									
Visits to GreenFeed																	
Primiparous	28.4	29.5	29.7	31.1	29.6	32.7	33.1	32.7	1.52	0.42	<0.01	<0.01	<0.01*	0.92	0.52	0.23	0.37
Multiparous	24.9	29.2	28.6	31.8	26.0	31.9	29.9	34.4									
CH_4 emissions																	
Production, g/d																	
Primiparous	348	238	272	208	316	215	263	200	10.7	<0.01	<0.01	<0.01	<0.01*	0.21	0.01	<0.01*	0.39
Multiparous	412	245	315	239	371	248	284	233									
Yield, g/kg DMI																	
Primiparous	17.0	13.1	13.8	11.5	14.9	11.8	14.4	12.0	0.39	0.80	0.35	<0.01	<0.01	<0.01	<0.01*	<0.01*	0.58
Multiparous	16.5	11.4	13.6	12.0	14.9	12.3	13.0	13.8									
Intensity, g/kg ECM																	
Primiparous	12.7	9.4	9.9	8.0	10.9	8.0	9.8	7.6	0.40	0.06	<0.01	<0.01	<0.01	<0.01	0.03	<0.01	0.39
Multiparous	11.9	8.0	9.2	7.7	10.0	7.7	8.0	7.7									
CO_2 emissions																	
Production, g/d																	
Primiparous	12,507	11,877	12,160	11,833	12,446	11,692	11,598	10,978	286.6	<0.01	<0.01	<0.01	<0.01*	<0.01	0.12	0.70	0.86
Multiparous	13,998	13,050	13,508	12,462	13,738	12,392	12,717	11,313									
Yield, g/kg DMI																	
Primiparous	610	654	614	652	587	628	629	653	11.4	<0.01	0.84	<0.01	<0.01*	0.01	0.35	0.81	1.0
Multiparous	560	605	581	628	552	619	583	663									
Intensity, g/kg ECM																	
Primiparous	456	472	443	460	431	430	432	415	14.1	<0.01	<0.01	0.02	0.05	0.56	0.06	0.58	0.90
Multiparous	403	424	394	407	374	381	363	375									
H_2 emissions																	
Production, g/d																	
Primiparous	1.83	5.75	3.69	5.51	1.86	5.10	3.24	4.80	0.241	0.05	0.01	<0.01	<0.01	0.30	<0.01	<0.01*	0.79
Multiparous	1.92	6.39	3.94	5.50	2.12	6.19	4.31	4.81									
Yield, g/kg DMI																	
Primiparous	0.088	0.321	0.185	0.308	0.089	0.277	0.176	0.289	0.013	0.59	0.56*	<0.01	<0.01	0.81	0.10	<0.01	1.0
Multiparous	0.076	0.296	0.171	0.280	0.085	0.312	0.197	0.276									
Intensity, g/kg ECM																	
Primiparous	0.065	0.232	0.135	0.217	0.065	0.189	0.121	0.182	0.010	0.10	<0.01	<0.01	<0.01	0.83	<0.01	<0.01	0.64
Multiparous	0.055	0.208	0.115	0.182	0.059	0.189	0.122	0.159									

[1]The PMR diets were 2 × 2 × 2 factorially arranged aiming for 2 levels of fat (Fat: 30 or 63 g of crude fat/kg DM; LF or HF, respectively), 2 levels of dietary nitrate (NITRATE: 0 or 10 g of nitrate/kg DM; UREA or NIT, respectively), and 2 levels of 3-nitrooxypropanol (3-NOP: 0 or 80 mg/kg DM; BLANK or NOP, respectively).

[2]Standard error of estimated marginal mean. The lowest value within the variable is reported as SEM.

