Feeding nitrate and docosahexaenoic acid affects enteric methane production and milk fatty acid composition in lactating dairy cows

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ABSTRACT

An experiment was conducted to study potential interaction between the effects of feeding nitrate and docosahexaenoic acid (DHA; C22:6 n-3) on enteric CH₄ production and performance of lactating dairy cows. Twenty-eight lactating Holstein dairy cows were grouped into 7 blocks of 4 cows. Within blocks, cows were randomly assigned to 1 of 4 treatments: control (CON; urea as alternative nonprotein N source to nitrate), NO₃ [21 g of nitrate/kg of dry matter (DM)], DHA (3 g of DHA/kg of DM and urea as alternative nonprotein N source to nitrate), or NO₃ + DHA (21 g of nitrate/kg of DM and 3 g of DHA/kg of DM, respectively). Cows were fed a total mixed ration consisting of 21% grass silage, 49% corn silage, and 30% concentrates on a DM basis. Feed additives were included in the concentrates. Cows assigned to a treatment including nitrate were gradually adapted to the treatment dose of nitrate over a period of 21 d during which no DHA was fed. The experimental period lasted 17 d, and CH₄ production was measured during the last 5 d in climate respiration chambers. Cows produced on average 363, 263, 369, and 298 g of CH₄/d on CON, NO₃, DHA, and NO₃ + DHA treatments, respectively, and a tendency for a nitrate × DHA interaction effect was found where the CH₄-mitigating effect of nitrate decreased when combined with DHA. This tendency was not obtained for CH₄ production relative to dry matter intake (DMI) or to fat- and protein corrected milk (FPCM). The NO₃ treatment decreased CH₄ production irrespective of the unit in which it was expressed, whereas DHA did not affect CH₄ production per kilogram of DMI, but resulted in a higher CH₄ production per kilogram of fat- and protein-corrected milk (FPCM) production. The FPCM production (27.9, 24.7, 24.2, and 23.8 kg/d for CON, NO₃, DHA, and NO₃ + DHA, respectively) was lower for DHA-fed cows because of decreased milk

fat concentration. The proportion of saturated fatty acids in milk fat was decreased by DHA, and the proportion of polyunsaturated fatty acids was increased by both nitrate and DHA. Milk protein concentration was lower for nitrate-fed cows. In conclusion, nitrate but not DHA decreased enteric CH₄ production and no interaction effects were found on CH₄ production per kilogram of DMI or per kilogram of FPCM.

Key words: methane, nitrate, docosahexaenoic acid, milk fatty acid

INTRODUCTION

Enteric CH₄ production in ruminants has received global interest (Hristov et al., 2013), and various feed additives have been suggested as a nutritional mitigation strategy. Feeding nitrate as alternative electron receptor effectively decreases CH₄ production in sheep (van Zijderveld et al., 2010), and a persistent effect was shown in lactating dairy cows (van Zijderveld et al., 2011). A sudden inclusion of high concentrations of nitrate in ruminant diets may result in a condition known as methemoglobinemia, which decreases the oxygen carrying capacity of the blood. Symptoms of nitrate toxicity depend on the level of methemoglobin in the blood and may include reduced intake and performance, brown discoloration of mucosae, and even death (Bruning-Fann and Kaneene, 1993). When animals are gradually adapted to higher concentrations of nitrate in their diets, no signs of (sub)clinical methemoglobinemia were observed (van Zijderveld et al., 2010, 2011; Lee and Beauchemin, 2014).

Supplementation of fat to ruminant diets also lowers CH₄ production (Grainger and Beauchemin, 2011). Specific fatty acids (FA) have been evaluated for their effect on rumen fermentation, and docosahexaenoic acid (DHA; an n-3 FA; C22:6n-3) has been shown to have a particularly marked effect on microbial metabolism in the rumen (Boeckaert et al., 2008a). Micro-algae enriched in DHA have been shown to decrease CH₄ production in vitro (Fievez et al., 2007), but this could not be confirmed in vivo (Moate et al., 2013).

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The VFA profile in rumen fluid may shift toward more acetate when nitrate is fed, whereas DHA may cause a shift toward a larger relative proportion of propionate (Boeckaert et al., 2008b; Guyader et al., 2015). Propionate production is an H_2 -consuming process and can therefore decrease CH_4 production. Because nitrate and DHA have different mechanisms of affecting ruminal methanogenesis, we hypothesize that their effects on CH_4 production are additive.

An additive, or positive, interaction effect of the 2 additives would be of interest because it would allow for a similar decrease in CH_4 emissions using lower doses of the separate additives. The latter would alleviate the risk of negative effects of the additives on cow health and performance. Moreover, feeding DHA to lactating dairy cows has been reported to increase the proportions of CLA and DHA in milk fat and decrease the SFA proportion (Boeckaert et al., 2008b). From a human health perspective, such an alteration in milk FA composition is of interest (Shingfield et al., 2013). To the best of our knowledge, the effect of feeding nitrate on milk FA profile is unknown.

The main objective of this study was to investigate whether the effects of nitrate and DHA on CH_4 production and animal performance in lactating dairy cows are additive or not. Milk FA profile is a potential indicator of CH_4 production (van Lingen et al., 2014), and therefore the effects of nitrate and DHA fed alone or in combination on milk FA composition were also evaluated.

MATERIALS AND METHODS

Experimental Design, Animals, and Housing

All experimental procedures were approved by the Animal Care and Use Committee of Wageningen University (Wageningen, the Netherlands). The experiment was set up as a completely randomized block design with 4 treatments. Eight primiparous and 20 multiparous lactating Holstein cows (125 ± 16 DIM at the start of the experimental period; mean $\pm$ SD) were blocked according to parity, lactation stage, milk production and presence or absence of a previously fitted rumen cannula. Within blocks, animals were randomly assigned to 1 of the 4 experimental diets. One of the 8 cows with a rumen cannula had to be culled because of foot injuries and was replaced by a nonfistulated reserve animal already adapted to the same experimental diet (NO_3).

