Effect of dietary nitrate level on enteric methane production, hydrogen emission, rumen fermentation, and nutrient digestibility in dairy cows

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ABSTRACT

Nitrate may lower methane production in ruminants by competing with methanogenesis for available hydrogen in the rumen. This study evaluated the effect of 4 levels of dietary nitrate addition on enteric methane production, hydrogen emission, feed intake, rumen fermentation, nutrient digestibility, microbial protein synthesis, and blood methemoglobin. In a 4 × 4 Latin square design 4 lactating Danish Holstein dairy cows fitted with rumen, duodenal, and ileal cannulas were assigned to 4 calcium ammonium nitrate addition levels: control, low, medium, and high [0, 5.3, 13.6, and 21.1 g of nitrate/kg of dry matter (DM), respectively]. Diets were made isonitrogenous by replacing urea. Cows were fed ad libitum and, after a 6-d period of gradual introduction of nitrate, adapted to the corn-silage-based total mixed ration (forage:concentrate ratio 50:50 on DM basis) for 16 d before sampling. Digesta content from duodenum, ileum, and feces, and rumen liquid were collected, after which methane production and hydrogen emissions were measured in respiration chambers. Methane production [L/kg of dry matter intake (DMI)] linearly decreased with increasing nitrate concentrations compared with the control, corresponding to a reduction of 6, 13, and 23% for the low, medium, and high diets, respectively. Methane production was lowered with apparent efficiencies (measured methane reduction relative to potential methane reduction) of 82.3, 71.9, and 79.4% for the low, medium, and high diets, respectively. Addition of nitrate increased hydrogen emissions (L/kg of DMI) quadratically by a factor of 2.5, 3.4, and 3.0 (as L/kg of DMI) for the low,

medium, and high diets, respectively, compared with the control. Blood methemoglobin levels and nitrate concentrations in milk and urine increased with increasing nitrate intake, but did not constitute a threat for animal health and human food safety. Microbial crude protein synthesis and efficiency were unaffected. Total volatile fatty acid concentration and molar proportions of acetate, butyrate, and propionate were unaffected, whereas molar proportions of formate increased. Milk yield, milk composition, DMI and digestibility of DM, organic matter, crude protein, and neutral detergent fiber in rumen, small intestine, hindgut, and total tract were unaffected by addition of nitrate. In conclusion, nitrate lowered methane production linearly with minor effects on rumen fermentation and no effects on nutrient digestibility.

Key words: methane, nitrate, hydrogen, digestibility, dairy cow

INTRODUCTION

Decreasing methane emission from agriculture is globally under investigation. Methane is a potent greenhouse gas having a 28 times higher warming potential over a 100-yr timespan than carbon dioxide (Myhre et al., 2013). Besides the environmental impact, methane eructed by ruminants is considered an energy loss. Methane has a gross energy (GE) value of 55 MJ/kg and between 2 to 12% of ingested GE is lost as methane (Johnson and Johnson, 1995).

In ruminants, methanogenic archaea produce methane by reducing principally carbon dioxide with hydrogen ($\text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$). Both carbon dioxide and hydrogen are end products from degradation of plant material (primarily carbohydrates) into VFA. Methanogenesis is considered to be the largest sink in

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the rumen to remove hydrogen, which is thought to inhibit fermentation (Ellis et al., 2008).

Nitrate (NO_3^-) acts as an alternative hydrogen sink and thereby lowers enteric methane production (Allison and Reddy, 1984). In the rumen, nitrate is first reduced to nitrite ($\text{NO}_3^- + \text{H}_2 \rightarrow \text{NO}_2^- + \text{H}_2\text{O}$) and is then further reduced to ammonium ($\text{NO}_2^- + 3\text{H}_2 + 2\text{H}^+ \rightarrow \text{NH}_4^+ + 2\text{H}_2\text{O}$). Based on change in Gibbs free energy (ΔG), nitrate reduction to nitrite ($\Delta G = -130$ kJ) followed by nitrite reduction to ammonium ($\Delta G = -371$ kJ) yields more energy compared with methanogenesis ($\Delta G = -67$ kJ; Ungerfeld and Kohn, 2006). Thus, nitrate reduction is highly competitive compared with methanogenesis with respect to available hydrogen and therefore lowers methane production. However, increased hydrogen emissions have been observed when cattle have been fed nitrate (van Zijderveld et al., 2011; Lund et al., 2014; Guyader et al., 2015).