*Indicates significant interaction ($P < 0.05$) to parity group (Parity; primiparous, multiparous).

meal time being used as actual eating time, although this was not the case for NIT supplemented cows. Additionally, daily eating rate was slower for these cows, summing up to only 0.6 and 0.3 kg of DM per day for NOP and NIT cows, respectively, which does not account for the observed reductions in DMI seen for these treatments (1.7 and 2.9 kg of DM per day, respectively). This indicates that other factors than slower eating rate explained cows' reduced DMI for these 2 treatments, whereas the greater eating rate in HF cows than in LF cows is merely explained by a slightly greater DM concentration of HF diets compared with LF diets. Cows on NIT and NOP were also having smaller, but more frequent meals, although meal frequency only increased significantly when NIT was combined with HF. These treatment differences were minor, but points in the same direction as what has been observed from nitrate fed beef cattle by Velazco et al. (2014). According to Lee et al. (2015) DMI reductions caused by nitrate, in feed restricted heifers, were more likely related to organoleptic properties of nitrate rather than physiological discomfort caused by nitrate poisoning indicated by increased MetHb levels of blood. In the present study, no NIT cows had MetHb (% of tHb) levels in blood sampled 3 h after returning from morning milking exceeding levels above 3.7%. According to Cockrum et al. (2010) signs of toxicity appears when the proportion of MetHb of tHb exceeds 20%, indicating that DMI reductions caused by acute nitrate poisoning seems unlikely, knowing that the formation of MetHb is dependent on feed consumption rate. However, Lee et al. (2015) reported that in heifers fed the same inclusion rate of nitrate as in the present study (1% of DM), MetHb never exceeded 2.73% of tHb 3 h after feeding, where 43% of the daily offered feed was consumed. For diets with 3-NOP, Kim et al. (2019) and Melgar et al. (2020b) suggested that cows were unaffected by possible organoleptic properties of 3-NOP, although data from the present experiment showed that cows to a greater extent seemed to seek other feed bins, than the one they were assigned to, as attempts to visit unassigned feed bins increased for all additives. Whether this behavior was motivated by palatability or physiological discomfort of eating the assigned ration is unknown, but lower or even negative BW changes in the same cows may as well reflect less gastrointestinal tract fill, which may suggest that cows experienced hunger, and therefore sought other bins. It could furthermore have been hypothesized that dietary inclusion of the additives would have affected drinking water intake in an attempt to overcome possible discomfort, however, the observed differences in drinking water intake merely reflected the decreased DMI, as we