Animals were housed in a freestall barn from which blocks of 4 cows consecutively entered a 17-d experimental period. This 17-d period consisted of 12 d in tie-stalls, and from 1500 h on d 13 until 0900 h on d

17, cows were housed individually in climate respiration chambers (CRC).

Diets and Feeding

The experimental diets consisted of 49% corn silage, 21% grass silage, and 30% concentrates on a DM basis. Treatments consisted of a control treatment (**CON**; no nitrate or DHA added), a nitrate treatment (**NO_3** ; 21 g of nitrate/kg of total DM), a DHA treatment (**DHA**; 3 g of DHA/kg of total DM), and a treatment including both nitrate and DHA in the diet (**$NO_3 + DHA$** ; 21 g of nitrate/kg of total DM and 3 g of DHA/kg of total DM). Nitrate, DHA, or both were included in the concentrates (Table 1). Diets were balanced for N content by isonitrogenous exchange of nitrate and urea. Cellulose and limestone were added to balance DM and Ca content of the concentrate mixtures. DHAgold (DSM Nutritional Products, Columbia, MD) was exchanged against wheat because of the similar CP content. The chemical composition of DHAgold was described by Boeckaert et al. (2007) where the DHA content was 198 g/kg of DM. In the present study, DHA content of DHAgold was 254 g/kg of DM. Chromium oxide (1.7 g/kg of DM) was included in all concentrates to estimate total-tract diet digestibility of energy and nutrients. Diets were offered to the cows as TMR (Table 2). Drinking water was continuously available during the entire experiment.

All animals that were assigned to either the NO_3 or the $NO_3 + DHA$ treatment, including 2 reserve animals, were gradually adapted to the experimental level of dietary nitrate (21 g/kg of DM) over a period of 21 d. Cows were group-fed once daily around 0900 h and received 25% of the experimental dose of dietary nitrate during the first week, followed by incremental steps of 25% per week and thereafter all cows received the full experimental dose of dietary nitrate. No DHA was fed during this period of adaptation to increasing levels of dietary nitrate.

During the experimental periods, cows were fed individually with 2 equal portions offered twice daily (at 0600 and 1600 h). A mixture of grass silage and corn silage was prepared twice weekly and weighed into crates that were stored in a cooling room ($\pm 7^\circ C$). The concentrates were in meal form and weighed separately into buckets and manually mixed into the roughage mixture at the moment of feeding. Until d 9 of the tie-stall period, each block of cows had free access to feed. Thereafter, DMI within a block was restricted to 95% of that of the animal with the lowest voluntary DMI between d 5 and 8, while ensuring that none of the animals in the block was restricted to less than 80% of its voluntary DMI.

Table 1. Ingredient composition (g/kg of DM) of the experimental concentrates containing no treatment additive (CON), nitrate (NO₃), docosahexaenoic acid (DHA), or NO₃ + DHA as feed additives

Ingredient	CON	NO ₃	DHA	NO ₃ + DHA
Wheat	194	194	155	155
Dry, ground corn	145	145	145	145
Beet pulp	165	165	165	165
Formaldehyde-treated soybean meal	321	321	321	321
Molasses	33	33	33	33
Trace mineral and vitamin premix	9	9	9	9
Monocalcium phosphate	17	17	17	17
NaCl	17	17	17	17
CaCO ₃	57	—	57	—
Nitrate source ¹	—	98	—	98
Urea	39	—	39	—
DHAgold ²	—	—	39	39
Cellulose	2	—	2	—
Cr ₂ O ₃	1.7	1.7	1.7	1.7

¹5Ca(NO₃)₂NH₄NO₃10H₂O, containing 75% nitrate.

²DHAgold (DSM Nutritional Products, Columbia, MD) = dried, whole cell algae product (seaweed meal), containing 25.4% DHA; trademark of Martek Biosciences Corporation, Royal DSM NV.

Measurements, Sampling, and Laboratory Analyses

Methane was measured in CRC with a volume of 35 m³ (for details of CRC, see van Gastelen et al., 2015). Briefly, temperature in the chambers was set at 16°C and the relative humidity was maintained at 65%. The ventilation rate was 43 m³/h per chamber, inlet and exhaust air of each compartment was sampled at 10-min intervals, and the light schedule allowed for 16 h of light per d, starting from 0530 h onward. Concentrations of CH₄, O₂, and CO₂ in inlet and exhaust air of each compartment were sampled, and ventilation rates were corrected for air pressure, temperature, and humidity to arrive at standard temperature pressure dew

point volumes of inlet and exhaust air. Heat production rates were calculated from gaseous exchange (Brouwer, 1965). Cows were weighed immediately after entering and just before leaving the CRC.

Representative samples of all individual TMR components were collected at the moments of feed preparation for measurement periods in the CRC. Orts were collected during the period that cows were in the CRC. If the amount composed more than 4% of the estimated DM supply, a representative subsample was analyzed for DM and ash content. If the amount was less than 4% of DM supply, composition of the Orts was assumed to be similar to the composition of the offered diet. During CRC periods, the total amount of manure was

Table 2. Average analyzed chemical composition of TMR ingredients (corn silage, grass silage, and concentrates) and calculated composition of complete TMR for the control (CON) diet and diets with nitrate (NO₃), docosahexaenoic acid (DHA), or NO₃ + DHA as feed additives (g/kg of DM unless otherwise stated)

Item	Roughage		Concentrate				TMR			
	Corn silage ¹	Grass silage ²	CON	NO ₃	DHA	NO ₃ + DHA	CON	NO ₃	DHA	NO ₃ + DHA
Inclusion (g/kg of DM)	490	210	300	300	300	300	—	—	—	—
DM (g/kg)	326	586	884	874	894	881	454	452	455	453
Gross energy (MJ/kg of DM)	18.6	18.5	16.4	15.7	16.8	16.1	17.9	17.7	18.0	17.8
Crude ash	36	76	134	135	135	134	74	74	74	74
CP	78	109	347	345	341	349	165	165	163	166
Crude fat	33	30	22	21	40	31	29	29	34	32
NDF	380	561	165	163	155	177	354	356	351	357
ADF	221	327	72	70	70	69	199	198	198	198
ADL	21	25	7	8	7	9	18	18	18	18
Starch	353	NA ³	239	231	209	209	245	242	236	236
Sugar	6	130	16	13	16	16	35	34	35	35
Nitrate	NA	NA	0	71	0	72	0	21	0	21

¹NE_L = 6.2 MJ/kg of DM.