Recent studies have established the effects of feeding high nitrate diets on lowering enteric methane production in dairy cattle (van Zijderveld et al., 2011; Lund et al., 2014; Klop et al., 2016). These studies quantified lower methane production of 16 to 25% as grams per kilogram of DMI or liters per kilogram of DMI at a nitrate inclusion level of 21 g of NO_3^- /kg of DM. In a recent meta-analysis including data from 8 studies with sheep, beef cattle, and dairy cattle, Lee and Beauchemin (2014) showed a linear decrease in methane production with increasing levels of dietary nitrate per kilogram of BW. Newbold et al. (2014) and Lee et al. (2015a) observed a linear decrease in methane production with increasing levels of dietary nitrate fed to steers and beef heifers, respectively. The response in methane production to different levels of dietary nitrate intake has not yet been investigated in vivo with lactating dairy cows adapted to dietary nitrate. Furthermore, previous studies (Lund et al., 2014; Guyader et al., 2015) indicate that nitrate or nitrite, or both, may be toxic toward rumen microbes, in particular through inhibition of fibrolytic bacteria and methanogens (Latham et al., 2016). Because of this possible toxicity it is important that microbial protein synthesis and nutrient digestibility in different sections of the gastrointestinal tract, especially rumen digestibility, are not affected by feeding nitrate. The rumen digestibility and microbial protein synthesis have not yet been studied in dairy cattle. Incomplete reduction of nitrate has been reported previously (Newbold et al., 2014) and might result in increased blood methemoglobin (**MetHb**) levels and increased excretion of nitrate and nitrite in milk and urine. The aim of this study was to quantify the methane mitigating effect of 4 different levels of dietary nitrate addition and the effects on hydrogen emission,

rumen fermentation, nutrient digestibility (in various sections of the digestive tract), rumen microbial protein synthesis, blood MetHb levels, and nitrate and nitrite concentrations in milk and urine. It was hypothesized that increasing levels of dietary nitrate will result in a linear decrease in methane production as also demonstrated in previous studies (Newbold et al., 2014; Lee et al., 2015a), an increase in hydrogen emission, and have no negative effects on rumen fermentation, nutrient digestibility, and microbial protein synthesis. However, feeding nitrate might increase blood MetHb levels, and nitrate and nitrite concentrations in milk and urine.

MATERIALS AND METHODS

Experimental Design

The experiment complied with the ethical requirements set out in the Danish Ministry of Justice Law No. 726 (September 9, 1993). The experiment was a 4×4 Latin square design with 4 lactating Danish Holstein dairy cows (2 in first parity, 1 in third parity, and 1 fourth parity), each fitted with rumen cannulas and duodenal and ileal simple T-cannulas, receiving 1 of 4 experimental diets in each period. Animals were housed in tie-stalls and beds were covered with rubber mats and sawdust. At the start of the experiment, the 2 first-parity cows were 72 and 97 DIM, the third-parity cow was 139 DIM, and the fourth-parity cow was 208 DIM. Average BW $\pm$ standard deviations at the start and end of the experiment were 592 ± 28 kg and 598 ± 28 kg, respectively. The treatments were a control diet (control) without nitrate and 3 diets with added nitrate: low, medium, and high, with calculated nitrate levels of 5.3, 13.6, and 21.1 g of NO_3^- /kg of DM, respectively. Period 1 was repeated because the cow receiving the high nitrate diet in period 1 had an abnormally low feed intake (DMI: 12.7 kg/d in period 1 vs. 20.7 kg/d in period 5) and data from this cow were excluded from analysis. Thus, 5 periods had 19 valid observations in total. Each experimental period lasted 28 d and was divided into 4 subperiods for adaptation to the diet and sample collection. On d 1 to 6, the level of dietary nitrate was gradually introduced (incremental increase of 3.5 g of NO_3^- /kg of DM per d) until the planned level was reached in 0, 2, 4, or 6 d for the control, low, medium, and high nitrate diets, respectively. From d 7 to 16, the animals were allowed to adapt to the experimental diet. Digesta and urine samples were collected from d 17 to 21. Days 17 to 21 will be referred to as the digesta sampling period. On d 22 and 23, no samples were collected. On d 24, the animals were moved to respiration chambers and gas