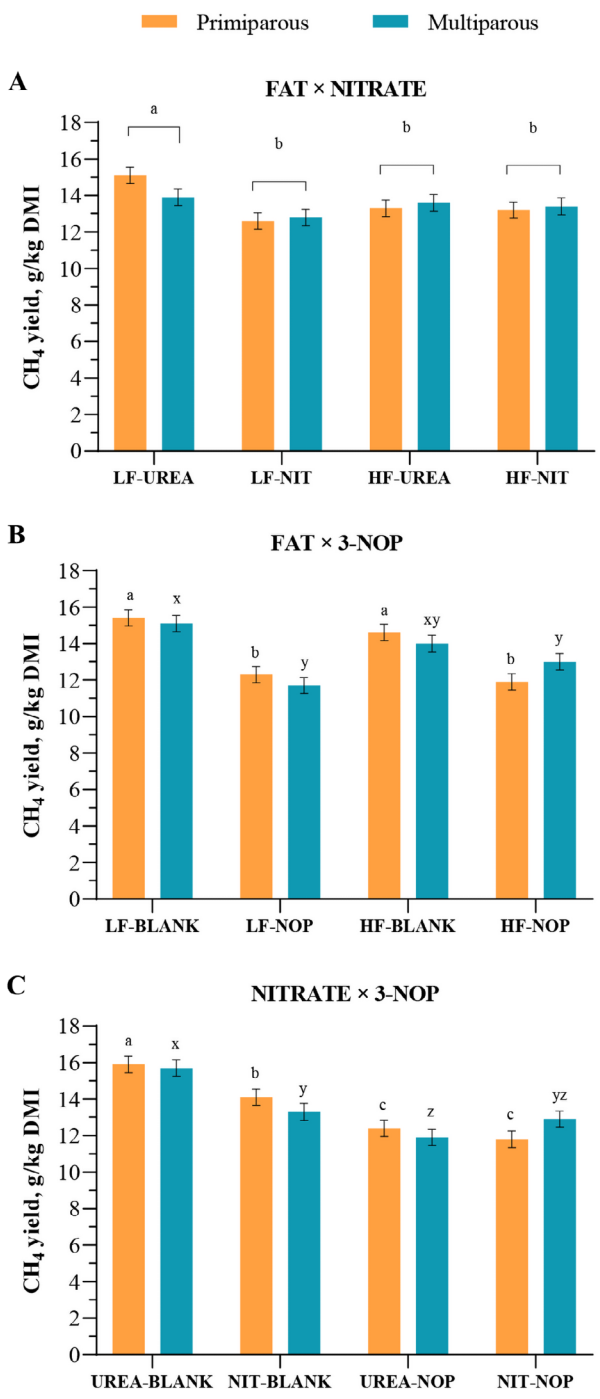

Figure 1. Methane (CH_4) yield (g/kg DMI) illustrated by treatment interactions between fat levels (FAT; LF, HF), nitrate levels (NITRATE; UREA, NIT), and 3-nitrooxypropanol levels (3-NOP; BLANK, NOP). (A) shows the FAT × NITRATE interaction ($P < 0.01$; averaged across levels of 3-NOP), (B) shows the FAT × 3-NOP interaction ($P < 0.01$; averaged across levels of NITRATE), and (C) shows the NITRATE × 3-NOP interaction ($P < 0.01$; averaged across levels of FAT). Orange and blue bars represent treatment estimated marginal means for primiparous and multiparous cows, respectively, and error bars represent standard error of estimated marginal means. Bars with no common letter differ ($P < 0.05$) within parity group (primiparous: a–c; multiparous: x–z), if parity group interacts with the 2-way interaction.

Table 7. Fecal DM concentration, fecal score, and apparent total-tract digestibility of dairy cows fed the 8 partial mixed ration (PMR) diets[1]

	LF				HF												
	UREA		NIT		UREA		NIT			*P*-value							
Item	BLANK	NOP	BLANK	NOP	BLANK	NOP	BLANK	NOP	SEM[2]	Parity	FAT	NITRATE	3-NOP	FAT × NITRATE	FAT × 3-NOP	NITRATE × 3-NOP	FAT × NITRATE × 3-NOP
Observations, n																	
Primiparous	9	9	9	9	9	8	9	9									
Multiparous	9	9	9	9	9	8	7	8									
Fecal DM concentration, g/kg																	
Primiparous	124	131	132	135	127	139	141	154	4.6	0.58	<0.01	<0.01	<0.01	0.09	0.05	0.21	0.60
Multiparous	128	129	124	137	129	138	129	152									
Fecal score																	
Primiparous	3.0	3.2	3.3	3.2	3.0	3.4	3.4	3.7	0.14	0.64	<0.01	<0.01	<0.01	0.24	0.05	0.46	0.72
Multiparous	3.2	3.2	3.1	3.4	3.2	3.4	3.2	3.8									
Digestibility, %																	
Ash																	
Primiparous	95.4	96.0	95.9	95.8	96.1	96.0	96.2	96.3	0.27	0.25	0.07	0.94	0.63	0.85	0.27	0.26	0.76
Multiparous	96.3	96.2	96.1	96.0	96.3	96.2	96.5	95.7									
OM																	
Primiparous	69.7	73.6	72.5	72.3	70.3	74.4	73.6	75.5	0.99	0.37	0.04	<0.01	<0.01	0.52	0.60	0.04	0.86
Multiparous	71.2	72.8	73.1	74.4	71.6	73.7	74.1	74.6									
NDF																	
Primiparous	54.1	57.5	55.1	56.1	55.2	58.2	60.7	61.6	1.46	0.41	<0.01	<0.01	0.03	0.14	0.96	0.11	0.87
Multiparous	52.0	54.6	57.8	57.6	54.6	56.9	59.7	59.9									

[1]The PMR diets were 2 × 2 × 2 factorially arranged aiming for 2 levels of fat (Fat: 30 or 63 g of crude fat/kg DM; LF or HF, respectively), 2 levels of dietary nitrate (NITRATE: 0 or 10 g of nitrate/kg DM; UREA or NIT, respectively), and 2 levels of 3-nitrooxypropanol (3-NOP: 0 or 80 mg/kg DM; BLANK or NOP, respectively).