²NE_L = 6.9 MJ/kg of DM.

³NA = not analyzed.

collected and mixed, and a representative subsample was taken for analysis of DM, gross energy (**GE**), and N content. Fecal grab samples were collected at each milking in the CRC for analysis of DM, GE, N, crude fat, starch, NDF, ash, and chromium content to estimate apparent total-tract digestibility of nutrients. Samples were stored at -20°C pending analysis. After thawing, samples were dried at 60°C until constant weight and ground to pass a 1-mm screen. The N concentrations in manure and of roughages were determined in fresh material. For the determination of NH_3 content, fresh silage samples were deproteinized by the addition of 10% (wt/vol) trichloroacetic acid solution followed by centrifugation. Subsequently, indophenol blue was formed using the Berthelot reaction with phenol and hypochlorite in an alkaline solution, which was determined spectroscopically at 623 nm. The DM content of air dry samples was gravimetrically determined by drying at 103°C until constant weight (ISO 6496; ISO, 1999b). Ash was determined after combustion at 550°C (ISO 5984; ISO, 2002). Crude protein content was calculated as $\text{N} \times 6.25$, where N was determined using the Kjeldahl method with CuSO_4 as catalyst (ISO 5983; ISO, 2005). Based on findings of Guo et al. (2007), N content of nitrate containing concentrates was corrected assuming a nitrate-N recovery of 53% after Kjeldahl analysis. The nitrate concentrations in all concentrates were analyzed at the Eurofins laboratory (Barendrecht, the Netherlands). Briefly, nitrate was extracted from the feed using Milli-Q water and converted into nitrite using a cadmium/copper column. Subsequently, the reaction product formed after combination of nitrite and sulfanilamide in an acidic environment was combined with N-1-naphtylethylene diamine dihydrochloride into a red/purple color, which was measured spectrophotometrically at 550 nm. Nitrite concentration of the original sample was analyzed separately to correct the result for nitrate. Hydrolysis with HCl and extraction with light petroleum was used to determine crude fat content of samples (ISO 6492; ISO, 1999a). Starch was determined enzymatically (ISO 15914; ISO, 2004). The NDF content of samples was analyzed according to Van Soest et al. (1991) after pretreatment with α -amylase, but without sodium sulfite. Methods described by Van Soest et al. (1991) were also used for analysis of ADF content and ADL was analyzed using sulfuric acid (Robertson and Van Soest, 1981). An adiabatic bomb calorimeter (IKA-C700, Janke and Kunkel, Heitersheim, Germany) was used for determination of GE content (ISO 9831; ISO, 1998). Chromium contents of concentrates and feces were analyzed using atomic absorption spectrophotometry (Williams et al., 1962).

Milk Production and Milk Composition. Cows were milked twice daily (0600 and 1600 h) throughout

the entire experiment. Milk production was recorded at each milking. A subsample of milk from each milking in the CRC was analyzed for fat, protein, lactose, and GE, and N and MUN content were analyzed in a pooled sample from all milkings in the CRC (5 g/kg of milk produced) according to methods described by Hatew et al. (2015a). Average milk composition for each cow was calculated from the weighted average of all samples taken during the 72-h measurement period in the CRC. Fat- and protein-corrected milk yield (**FPCM**) was calculated according to the formula $\text{FPCM (kg/d)} = (0.337 + 0.116 \times \text{fat \%} + 0.06 \times \text{protein \%}) \times \text{milk yield (kg/d)}$ (CVB, 2008). For each cow, an additional milk sample was collected (5 g/kg of milk at each milking in the chambers) and analyzed for milk FA composition through gas chromatography as described by van Gastelen et al. (2015). Milk FA were expressed in grams per 100 g of total FA.

Blood Samples. During the 21 d of pre-experimental period of adaptation to the final inclusion level of dietary nitrate, a blood sample was collected from all 16 cows fed nitrate after each incremental dose of nitrate in the diet (i.e., d 1, 7, 14, and 21 of this pre-experimental period). Blood was collected from the tail vein in heparinized collection tubes at 3 h postfeeding. Blood samples were analyzed for hemoglobin (**Hb**) and methemoglobin (**MetHb**) content within 1.5 h after sampling in the laboratory of Hospital Gelderse Vallei (Ede, the Netherlands) using a blood gas analyzer ABL-825 (Radiometer, Copenhagen, Denmark).

Statistical Analysis

Data on DMI, milk production, milk composition, and CH_4 production are based on measurements during the last 72 h of the measurement period when cows were in the CRC. For one cow (DHA treatment) only the last 48 h of the measurement period were used, because this cow had an extremely low DMI and water intake during the first 24 h of the measurement period. Two cows (CON and NO_3 treatment) were excluded from the analyses because of a feeding error in the CRC. Energy and N retention and digestibility values were calculated based on the entire period in the CRC and averaged per day. For milk FA composition, values below the detection limit (<0.02 g/100 g of FA) were considered missing values.

All data were analyzed using PROC MIXED (SAS 9.2, SAS Inst. Inc., Cary, NC). The model contained main and interaction effects of dietary treatment factors (nitrate and DHA) as fixed effects and the effect of period (which is equal to block) as a random factor using a variance components (VC) covariance structure. The effect of chamber was initially included as fixed

effect in the model, but was removed because it was not significant. Denominator degrees of freedom were estimated using the Kenward-Roger option. Multiple comparisons between treatments were made using the Tukey-Kramer method. Results are reported as least squares means, and significance of effects was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Methane Production and Cow Performance

The main objective of this study was to examine if the effects of dietary nitrate and DHA on enteric CH_4 production of lactating dairy cows are additive. For CH_4 production in grams per day, a tendency for a nitrate $\times$ DHA interaction was found (Table 3), showing that the effect of nitrate and DHA is different when combined. This was most likely a result of the lower DMI of cows receiving the NO_3 treatment, despite the restricted feeding regimen. Nevertheless, if DMI would have been equal across all treatments, the CH_4 production per kilogram of DMI might have been slightly higher for cows on the NO_3 treatment, but not to such an extent that it would have altered the overall conclusions of this experiment because the feed intake of the NO_3 treatment is still $\sim 95\%$ of the intake of the other treatments. Decreased feed intake after feeding dietary nitrate to ruminant animals has been reported previously (Newbold et al., 2014; Lee et al., 2015b).