measurements were taken from d 24 to 28. Days 24 to 28 will be referred to as the gas measurement period.

Diets and Feeding. The rations were formulated using NorFor (Nordic Feed Evaluation System; Volden, 2011) and based on a milk yield of 9,500 kg of ECM per yr. The forage-to-concentrate ratio of the TMR was 50:50 on DM basis. The TMR consisted of corn silage, grass clover silage, concentrate, vitamin and mineral supplements, and either urea (control) or calcium ammonium nitrate [$5\text{Ca}(\text{NO}_3)_2 \cdot \text{NH}_4\text{NO}_3 \cdot 10\text{H}_2\text{O}$; Bolifor CNF, Yara, Oslo, Norway] supplemented with urea to make the rations iso-nitrogenous (Table 1). The 4 rations fulfilled protein requirements of the cows and were balanced for energy, nitrogen, and calcium. Cows were fed ad libitum with TMR twice a day (0630 and 1630 h). Orts were collected daily before the morning feeding, and feed intake was determined on a daily basis. Fresh water was provided ad libitum and daily water intake was not corrected for eventual spill by the cows. Feed intake was monitored continuously on a built-in scale in the feeding trough of the respiration chamber during gas measurements. Four subsamples per period of TMR (19 in total) and Orts were collected and pooled. One pooled sample for each period was analyzed for DM, nitrogen, NDF, and ash.

Rumen, Digesta, Feces, and Urine Sampling. Chromic oxide (Cr_2O_3) and titanium dioxide (TiO_2) were both used as markers to determine nutrient flow because using 2 markers gives more reliable results than using a single marker. Disintegrable bags with 10 g of chromic oxide and 13 g of titanium dioxide were placed directly into the rumen twice a day after feeding. Rumen liquid, duodenal and ileal digesta content, and feces were sampled 12 times over 5 d [d 17–21: 1000 h, 1800 h (d 17), 0200 h, 1200 h, 2000 h (d 18), 0400 h, 1400 h, 2200 h (d 19), 0600 h, 1600 h, 2400 h (d 20), and 0800 h (d 21)]. Six out of 228 observations were not used in the statistical analysis because of a technical problem with the duodenum fistula of one cow. Rumen liquid was sampled from the ventral rumen using a 90-cm steel rumen sampler (Bar Diamond Inc., Parma, ID) with an attached 50-mL syringe. Urine spot samples were collected during urination or by stimulation of the urogenital area at 1000 h on d 17, 1200 h on d 18, 1400 h on d 19, and 1600 h on d 20 of each collection period resulting in 76 observations in total and 74 observations used in the statistical analysis due to the aforementioned problem with one cow. Rumen liquid and urine samples were analyzed for pH directly after sampling using a digital pH meter (Meterlab PHM 220, Radiometer, Brønshøj, Denmark). Rumen samples were analyzed for VFA, lactate, and ammonia concentrations. The 4 urine samples were pooled per

Table 1. Dietary and chemical composition (g/kg of DM unless noted otherwise) as analyzed or calculated for the control (control: 0 g of NO_3^- /kg of DM) and experimental diets (low: 5.3 g of NO_3^- /kg of DM; medium: 13.6 g of NO_3^- /kg of DM; and high: 21.1 g of NO_3^- /kg of DM)