[2]Standard error of estimated marginal mean. The lowest value within the variable is reported as SEM.

observed no treatment differences in drinking water intake when corrected for DMI (Supplemental Table S1).

Along with CH_4 reductions caused by NIT and NOP, elevated H_2 emissions were concurrently observed in the present study. It has been suggested that increased ruminal dissolved hydrogen concentration (dH_2) may impair rumen fermentation, through increased ratio of the co-factors NADH to NAD^+ in the rumen environment, although it is unclear if dH_2 during CH_4 inhibition ever reach sufficient levels to thermodynamically control reoxidation of NADH to NAD^+ (Ungerfeld, 2020). The considerable improvements in apparent total-tract digestibility of OM in cows fed HF, NIT, or NOP in the present study, indicated that rumen fermentation was not impaired, knowing that these digestibility measures reflect total-tract digestibility and not rumen digestibility only, hence a theoretical compensatory postruminal digestion cannot be ruled out. Additionally, the fact that multiparous cows' DMI were more negatively affected by the treatments than primiparous cows, may accordingly support that the mechanism behind the reduced DMI does not relate to increased rumen fill, because increased rumen fill, as a consequence of impaired ruminal fermentation and hence slower passage rate, would be expected to limit DMI more in primiparous cows than in multiparous cows (Dado and Allen, 1994). Furthermore, it has been reported that increased H_2 emission during CH_4 inhibition was accompanied by increased ruminal concentrations of lactate, formate, and some primary alcohols such as ethanol (Melgar et al., 2020a). Such metabolites may facilitate systemic responses associated with discomfort or in other ways negatively affect DMI of cows. Independent on whether the mechanism behind DMI reductions in the present study was caused by palatability, physiological discomfort, or something else, it remains unclear why combinations of diets with HF (HF with NIT or HF with NOP) decreased DMI in a somewhat synergistic manner, whereas the combination of nitrate and 3-NOP additively decreased DMI.

Milk Production

According to Huhtanen and Hetta (2012), responses in milk production can be underestimated if changes in daily DMI of cows exceed 5 kg/d, by using crossover designs as in the present study. Total DMI in this experiment varied from 17.0 to 21.2 kg/d and from 17.3 to 25.0 kg/d for primiparous and multiparous cows, respectively. This suggest that potential responses in milk production for multiparous cows in particular may not have been fully reflected within the 3-wk periods for some treatments, although the balancing of the treatments across the periods enables evaluation of relative differences between treatments. Therefore, feed conversion efficiencies were not reported in the present study. Though, an analysis of carry-over effects showed no effect of previous period's treatment (results not shown).

Cows fed HF-UREA diets compared with LF-UREA diets responded positively in milk and ECM production (+9% and 6%, respectively), due to greater energy intake. Increased milk production by fat supplementation below 6% of DM was also concluded from a meta-analysis by Patra (2013) and concurrent decreased milk fat and protein concentration was similarly reported by Hellwing et al. (2014) also by using rapeseed as fat source. In the present study, HF supplementation also significantly increased lactose concentration of milk, which is regarded as the main osmotic driver for milk volume, hence increased milk volume most likely diluted fat and protein concentration of milk. The increase in milk production was only present if HF was not combined with NIT, which, supplemented alone, did not decrease milk production, although separate NIT supplementation negatively affected DMI (LF-NIT; −5%). Cows fed NOP, reduced milk and ECM production with 12% and 9% across parities, respectively, which again reflected decreasing effects on DMI. In general, the discrepancy between response in milk yield and ECM yield, was mainly driven by increased milk fat concentration of cows supplemented NIT or NOP, because protein and lactose concentration in milk was either decreased or unaffected by the treatments. Increased fat concentration may be a result of increased body fat mobilization due to lower DMI, as well as changes in rumen fermentation products, altering availability of precursors for mammary de novo fatty acid synthesis. However, yet unpublished data showed no or decreasing effects of treatments on ruminal concentrations of acetic acid, but increasing effects on butyric acid, from cows in the present study. Nevertheless, evaluating the sum of acetic acid and butyric acid revealed that only supplementation of NIT, alone, or in combination with NOP, resulted in increased total concentration of such precursors for milk fat, which illustrates that this cannot be the explanation for increased milk fat concentration in NOP supplemented cows. This suggests that NOP supplemented cows partly compensated for decreased DMI by increased body mobilization to maintain milk production, despite concurrent increased apparent total-tract digestibility of nutrients.