With CH_4 production expressed in grams per kilogram of DMI or grams per kilogram of FPCM, the nitrate $\times$ DHA interaction term was not significant, showing an additive effect between nitrate and DHA. Nitrate decreased CH_4 irrespective of the unit in which it was expressed, whereas DHA had no effect on CH_4 per kilogram of DMI or CH_4 per kilogram of digestible OM intake, but resulted in a higher CH_4 production per kilogram of FPCM (Table 3). Moate et al. (2013) reported increased CH_4 emissions per kilogram of DMI and per kilogram of ECM in response to increasing levels of DHA in the diet. Cows in the study of Moate et al. (2013) had unrestricted access to roughage, whereas in the present experiment a restricted feeding regimen was applied. The latter may explain the absence of an effect of DHA on DMI in the present study, whereas in the study of Moate et al. (2013), DMI was significantly reduced at higher doses of DHA (22.1, 22.4, 21.3, and 20.5 kg of DMI/d for the treatments receiving 0, 25, 50, or 75 g of DHA/d, respectively). Previously, DHA has been found to reduce CH_4 production in vitro (Fievez et al., 2007), but this reduction could not be confirmed in vivo by Moate et al. (2013) and in the present trial. Hatew et al. (2015b) showed that effects of starch source and level on in vitro CH_4 production were not observed in vivo in animals adapted to the various starch sources and levels when CH_4 production was expressed per unit of OM intake. We hypothesize that in the present trial the rumen microbial ecosystem adapted to DHA supply resulting in unchanged

Table 3. Dry matter intake, milk production, milk composition, and CH_4 production of dairy cattle fed the control (CON) diet or diets with nitrate (NO_3), docosahexaenoic acid (DHA), or NO_3 + DHA as feed additives

Item	Treatment ¹				SEM	P-value		
	CON	NO_3	DHA	NO_3 + DHA		NO_3	DHA	$\text{NO}_3 \times \text{DHA}$
DMI (kg/d)	16.5	15.7	16.5	16.4	0.81	0.020	0.044	0.060
Milk production (kg/d)	27.8	25.1	28.0	28.0	1.64	0.201	0.180	0.228
FPCM ² (kg/d)	27.9	24.7	24.2	23.8	1.58	0.128	0.062	0.233
Fat (g/kg)	40.9	39.5	29.8	29.4	2.12	0.602	<0.001	0.744
Fat (g/d)	1,147	1,008	824	814	76.4	0.231	<0.001	0.296
Protein (g/kg)	31.2	30.4	31.0	29.5	0.68	0.047	0.369	0.561
Protein (g/d)	869	765	869	826	53.0	0.030	0.354	0.338
Lactose (g/kg)	44.8	45.6	46.6	46.2	0.69	0.728	0.043	0.281
MUN (mg/dL)	11.4	11.4	13.1	11.2	0.88	0.288	0.393	0.311
CH_4 (g/d)	363	263	369	298	14.5	<0.001	0.016	0.069
CH_4 (g/kg of DMI)	22.0	16.9	22.4	18.2	0.52	<0.001	0.086	0.305
CH_4 (g/kg of DOMI ³)	31.4	24.7	31.9	25.5	0.69	<0.001	0.352	0.759
CH_4 (g/kg of FPCM)	13.1	10.8	15.4	12.6	0.57	<0.001	0.001	0.629
CH_4 (% of GEI ⁴)	6.8	5.3	7.0	5.7	0.35	<0.001	0.090	0.487

¹CON (urea as nonprotein N source), NO_3 (21 g of nitrate/kg of DM), DHA (3 g of DHA/kg of DM and urea as nonprotein N source), NO_3 + DHA (21 g of nitrate/kg of DM and 3 g of DHA/kg of DM). For CON and NO_3 treatments $n = 6$, for DHA and NO_3 + DHA treatments $n = 7$.

²Fat- and protein-corrected milk (FPCM) = $(0.337 + 0.116 \times \text{fat } \% + 0.06 \times \text{protein } \%) \times \text{milk yield (kg/d)}$ (CVB, 2008).

³DOMI = digestible organic matter intake.

⁴GEI = gross energy intake.

CH₄ production compared with the control. If 21 g of nitrate/kg of DMI is completely reduced to ammonia, CH₄ emission should be lowered by 5.4 g/kg of DMI based on stoichiometry. With an average CH₄ production of 17.6 and 22.2 g/kg of DMI for cows receiving a diet with and without nitrate, respectively, the average decline in CH₄ reduction corresponds to 85% of the stoichiometric potential to decrease CH₄. This agrees with findings from previous studies in which similar dietary inclusion levels of nitrate were fed to lactating dairy cows (Lund et al., 2014) or beef cattle (Hulshof et al., 2012) and where CH₄ production was lowered by 86% and 87% of the stoichiometric potential, respectively. The present decrease in CH₄ production is higher compared with the study of van Zijderveld et al. (2011), who found a decrease of 59% of the theoretical potential. The feed intake of cows in the study of van Zijderveld et al. (2011) was higher (± 19 kg of DMI/d) than the DMI of cows in the current experiment (± 16 kg of DMI/d). The lower DMI in the present study may have resulted in a longer retention time of feed or fluid, and of nitrate, in the rumen and thus more time for nitrate to be completely reduced to ammonia. Although this argument seems to be in contrast with findings of Lund et al. (2014), who reported a similar decline in CH₄ yield at DMI values above 19 kg/d when nitrate was fed, this contrast may be partly explained by the differences in experimental setup. In the study of van Zijderveld et al. (2011) methods of adaptation and feed restriction were similar to the present study, whereas in the study of Lund et al. (2014) no feed restriction was imposed and cows were also not gradually adapted to the experimental level of nitrate in their diet. Such differences in experimental setup may have affected rumen metabolism differently. Moreover, based on visual observations in the tie-stalls, cows receiving any of the additive treatments in the present study also seemed to have a more gradual feed intake pattern than cows on the CON treatment. Based on visual observations of the diurnal patterns of the respiration quotient (RQ; data not included), we noticed that the RQ value showed a sharp increase for the CON treatment shortly after feeding, whereas the other treatments had lower RQ peak values after feeding. This numerical difference supports the visual observations in tie-stalls and CRC that the feed intake pattern was different across treatments. Alteration of feeding behavior as a result of dietary nitrate supplementation has been reported previously for beef calves (Lichtenwalner et al., 1973). Such a difference in feed intake pattern could not be quantified in the present study, but a more gradual feed intake, with smaller portions per meal, may have contributed as well to a longer retention time of nitrate in the rumen. Guyader et al. (2015) fed nitrate (22.5 g/