Item	Control	Low	Medium	High
Dietary composition				
Corn silage	413	413	412	411
Grass/clover silage	87	87	87	87
Spring barley	196	196	195	195
Sugar beet pulp	109	109	108	108
Rapeseed cake	48	48	48	48
Molasses	65	65	65	65
SoyPass	48	48	48	48
Monocalcium phosphate	2.6	2.6	2.6	2.6
Sodium sulfate	0.9	0.9	0.9	0.9
Mineral premix ¹	6.5	6.5	6.5	6.5
Vitamin premix ²	1.3	1.3	1.3	1.3
Calcium carbonate	13.9	10.4	5.0	0.0
Urea	9.3	7.0	3.3	0.0
Nitrate source ³	0.0	7.0	18.0	27.9
Chemical composition				
DM (g/kg)	391	393	396	395
Ash	64.5	64.5	64.6	66.4
CP	156	157	159	159
Crude fat ⁴	24.9	25.1	27.5	26.9
NDF	352	349	340	335
Starch ⁴	221	221	220	220
Calcium ⁴	10.0	10.2	10.5	10.9
Gross energy ⁵ (MJ/kg of DM)	18.3	18.3	18.4	18.5

¹Concentration of vitamins and minerals per kilogram of DM of feed-stuff: 176 g of chloride, 140 g of magnesium, 120 g of calcium, 120 g of sodium, 1.5 g of potassium, 0.5 g of sulfur, 4,500 mg of zinc, 4,000 mg of manganese, 1,500 mg of copper, 225 mg of iodine, 50 mg of selenium, 25 mg of cobalt, 600,000 IE of vitamin A, 190,000 IE of vitamin D₃, 4,000 IE of vitamin E.

²Concentration of vitamins and minerals per kilogram of DM of feed-stuff: 10 mg of selenium, 5,000,000 IE of vitamin A, 200,000 IE of vitamin D, 10,000 IE of vitamin E.

³Calcium ammonium nitrate; chemical formula: $5\text{Ca}(\text{NO}_3)_2 \cdot \text{NH}_4\text{NO}_3 \cdot 10\text{H}_2\text{O}$; 75% NO_3^- in DM; calculated composition of 0, 5.3, 13.6, and 21.1 g of NO_3^- /kg of DM for the treatments control, low, medium, and high, respectively.

⁴Calculated values based on table values from NorFor (Volden, 2011).

⁵Calculated according to NorFor (Volden, 2011).

cow per period and analyzed for nitrogen, nitrate, and nitrite concentration.

Duodenal (0.5 L) and ileal (0.2 L) samples were collected by placing plastic collection bags on the T-cannula whereby the bags were filled by bowel movements. Feces (approximately 350 g) were sampled during defecation or from the rectum. The 12 samples for duodenal and ileal contents and feces were pooled and analyzed for markers, DM, nitrogen, NDF, and ash. Purines were analyzed in duodenal chyme to calculate microbial CP synthesis.

Two liters of rumen liquid was collected at 1200 h on d 21 with a cup and filtered in 2 layers of cheese cloth into pre-warmed thermos bottles to analyze microbial

CP content and synthesis. Samples were immediately handled after collection as described by Brask et al. (2015). The method involved the removal of feed particles and protozoa by centrifuging twice at $500 \times g$ for 5 min at 3°C. Next, the supernatant was centrifuged at $17,300 \times g$ for 20 min at 3°C. The pellet was resuspended in 200 mL of saline solution (9% NaCl) and centrifuged at $17,300 \times g$ for 20 min at 3°C. The washed pellet (microbial matter) was frozen (−20°C), freeze-dried, and then analyzed for DM, purines, nitrogen, and ash content.

Blood Sampling. Nine milliliters of blood was sampled from the tail vein 5 times on d 21 (except for period 1 which was on d 19) with 2-h intervals (0600, 0800, 1000, 1200, and 1400 h) to analyze for hemoglobin (Hb) and MetHb levels. This resulted in 95 observations and 90 observations used in the statistical analysis due to the aforementioned problem with the duodenum fistula of one cow. Further, samples were taken once a wk 3.5 h after morning feeding throughout the experiment to ensure that no toxic levels of MetHb were reached. These samples were not included in the statistical analysis.