In line with results from Alstrup et al. (2015), FAT in particular had a positive effect on cows' deposition of N in milk as shown by a 2.8 percentage point increased milk N efficiency (milk N/dietary N) in HF cows compared with LF cows. Also supported by a lower milk urea concentration in fat supplemented cows, this is a

result of a lower total N intake along with an unaffected daily milk protein yield (kg/d) in HF supplemented cows. Increases in N efficiency by NIT and NOP, despite lower N intake and lower N output in milk, may be due to increased mobilization of body protein or increased utilization of N in feed, also supported by an absence of a response in milk urea concentration of separate NIT or NOP supplementation.

Gas Emissions

In the present study, all 3 additives reduced absolute CH_4 emission. However, with reductions in feed intake and milk production responses that may not fully reflect changes in feed intake within the course of an experimental period, only emissions related to DMI (gas yield) will be discussed in the following section.

Combining additives to reduce CH_4 emission revealed interactions between the additives, why any main effects of the additives in such cases should be interpreted with caution. Therefore, as an example, the effect of separate fat addition should be evaluated based on significant interactions, wherein FAT is present. Based on the FAT × NITRATE and the FAT × 3-NOP interactions, the CH_4 reduction potential of fat alone, in the present study, can be ascribed to 6% to 7%, corresponding to 1.6% to 1.9% reduction in CH_4 yield per percent added fat. This reduction potential appears to be in the lower range of what previously has been reported in studies by using rapeseed as fat source (Martin et al., 2010; Brask et al., 2013b), although greater than in studies reporting no or even increasing effect of rapeseed supplementation on CH_4 yield (Johnson et al., 2002; Hellwing et al., 2014). Based on interactions including NITRATE, around 12% to 13% reduction in CH_4 yield can be ascribed to NIT, in the present study, which is in line with reductions reported by Olijhoek et al. (2016; 13%), but lower than reductions suggested in the review by van Gastelen et al. (2020; 22%). Theoretically, supplementation of 10 g of nitrate /kg of DM would yield a reduction of 2.58 g/kg DMI (stoichiometric calculations not shown), assuming a complete reduction of NO_3^- to NH_4^+. The reduction obtained by NIT in the present study was 1.95 g/kg DMI, which is equivalent to an apparent efficiency of 75.6%, even though DMI was reduced by NIT. This indicates that nitrate as CH_4 mitigating additive was as effective in the present study as in other studies, although variation between studies and between animals within studies must be expected (apparent efficiency of 59%, van Zijderveld et al., 2011; 72% to 82%, Olijhoek et al., 2016; and 86%, Lund et al., 2014). Evaluating interactions including 3-NOP, the CH_4 reduction potential of separate NOP supplementation was around 21% to 23% in the present study, which, similar to nitrate, appeared to be in the lower range of the elsewhere reported reduction potentials (Lopes et al., 2016; van Gastelen et al., 2020; Melgar et al., 2021). In general, these lower-than-expected reduction potentials could be a potential lack of microbial adaptation within the experimental period or a result of the negative effect on DMI, although total-tract digestibility increased, which may have given rise to compensatory rumen fermentation. This counteracted the obtained absolute effect of the additives per kg of DMI. Nevertheless, the observed treatment interaction to parity group on CH_4 yield was also a result of the more pronounced effect on DMI in multiparous cows, which illustrates that the efficiency of additives per se was similar across parities.