kg of DMI) to nonlactating cows with an average DMI of 12.3 kg/d and found a decrease in CH₄ production of 5.6 g/kg of DM compared with the control diet. This corresponds to 96% of the stoichiometric potential of 5.8 g/kg of DM. The difference in physiological state (nonlactating), as well as an increased rumen retention time of nitrate as a result of the lower feed intake in the former study, may explain the larger decrease in CH₄ as compared with the present experiment.

Milk production was not affected by dietary treatment, but FPCM production tended to be decreased by DHA as a result of a significantly lower milk fat production (Table 3). Several rumen biohydrogenation intermediates, including *trans*-10 FA, increase upon feeding DHA, and after absorption such intermediates may decrease de novo FA synthesis in the mammary gland (Boeckaert et al., 2008b). Feeding DHA decreased SFA concentrations (expressed as g/100 g of total FA) in milk and increased concentrations of PUFA (Table 4). The latter is comparable to findings of Boeckaert et al. (2008b) and Moate et al. (2013). To our knowledge, the effect of dietary nitrate on milk FA composition has not yet been reported. Nitrate had no effect on SFA proportion and proportion of MUFA, but increased the proportion of PUFA in milk FA. The proportion of C4:0 in milk FA was increased by feeding nitrate. Unlike other saturated short-chain FA, C4:0 in milk fat does not require acetate for its production as it can be produced directly from β -hydroxybutyrate derived from the blood. Nitrate also increased the proportion of C18:0 in milk fat (Table 4), which is indicative for more biohydrogenation in the rumen. This may be a consequence of the aforementioned longer retention time of feed in the rumen as compared with cows on the CON treatment. The proportions of C14:0 *iso* and C15:0 *iso* were also increased by nitrate, whereas CH₄ was decreased. This is in contrast with findings of Castro-Montoya et al. (2011), who reported a positive relationship between *iso*-FA and CH₄ yield. This relationship was associated with the higher abundance of *iso*-FA in fibrolytic microbes (Vlaeminck et al. 2006), which in turn are associated with a higher CH₄ yield. However, feeding nitrate only was observed to decrease total-tract apparent fiber digestion, and the increased levels of C14:0 *iso* and C15:0 *iso* in milk fat, indicative of increased abundance of fibrolytic bacteria, are not in line with the reduced fiber digestion observed when feeding nitrate without DHA. The increase in *trans*-11 FA together with a decline in CH₄ production in cows receiving nitrate is in line with van Lingen et al. (2014). In contrast to feeding nitrate, feeding DHA decreased the proportion of C18:0 in milk fat. This agrees qualitatively with in vitro studies with DHA added to rumen fluid of cows adapted to DHA, where

biohydrogenation of C18:2 *trans*-11,*cis*-15 was hindered and no biohydrogenation of C18:1 *trans*-11 to C18:0 occurred (Vlaeminck et al., 2008). Feeding DHA increased proportions of several MUFA, including C18:1 *trans*-10 and C18:1 *trans*-11. The alteration in milk FA profile is in line with findings of Boeckaert et al. (2008b). However, contrary to the present study, these FA are often associated with a decrease in CH₄ per unit of feed and per unit of FPCM (van Lingen et al. 2014). If the best performing equations (viz. equations 3 and 4) of van Lingen et al. (2014) are used to predict CH₄/kg of DMI and CH₄/kg of FPCM, respectively, from the present milk FA data, a considerable deviation is present between observed and predicted values. Predicted CH₄ production is 20.8, 19.9, 12.4, and 14.8 g/kg of DMI and 12.1, 11.5, 9.7, and 9.4 g/kg of FPCM for the CON, NO₃, DHA, and NO₃ + DHA treatments, respectively. Based on the prediction equations, CH₄ production should be decreased by DHA and nitrate would have almost no effect, which is in contrast with the present observations. These comparisons indicate that relationships between CH₄ production and milk FA profile, obtained on a wide variety of diets (van Lingen et al. 2014), differ from relationships between CH₄ production and milk FA profile when CH₄-mitigating supplements such as nitrate and DHA are included in the diet, and thus also limit the general potential of milk FA to predict CH₄ production. No DHA was detected in milk from cows receiving the CON or NO₃ treatment. The absence of DHA levels above the detection limit of 0.02 g/100 g of FA in milk of cows that were not supplemented with DHA corresponds to the findings of van Valenberg et al. (2013), who investigated milk FA composition of representative Dutch bovine milk samples that were collected weekly for a period of 1 yr. On average 0.67 g of DHA/100 g of FA was detected in milk from cows receiving DHA. The DHA content of the TMR (3 g/kg of DM) resulted in a daily intake of almost 50 g of DHA/cow. This intake is comparable to the 50 g of DHA/cow (D50) dose fed by Moate et al. (2013) who found a similar amount of 0.60 g of DHA/100 g of FA in milk.