Gas Exchange. Gas exchange was measured (d 24 to 28) in 4 individual transparent polycarbonate respiration chambers by means of open-circuit indirect calorimetry as described by Hellwing et al. (2012). The chambers were positioned in the same barn as the digestibility trial to minimize the effect of changed environment. Further, the chambers were positioned in a square allowing cows to face each other. The cows switched chambers diagonally, halfway through the 4-d measurements to balance out any possible differences in inlet air composition. Flow and concentrations for the outlet gases (methane, carbon dioxide, oxygen, and hydrogen) were measured continuously over the day (Columbus Instruments, Columbus, OH). Further, temperature, humidity, and pressure were measured continuously (Veng Systems, Roslev, Denmark).

Calculations for gas exchange were based on standard conditions for temperature and pressure (0°C and 101.325 kPa). Cows were fed, milked, and cleaned concurrently to minimize accessing the chambers.

Milk Yield and Composition. Cows were milked twice a day at 0630 h and 1630 h. Milk yield was recorded daily when cows were in the respiration chambers (d 24 to 28). Milk (800 mL) was sampled on 2 subsequent days during digesta collection (d 18 and 19) and gas measurements (d 25 and 26) resulting in 4 observations per cow per period. Protein, fat, lactose, nitrate, and nitrite concentrations were determined for all separate milk samples as described below, and the results were averaged per cow per period before statistical analysis.

Laboratory Analyses

All feed and digesta samples were stored frozen at −20°C and subsequently freeze-dried (except urine and milk) before laboratory analysis. Feed, duodenal, ileal, and fecal samples were dried at 60°C for 48 h to determine DM (AOAC International, 2000). The ash content was determined in the feed, digesta, and microbial matter samples by combustion at 525°C for 6 h. The Dumas method (Hansen, 1989) was used to determine the nitrogen content of feed, digesta, and microbial matter using a Vario MAX CN (Elementar Analysensysteme GmbH, Hanau, Germany). Crude protein content was calculated by multiplying the nitrogen content by 6.25. Total urinary nitrogen was determined by the Kjeldahl method using a Kjeltec 2400 distillation unit with autosampler (Foss Analytical, Hillerød, Denmark). Neutral detergent fiber was analyzed according to the method used by Mertens (2002) with a Fibertec M6 System (Foss Analytical) using heat-stable amylase and sodium sulfite and reported as ash-free NDF. Ammonia in rumen liquid was alkalized with potassium hydroxide followed by distillation and titration using a Kjeltec 2400 (Foss Analytical; Brask et al., 2013). Volatile fatty acids and lactate concentrations were analyzed as described by Jensen et al. (1995) and Canibe et al. (2007) by a Hewlett Packard gas chromatograph (model 6890, Agilent Technologies Inc., Wilmington, DE) with a flame ionization detector and a 30-m SGE BP1 column (Scientific Instrument Services, NJ). Chromic oxide in digesta and feces was oxidized to chromate and determined colorimetrically (Schürch et al., 1950). Titanium dioxide was digested with sulfuric acid, hydrogen peroxide was added, and absorbance was measured spectrophotometrically according to Myers et al. (2004) with the modification that 15 mL of 30% hydrogen peroxide was added instead of 10 mL, and before measuring the absorbance an additional 5 drops were added. Microbial matter and duodenal chyme were analyzed for total purine content by the method of Zinn and Owens (1986) as modified by Thode (1997). Briefly, the nucleotides were hydrolyzed by the addition of perchloric acid after which purines precipitated into complexes with silver nitrate and were measured spectrophotometrically. Protein, fat, and lactose concentrations in milk were measured by an infrared analyzer (Milkoscan Msc4000, Foss Analytical) at Eurofins Steins Laboratories (Holstebro, Denmark). Nitrate and nitrite concentrations in milk and urine were determined according to the method developed for milk as described by Woollard and Indyk (2014) using a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA). Blood was analyzed for Hb

and MetHb directly after sampling by oximetry using an acid base laboratory analyzer (Radiometer ABL 725 Flex analyzer; Radiometer).