The objective of the present study was to evaluate interactions when combining additives. Knowing that the effects on CH_4 reduction of such combinations may have been different, if DMI was not decreased, it is still possible to reflect on the observed interactions. Assuming that most of the reduction potential of fat supplementation can be ascribed to the replacement of rumen fermentable OM by nonfermentable OM, it was expected that combining HF with NIT or NOP would have reduced CH_4 yield in an at least additive manner. Synergistic effects on CH_4 yield reduction of fat and nitrate was reported by Villar et al. (2020), whereas an additive effect of fat and nitrate and fat and 3-NOP was reported in a study by Guyader et al. (2015) and Zhang et al. (2021), respectively. This was not the case in the present study, as combinations of HF with either NIT or NOP as well as the combination of NIT and NOP did not lead to further significant reductions other than what could be achieved by separate supplementation of the most effective of the 2, indicating antagonistic effects of the specific combination on CH_4 yield reduction due to the absence of additivity and synergistic effects. It is expected that such antagonistic effects are related to the somewhat synergistically decreasing effects on DMI in cows fed combinations of HF with NIT or NOP, or the additive decreasing effect on DMI in cows fed combinations of NIT and NOP. Moreover, as 3-NOP in the present and in previous studies (Melgar et al., 2020a; van Gastelen et al., 2022) have resulted in increased H_2 emission, one could expect that combined use of 3-NOP with either fat or nitrate would result in net-decreased H_2 emission, compared with separate supplementation of 3-NOP, due to possible biohydrogenation of fatty acids or reduction of NO_3^- to NH_4^+ consuming H_2 (Ungerfeld and Kohn, 2006; Jenkins et al., 2008), although Jenkins et al. (2008) suggested that biohydrogenation occupy only small amounts of the total rumen hydrogen pool. This has been illustrated previously in vivo by Zhang et al. (2021), who found

lower H_2 emission from beef cattle fed combinations of rapeseed oil and 3-NOP compared with separate supplementation. In the present study, combined use of NIT and NOP did not change H_2 emissions significantly compared with separate supplementation, although numerical reductions appeared. Similarly, FAT had no measurable effect neither. The lack of such effects may as well be related to reductions in DMI that may have affected total H_2 production or a possible underestimation (or biased estimation) of H_2 emission if a large amount of emitted H_2 is missed if an animal does not visit the GreenFeed relatively shortly after a meal (van Lingen et al., 2017). Gas measurements may also have been biased for some treatments, due to relatively more visits in GreenFeed when cows were supplemented with HF, NIT, or NOP, thereby increasing the likelihood of measuring the peaks in emission.

Increases in CO_2 yield were observed in the present study, when CH_4 yield was reduced. Based on significant effects only, yield of CO_2 was 25 g/kg DMI greater in HF-NIT cows compared with LF-UREA cows and 48 g/kg DMI greater in cows on NOP compared with BLANK. Corresponding reductions in CH_4 yield in the same groups were 1.2 and approximately 3.5 g/kg DMI for HF-NIT and NOP, respectively. Assuming that 1 mol of CO_2 is used to produce 1 mol of CH_4 during methanogenesis (McAllister and Newbold, 2008), reductions of 1.2 and 3.5 g of CH_4/kg DMI in the present study would correspond to 3.3 and 9.6 g of CO_2/kg DMI. Thus, a greater than theoretically expected CO_2 yield was observed in these groups, which may suggest that additional CO_2 originated from other either fermentative or metabolic processes. However, because improved total-tract digestibility could not fully compensate for decreased OM intake in these groups (see discussion of apparent total-tract digestibility), it appears likely that part of the increased CO_2 yield originates from metabolic processes such as mobilization of body reserves.