Milk protein content was not affected by DHA, but feeding nitrate resulted in a small, but significant reduction in milk protein content and yield (Table 3). Dietary nitrate also resulted in a lower protein concentration in the study of van Zijderveld et al. (2011). However, protein yield was not affected by nitrate in their study in contrast to the present study where protein yield was 796 and 869 g/d for diets with or without nitrate, respectively ($P = 0.030$). In the Dutch protein evaluation system (DVE/OEB system), DVE indicates digestible feed and microbial true protein digested in the small intestine (Tamminga et al., 1994).

In the current experiment, the calculated DVE supply based on diet composition exceeded 100% of the calculated DVE requirements, indicating that supply of protein did not limit milk protein synthesis. Incomplete reduction of dietary nitrate may decrease the amount of rumen-available N and consequently impair microbial protein synthesis and result in a lower DVE supply than expected based on standard feed values. The resulting lower DVE supply would then negatively affect milk protein yield. However, the actual decline in CH₄ production in the current study was rather close to stoichiometric potential of nitrate, which implies that most of the nitrate must have been decreased to ammonia and has contributed to rumen available N. Alternatively, the negative effect of nitrate on milk protein yield may be related to a decreased supply of gluconeogenic precursors. Nitrate has been shown to increase the acetate:propionate ratio in the rumen (Guyader et al., 2015), which could also affect milk protein content. Rigout et al. (2003) reported an experiment and bibliographical study showing a positive linear relationship between the supply of glucogenic precursors and milk protein content. Glucose is an important factor in signaling pathways thought to regulate milk protein synthesis (Rius et al., 2010).

No treatment effects were found for MUN content of milk (Table 3), and values were comparable to those found by van Zijderveld et al. (2011), who fed a similar diet as in the present study.

Blood Methemoglobin

The average Hb content (mmol/L) of blood of the 16 cows that were gradually adapted to increasing levels of dietary nitrate was 5.9 on d 1 and 7, and 5.6 on d 14 and 21 of the adaptation period before the experimental period. Blood MetHb (% of total Hb) was on average 1.3% on both d 1 and d 7, 2.5% on d 14, and 3.4% on d 21. The highest MetHb value measured for an individual animal was 11.8% on d 21. This level is substantially below the level of 30% that is considered to cause subclinical methemoglobinemia (Bruning-Fann and Kaneene, 1993).

Energy and Nitrogen Retention

No nitrate $\times$ DHA interaction effects on energy and N retention were found (Table 5). The MEI:GEI ratio was higher for the diets containing DHA (Table 5). The calculated energy retention was positive for cows receiving DHA and negative for cows on the CON or NO₃ treatment. The tendency for decreased energy output in milk may explain the positive energy retention of the cows receiving DHA. The absence of a significant

effect of nitrate on FPCM production or milk energy output is in line with results from a recent review by Lee and Beauchemin (2014), who also reported that the

consistent decline in CH₄ yield by dietary nitrate appears to be without directing additional energy toward animal production.

Table 4. Fatty acid composition of milk from dairy cattle fed the control (CON) diet or diets with nitrate (NO₃), docosahexaenoic acid (DHA), or NO₃ + DHA as feed additives

Fatty acid (g/100 g of FA)	Treatment ¹				SEM	P-value		
	CON	NO ₃	DHA	NO ₃ + DHA		NO ₃	DHA	NO ₃ × DHA
C4:0	3.98	4.16	3.85	4.42	0.165	0.034	0.709	0.249
C6:0	2.40	2.41	2.35	2.33	0.071	0.962	0.367	0.833
C8:0	1.22	1.22	1.25	1.14	0.070	0.426	0.736	0.426
C10:0	2.59	2.54	2.92	2.35	0.457	0.188	0.754	0.264
C12:0	2.88	2.79	3.37	2.68	0.263	0.157	0.472	0.265
C14:0	10.62	10.72	12.10	10.87	0.532	0.295	0.141	0.222
C14:0 <i>iso</i>	0.06	0.08	0.05	0.07	0.006	0.004	0.056	0.587
C14:1 <i>cis</i> -9	1.07	1.00	1.19	1.14	0.099	0.532	0.198	0.943
C15:0	0.78	0.83	0.86	0.83	0.050	0.850	0.425	0.344
C15:0 <i>iso</i>	0.18	0.23	0.18	0.19	0.014	0.034	0.093	0.109
C15:0 <i>anteiso</i>	0.30	0.38	0.39	0.40	0.022	0.027	0.009	0.070
C16:0	35.00	32.03	33.73	32.22	1.354	0.080	0.666	0.553
C16:0 <i>iso</i>	0.15	0.16	0.16	0.14	0.011	0.902	0.976	0.079
C16:1 <i>cis</i> -9	2.20	1.84	1.69	1.59	0.149	0.133	0.018	0.375
C16:1 <i>trans</i> -9	0.19	0.23	0.19	0.21	0.016	0.045	0.485	0.562
C17:0	0.51 ^b	0.58 ^a	0.53 ^{ab}	0.53 ^{ab}	0.020	0.036	0.411	0.029
C17:0 <i>iso</i>	0.36	0.40	0.42	0.39	0.023	0.762	0.332	0.185
C17:0 <i>anteiso</i>	0.36	0.39	0.43	0.41	0.019	0.731	0.027	0.218
C17:1 <i>cis</i> -9	0.29	0.28	0.21	0.22	0.023	0.886	0.003	0.721
C18:0	7.23	8.68	2.11	3.54	0.423	0.003	<0.001	0.977
C18:1 <i>cis</i> -9 ²	19.38	19.46	10.20	14.13	1.397	0.054	<0.001	0.063
C18:1 <i>cis</i> -12	0.29	0.31	0.13	0.16	0.017	0.097	<0.001	0.919
C18:1 <i>cis</i> -13	0.14	0.16	0.23	0.21	0.021	0.951	0.001	0.367
C18:1 <i>trans</i> -9	0.16	0.20	0.79	0.82	0.065	0.583	<0.001	0.966
C18:1 <i>trans</i> -10	0.30 ^c	0.48 ^c	5.60 ^a	1.90 ^b	0.336	<0.001	<0.001	<0.001
C18:1 <i>trans</i> -11	0.72	1.24	3.20	4.40	0.421	0.037	<0.001	0.380
C18:1 <i>trans</i> -15 + C18:1 <i>cis</i> -11	0.77	0.82	1.08	1.15	0.071	0.345	<0.001	0.878
C18:2 <i>cis</i> -9, <i>trans</i> -11	0.40	0.55	1.35	2.08	0.187	0.027	<0.001	0.126
C18:2n-6	1.72	1.86	1.86	1.88	0.080	0.340	0.327	0.509
C18:3n-3	0.32	0.33	0.31	0.31	0.018	0.509	0.348	0.612
C18:3n-6	0.07	0.08	0.07	0.08	0.003	0.002	0.125	0.207
C20:0	0.10	0.12	0.06	0.08	0.007	0.043	<0.001	0.915
C20:1 <i>cis</i> -11	0.06	0.06	0.09	0.11	0.008	0.125	<0.001	0.365
C20:2n-6	0.04	0.04	0.05	0.05	0.003	0.690	<0.001	0.155
C20:3n-6	0.07	0.07	0.10	0.11	0.007	0.943	<0.001	0.567
C20:4n-6	0.12	0.12	0.25	0.24	0.010	0.506	<0.001	0.906
C20:5n-3	0.05	0.05	0.15	0.14	0.008	0.494	<0.001	0.494
C22:0	0.04	0.04	0.03	0.04	0.004	0.039	0.376	0.335
C22:5n-3	0.08	0.08	0.18	0.17	0.007	0.094	<0.001	0.873
C22:6n-3	ND ³	ND	0.65	0.70	—	—	—	—
C24:0	0.03	0.03	0.04	0.04	0.003	0.411	0.021	0.411
SFA ⁴	68.76	67.75	64.84	62.61	1.578	0.282	0.007	0.680
MUFA ⁵	25.62	26.14	24.58	26.04	1.440	0.402	0.636	0.688
PUFA ⁶	2.87	3.18	4.97	5.76	0.258	0.046	<0.001	0.352
n-6:n-3 ratio ⁷	4.59	4.76	1.82	1.79	0.010	0.472	<0.001	0.327