Calculations and Statistical Analyses

Data on gas exchange, DMI, water intake, digestibility, milk yield and composition, nitrate concentrations in milk and urine, microbial CP synthesis, and microbial efficiency were averaged per cow per period. Exclusion of 1 cow in period 1 resulted in a total of 19 observations for these variables. If different sample sizes were used, it is stated otherwise.

The stoichiometric potential of nitrate for lowering methane production was based on the preference for complete nitrate reduction, due to it being more energetically favorable than carbon dioxide reduction (Ungerfeld and Kohn, 2006). Stoichiometrically, the addition of 1 mol of nitrate will lower methane production with 1 mol, corresponding to a decline in methane production of 25.8 g or 36.0 L for each 100 g of nitrate fed. Addition of nitrate should in theory lower methane production by 1.9, 4.9, and 7.6 L of CH₄/kg of DM for the low (5.3 g of NO₃⁻/kg of DM), medium (13.6 g of NO₃⁻/kg of DM), and high nitrate (21.1 g of NO₃⁻/kg of DM) diets, respectively. Thus, the apparent efficiency with which enteric methane is mitigated is the measured methane reduction relative to the potential methane reduction (14 observations).

Daily DMI was calculated as DM offered with DM in refusals subtracted. Hourly DMI during gas measurements was calculated as hourly change in residual feed multiplied with the DM content of feed. Digesta DM flows calculated for each of the markers separately gave similar results, therefore mean flows were reported for DM, OM, CP, and NDF in duodenum, ileum, and feces. Apparent digestibility coefficients for DM, OM, CP, and NDF for rumen, small intestine, hindgut, and total-tract were calculated from the respective intake and flow at duodenum, ileum, and feces. Microbial CP synthesis was calculated according to Lund et al. (2003) using the concentration of nitrogen and purines in rumen-isolated bacteria, the concentration of purines in duodenal contents, and duodenal DM flow. The calculation of microbial efficiency was expressed as the amount of microbial CP synthesized relative to the true rumen digested OM (g of CP/kg of true rumen digested OM; Lund et al., 2003). The amount of true rumen digested OM was calculated by subtracting duodenal OM flow (corrected for duodenal microbial OM flow) from feed OM intake.

Gross energy intake (**GEI**) of the 4 TMR diets was calculated according to the Nordic feed evaluation sys-

tem (Volden and Nielsen, 2011) with an additional correction of nitrogen content in feed with urea-nitrogen and nitrate-nitrogen content. Energy-corrected milk yield (3.14 MJ/kg) was calculated according to Sjaunja et al. (1991) using the equation: ECM yield (kg/d) = milk yield (kg/d) × [(38.3 × fat (g/kg) + 24.2 × protein (g/kg) + 15.71 × lactose (g/kg) + 20.7)/3,140].

The VFA hydrogen ratio represents the amount of hydrogen originating from VFA fermentation and which can potentially be emitted. The VFA hydrogen ratio was estimated from the concentration of acetate and butyrate in rumen liquid and divided by the concentration of propionate and valerate and multiplied by 2 (Brask et al., 2015).