Apparent Total-Tract Digestibility

The observed improvements of apparent total-tract digestibility of nutrients caused by HF, NIT, and NOP in the present study were rather substantial. Fed separately, HF, NIT (NIT-BLANK within NITRATE × 3-NOP), and NOP (UREA-NOP within NITRATE × 3-NOP) improved total-tract OM digestibility with 1.1, 2.6, and 2.9 percentage points, respectively. The improved OM digestibility increased the total amount of digested OM relatively, although it was not enough to fully compensate for the reduced OM intake. Across parities, total intake of OM was reduced by 4.6% and 13.4% respectively by NIT and NOP supplementation, whereas we observed no effect of FAT. Accordingly, increased digestibility of OM gave rise to 1% increase in digested OM (kg/d) by HF supplementation, but 1.0% and 10.2% decrease in digested OM by NIT and NOP, respectively (data not shown). For NOP supplemented cows compared with BLANK for example, this difference in digested OM corresponded roughly to the observed reductions in milk production.

Averaged per treatment and across parities, the obtained improvements in apparent total-tract digestibility of OM was negatively correlated with DMI ($r = -0.94$; data not shown), supporting the hypothesis that decreased DMI led to slower gastrointestinal tract passage rate and most likely also increased rumen retention time, as documented by Robinson et al. (1987). Across parities, DMI in cows fed the combination of HF, NIT, and NOP (HF-NIT-NOP) was 5.7 kg less than in cows receiving no additives (LF-UREA-BLANK). Comparing the same groups, OM digestibility was increased by 4.6 percentage points, whereas NDF digestibility was increased by 7.7 percentage points, when DMI was decreased. Robinson et al. (1987) investigated the effect of feed intake on total-tract digestibility in cows during a full lactation and reported that change in digestibility of NDF depends on feed intake level and thus reported a decrease in NDF digestibility of 5.4 percentage points by an increase in DMI from 6 to 15 kg and an additional decrease of 8.3 percentage points by increasing DMI from 15 to 24 kg. This supports that the rather substantial increase in apparent total-tract digestibility obtained in the present study may be explained by decreased DMI, hence decreased passage rate. An observed higher fecal DM concentration and feces score of treatment groups with greatest reductions in DMI, further support this hypothesis. Moreover, it has been reported in a review by Patra (2013) that inclusion of dietary fat negatively affected fiber digestion, though Doreau and Chilliard (1997) suggested that this is dependent on fatty acid composition and the degree of unsaturation of fatty acids. Fat from rapeseed is mainly constituted of monounsaturated fatty acids and Patra (2013) suggested that the negative effect of fat on fibrolytic bacteria in the rumen were more pronounced if fat was supplemented as oils compared with oil seeds, as in the present study. However, Brask et al. (2013b) reported no effects on rumen digestibility nor total-tract digestibility of OM and NDF, when rapeseed were supplemented as cake, whole cracked or as oil, whereas Zhang et al. (2021) reported decreased DM and NDF total-tract digestibility from rapeseed oil supplemented to steers. Digestibility of NDF did not decrease by HF supplementation in the present study. Due to confounding effects of decreased DMI in the present study, it cannot be concluded if NIT or NOP as such affected digestibility measures, however, recent

literature have not reported any negative effects, not ascribed to changes in DMI (nitrate: Olijhoek et al., 2016; Almeida et al., 2022; and 3-NOP: Melgar et al., 2020a; Zhang et al., 2021).

CONCLUSIONS

Observed interactions for CH_4 yield (g/kg DMI) between dietary fat, nitrate, and 3-NOP illustrated antagonistic effects of combinations of additives. No combinations of treatments resulted in CH_4 emission reductions that were greater than what were achieved by separate supplementation of the most effective additive within the combination. Additionally, interactions between the additives for DMI revealed that combined supplementation of HF and NIT decreased DMI in a synergistic manner, whereas NIT and NOP, alone and in combination, and the 3 in combination, decreased DMI in an additive manner. Accordingly, eating behavior data showed that cows' attempts to seek unassigned feed increased, when HF, NIT, and NOP were supplemented, and this effect was additive. Milk production was increased by HF supplementation, however, the negative effects of NIT and NOP on DMI were similarly reflected in milk production parameters, despite remarkably improved apparent total-tract digestibility. Based on the findings of the present study, it can be concluded that the separate CH_4 mitigating effect of fat, nitrate, and 3-NOP were not additive, and that supplementation of combinations of these additives negatively affected production parameters.

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