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹CON (urea as nonprotein N source), NO₃ (21 g of nitrate/kg of DM), DHA (3 g of DHA/kg of DM and urea as nonprotein N source), NO₃ + DHA (21 g of nitrate/kg of DM and 3 g of DHA/kg of DM). For CON and NO₃ treatments, n = 6; for DHA and NO₃ + DHA treatments, n = 7.

²C18:1 *cis*-9 consists of the sum of C18:1 *cis*-9 and C18:1 *trans*-12, because these 2 fatty acids could not be separated in the analysis. The proportion of C18:1 *trans*-12 is usually negligible.

³ND = not detected (detection limit = 0.02 g/100 g of fatty acids).

⁴Sum of SFA reported in this table.

⁵Sum of MUFA reported in this table.

⁶Sum of PUFA reported in this table.

⁷Ratio between the sum of C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, and C20:4n-6, and the sum of C18:3n-3, C20:5n-3, C22:5n-3, and for treatments DHA and NO₃ + DHA also C22:6n-3.

Table 5. Daily energy and N balance of lactating cows fed the control (CON) diet or diets with nitrate (NO₃), docosahexaenoic acid (DHA), or NO₃ + DHA as feed additives

Item	Treatment ¹					P-value		
	CON	NO ₃	DHA	NO ₃ + DHA	SEM	NO ₃	DHA	NO ₃ × DHA
Metabolic BW (kg of BW ^{0.75})	121	117	120	124	5.3	0.904	0.340	0.235
Gross energy intake (kJ/kg of BW ^{0.75})	2,476	2,378	2,464	2,367	109.4	0.083	0.834	0.995
ME intake ² (kJ/kg of BW ^{0.75})	1,530	1,478	1,561	1,509	10.2	0.196	0.453	0.995
MEI:GEI ratio ³	61.9	62.2	63.3	63.9	20.9	0.416	0.025	0.830
Methane production (kJ/kg of BW ^{0.75})	167	124	172	135	17.9	<0.001	0.134	0.598
Heat production (kJ/kg of BW ^{0.75})	862	873	897	893	26.3	0.838	0.138	0.657
Energy in milk (kJ/kg of BW ^{0.75})	715	646	632	602	32.0	0.136	0.060	0.533
Energy retention total ⁴ (kJ/kg of BW ^{0.75})	-51	-44	32	13	35.6	0.777	0.005	0.549
Energy retention protein ⁵ (kJ/kg of BW ^{0.75})	30	41	27	41	71.2	0.048	0.866	0.829
Calculated energy retention fat ⁶ (kJ/kg of BW ^{0.75})	-81	-85	5	-28	29.0	0.315	0.001	0.438
N intake (g/kg of BW ^{0.75})	3.67	3.57	3.60	3.52	0.171	0.244	0.459	0.852
N manure (g/kg of BW ^{0.75})	2.29	2.22	2.24	2.18	0.101	0.293	0.448	0.911
N milk (g/kg of BW ^{0.75})	1.14	1.02	1.14	1.02	0.055	0.019	0.946	0.908
N retention ⁷ (g/kg of BW ^{0.75})	0.20	0.28	0.19	0.28	0.115	0.048	0.866	0.829
N efficiency ⁸	31.7	29.0	31.6	29.2	1.111	0.025	0.967	0.891

¹CON (urea as nonprotein N source), NO₃ (21 g of nitrate/kg of DM), DHA (3 g of DHA/kg of DM and urea as nonprotein N source), NO₃ + DHA (21 g of nitrate/kg of DM and 3 g of DHA/kg of DM). For CON and NO₃ treatments, n = 6; for DHA and NO₃ + DHA treatments, n = 7.

²ME intake = gross energy intake - CH₄ production - energy in manure.

³MEI:GEI = ratio between ME intake and gross energy intake.

⁴Energy retention total = ME intake - heat production - energy in milk.

⁵Energy retention protein = protein gain × 23.6 kJ/g of protein.

⁶Calculated energy retention fat = energy retention total - energy retention protein.