The mean values for gas emissions, methane efficiency, digestibility, DMI, water intake, milk yield and composition, ECM yield, nitrite and log-transformed nitrate concentrations in milk and urine, microbial CP synthesis, and microbial efficiency were analyzed using the PROC MIXED procedure in SAS (version 9.3, SAS Institute Inc., Cary, NC) using treatment (control, low, medium, and high) and period as fixed effects and cow as random effect. The PROC IML was used to generate contrast coefficients because nitrate levels were not equidistant across treatments. Orthogonal polynomial contrasts (linear and quadratic) were used to examine treatment effects. Differences of least squares means were adjusted by the Tukey-Kramer test. The ammonia concentration and pH in rumen liquid and urine were analyzed using PROC MIXED with treatment, period, sampling time, and the interaction between treatment and sampling time as fixed effects, cow as random effect, and repeated measures for sampling time. The used covariance structure was heterogeneous autoregressive [ARH(1)]. Covariance structures were based on lowest Akaike's information criterion values to optimize the statistical analysis. Similar mixed models were used for (1) lactate, total VFA, molar proportions of VFA, acetate:propionate ratio and VFA hydrogen ratio, but using Toeplitz as covariance structure; (2) VFA hydrogen ratio per 2 h using ARH(1) as covariance structure and adjusting differences of least squares means by the Tukey-Kramer test; and (3) Hb and MetHb levels in blood using autoregressive [AR(1)] as covariance structure and for MetHb, the differences of least squares means were adjusted by the Tukey-Kramer test. The previously described mixed model for ammonia concentration and pH in rumen liquid and urine was also used for the hourly methane production, hourly hydrogen emission, and hourly DMI, but with day, chamber number, and the interaction between treatment and day, and time and day as additional fixed factors (1,820 observations, 100 excluded). Differences in least squares means were adjusted by the Tukey-Kramer test.

Mean values presented in tables are least squares means with corresponding SEM. Significance was declared at $P < 0.05$ and a tendency at $0.05 < P < 0.10$.

RESULTS

Dry Matter Intake

Daily DMI during digesta collection (Table 2) tended to increase linearly ($P = 0.09$) with increased dietary nitrate levels. Hourly DMI did not differ between dietary treatments (Figure 1a). Further, water intake was unaffected by feeding nitrate (Table 2).

Rumen Fermentation Characteristics, Microbial Protein Synthesis, Microbial Efficiency, and Digestibility

The total VFA concentration and molar proportions of acetate, butyrate, and propionate were unaffected by feeding nitrate (Table 2), although there was a

linear tendency to increased total VFA concentrations ($P = 0.06$) and butyrate proportions ($P = 0.09$), and decreased propionate proportions ($P = 0.07$) with increased nitrate level in the diet. Further, an interaction was found between dietary treatment and sampling time for the acetate:propionate ratio ($P = 0.04$) and a tendency for acetate proportions ($P = 0.06$). The VFA hydrogen ratio tended to increase linearly ($P = 0.09$) with increasing nitrate in the diet and also showed a tendency ($P = 0.09$) for interaction between treatment and sampling time. Further, the VFA hydrogen ratio (Figure 1d) did not show any differences among treatments; however, there seemed to be a dissimilar diurnal pattern between the control and high treatment. The VFA hydrogen ratio for the control diet showed only minor diurnal fluctuations, whereas for the high nitrate treatment the diurnal fluctuations were much more pronounced: a steep increase after feeding, especially after the morning feeding, followed by a decline until the next meal. Molar proportions of formate increased linearly ($P < 0.001$) across nitrate treatments from

Table 2. Dry matter intake during digesta collection¹, water intake, rumen fermentation characteristics, total nitrogen, nitrite (NO_2^-), and pH in urine, and hemoglobin (Hb) levels in venous blood per sampling time and treatment (control: 0 g of NO_3^- /kg of DM; low: 5.3 g of NO_3^- /kg of DM; medium: 13.6 g of NO_3^- /kg of DM; and high: 21.1 g of NO_3^- /kg of DM)