⁷N retention = N intake - N in manure - N in milk - N from condensate that was collected from heat exchanger - N trapped from the outflowing air.

⁸Fraction of N intake that is incorporated in milk (N milk/N intake; in % of N intake).

Nitrogen retention was positive for all treatments (Table 5). The average N retention was 28 g/d, which is in line with the generally small positive N retention reported for dairy cattle N balance trials as reviewed by Spanghero and Kowalski (1997). Intake and excretion of N was similar among treatments. As expected based on results for milk protein, the N output in milk and N efficiency of milk production were lower for cows receiving nitrate (Table 5). Nitrogen retention was significantly higher for cows receiving nitrate, whereas N in manure was not affected.

Digestibility of Nutrients

Supplementation of DHA generally resulted in higher total-tract digestibility of various nutrients (Table 6). The higher fat digestibility on treatments with DHA is probably caused by the slight difference in fat content of the TMR with and without DHA (Table 2). If fat supplementation is higher, the calculated digestibility values are less affected by fecal excretion of endogenous fat sources (Kil et al., 2010). This difference in dietary fat content could not be prevented in the experimental set-up as exchanging DHA against another fat source would not allow to distinguish between the effect of fat or a specific FA on CH₄ emissions.

Unlike the results for CH₄ production, effects of nitrate and DHA on apparent total-tract digestibility of nutrients were often not additive (Table 6). Digestibility of CP was not different between treatments and does therefore not provide an explanation for the difference in milk protein yield and N utilization for milk production that was observed between treatments with and without nitrate. Moreover, a reduction in DMI and nutrient digestibility was only found for the NO₃ treatment and not for the NO₃ + DHA treatment (Table 6). The effect of DHA on NDF digestibility and signifi-

cance of the interaction term seems mainly to be the result of the low NDF digestibility value obtained for the NO₃ treatment (Table 6). The significantly lower NDF digestion may be related to a decreased functioning of cell wall degrading microorganisms as a result of a temporarily increased ruminal concentrations of H₂. Such increases in H₂ concentration after nitrate supplementation have been reported previously (van Zijderveld et al., 2011; Lund et al., 2014). Accumulation of H₂ in the rumen may impair regeneration of NAD⁺ from NADH (McAllister and Newbold, 2008), and this may negatively affect cell wall degradation by rumen microbes. Nitrite, as intermediate in the reduction of nitrate to ammonia, decreased *in vitro* cell wall digestion and inhibited growth of cellulolytic bacteria (Marais et al., 1988) and may also have negatively affected NDF digestibility. However, the MetHb concentrations in blood of cows receiving nitrate were relatively low in the present study, and it is thus less likely that nitrite accumulated to substantial amounts in the rumen. Nevertheless, a possible negative effect of nitrite on fiber digestion cannot be excluded.

The findings of Lee et al. (2015b) suggest that a restricted feeding regimen influences the potential adverse effects of nitrate on animal health and performance. Despite the poor palatability of nitrate, cows may consume relatively large amounts of nitrate in one meal under restricted feeding, which may exert negative effects in the rumen. Lee et al. (2015a) observed no effect of nitrate on NDF digestibility in beef cattle that had free access to feed. However, ADF digestibility was significantly decreased by nitrate, which indicated that also in their study, fiber degradability did not remain completely unaffected. Better NDF degradation in the rumen and thus more fermentation, probably explains the numerically smaller decrease in CH₄ per kilogram of DMI for cows on the NO₃ + DHA treatment as compared with cows on the NO₃ treatment (Table 3).

Table 6. Apparent total-tract digestibility of nutrients in lactating dairy cows fed the control (CON) diet or diets with nitrate (NO₃), docosahexaenoic acid (DHA), or NO₃ + DHA as feed additives

Digestibility (%)	Treatment ¹				SEM	P-value		
	CON	NO ₃	DHA	NO ₃ + DHA		NO ₃	DHA	NO ₃ × DHA
DM	73.9	71.2	75.2	75.6	0.72	0.133	0.001	0.051
OM	75.6 ^{ab}	73.2 ^b	76.9 ^a	77.3 ^a	0.67	0.143	<0.001	0.048
CP	70.8	69.7	68.6	69.8	1.10	0.989	0.313	0.265
NDF	61.0 ^a	55.3 ^b	63.6 ^a	65.1 ^a	1.31	0.132	<0.001	0.011
Crude fat	70.2	71.3	74.4	76.0	1.37	0.330	0.004	0.884
Starch	99.0	99.3	99.7	99.6	0.18	0.597	0.014	0.193
Gross energy	73.4 ^a	70.3 ^b	74.6 ^a	75.1 ^a	0.75	0.099	<0.001	0.027

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

¹CON (urea as nonprotein N source), NO₃ (21 g of nitrate/kg of DM), DHA (3 g of DHA/kg of DM and urea as nonprotein N source), NO₃ + DHA (21 g of nitrate/kg of DM and 3 g of DHA/kg of DM). For CON and NO₃ treatments, n = 6; for DHA and NO₃ + DHA treatments, n = 7.

CONCLUSIONS

Additive CH₄-mitigating effects, or a positive interaction, of nitrate and DHA fed together would have allowed for a significant decrease in CH₄ at lower doses of individual additives. Feeding DHA strongly affected milk FA composition, but did not decrease CH₄ production per kilogram of DMI and increased CH₄ production per kilogram of FPCM, whereas nitrate showed a large and consistent decrease in CH₄ production irrespective of the unit in which it was expressed. No interaction effect was found for the effects of nitrate and DHA on CH₄ in grams per kilogram of DMI and CH₄ in grams per kilogram of FPCM. A significant interaction effect between nitrate and DHA on NDF digestibility indicated that negative effects of nitrate on apparent total-tract digestibility of nutrients were alleviated by DHA. Such an interaction effect between nitrate and DHA could be of interest if nitrate is fed to decrease CH₄ production, because a decrease in CH₄ production should not be accompanied by reduced animal performance. Given the significant reductions in milk fat and protein yield by DHA and nitrate, respectively, the current doses of the additives are not recommended for application in practice.

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