Item	Treatment (Trt)				SEM	P-value ²				
	Control	Low	Medium	High		Trt	Time	Trt × Time	L	Q
DMI during digesta collection (kg/d)	17.0	17.3	18.7	18.5	0.79	0.26	—	—	0.09	0.58
Water intake (L/d)	56.0	57.3	59.2	59.2	3.73	0.76	—	—	0.35	0.73
NH_3 (mmol/L)	4.27	4.44	4.75	5.63	0.31	0.01	<0.001	0.50	0.001	0.26
pH rumen	6.38	6.30	6.38	6.28	0.11	0.24	<0.001	0.65	0.30	0.68
Lactate (mmol/L)	2.58	2.29	3.31	2.09	1.12	0.75	<0.001	0.71	0.92	0.52
Total VFA ³ (mmol/L)	107	112	110	115	4.38	0.16	<0.001	0.97	0.06	0.99
Acetate (A)	60.2	60.2	60.0	60.0	1.21	0.99	<0.001	0.06	0.80	0.94
Butyrate	14.4	14.9	15.6	16.0	0.76	0.34	<0.001	0.76	0.09	0.80
Propionate (P)	21.4	20.9	20.0	19.6	0.94	0.29	0.01	0.14	0.07	0.78
Valerate	2.12	2.15	2.32	2.08	0.60	0.57	<0.01	0.43	0.93	0.27
Formate	0.08	0.13	0.35	0.45	0.07	<0.01	<0.001	<0.001	<0.001	0.98
Isobutyrate	0.55	0.52	0.50	0.50	0.03	0.33	<0.001	0.67	0.11	0.47
Isovalerate	0.22	0.14	0.12	0.20	0.20	0.14	<0.001	0.48	0.72	0.03
A:P	2.86	2.94	3.05	3.11	0.17	0.36	<0.001	0.04	0.10	0.80
VFA hydrogen ratio ⁴	6.47	6.68	6.93	7.14	0.50	0.34	<0.001	0.09	0.09	0.89
Microbial CP synthesis (g of CP/d)	1,159	1,182	1,284	1,307	77.1	0.35	—	—	0.10	0.86
Microbial efficiency (g of CP/kg of true rumen digested OM)	128	132	130	131	7.46	0.89	—	—	0.76	0.81
pH urine	8.10	7.99	8.24	8.26	0.12	0.24	0.36	0.47	0.12	0.73
Total nitrogen in urine (g/L)	12.2	11.0	11.0	12.8	0.78	0.25	—	—	0.52	0.06
NO_2^- in urine ($\mu\text{g/L}$)	251	225	253	275	22.6	0.43	—	—	0.26	0.33
Hb (mmol/L)	5.82	5.91	5.77	5.92	0.29	0.50	0.17	0.37	0.65	0.58

¹Digesta were collected on d 17 to 21 of each period.

²Time = sampling time, for NH_3 and rumen pH 0200 to 2400 h (12 sampling moments); for urine pH 1000, 1200, 1400, and 1600 h; for Hb 0600, 0800, 1000, 1200, and 1400 h. L = linear; Q = quadratic.

³Total VFA is the sum of the concentrations of the following VFA: acetate, butyrate, propionate, formate, isobutyrate, isovalerate, valerate, caproate, heptanate, and succinate. Individual VFA are shown in mol/100 mol.

⁴VFA hydrogen ratio = $2 \times (\text{acetate} + \text{butyrate})/(\text{propionate} + \text{valerate})$.

Figure 1. Least squares means for DMI (a), methane production (b), hydrogen emission (c), and VFA hydrogen ratio [$2 \times (\text{acetate} + \text{butyrate}) / (\text{propionate} + \text{valerate})$] (d) over a 24-h period for dairy cows fed a control diet (■), control: 0 g of NO_3^-/kg of DM; or nitrate diet (◆, low: 5.3 g of NO_3^-/kg of DM; ▲, medium: 13.6 g of NO_3^-/kg of DM; and ×, high: 21.1 g of NO_3^-/kg of DM). Arrows indicate the time of feeding. Each time point represents the average gas production for 1 h around that time (i.e., time point 0130 h represents the average gas production from 0100 to 0200 h). Above the figure, different letters for different treatments at the same time point are significantly different at $P < 0.05$.

0.08 to 0.45 mol/100 mol. The ammonia concentration in rumen liquid increased linearly ($P = 0.001$) with increasing dietary nitrate levels (Table 2) with highest levels observed for the high nitrate treatment (5.63 ± 0.31 mmol/L).

Daily microbial CP synthesis was unaffected by nitrate levels in the diet, though a numerical linear increase (1,159 to 1,307 g of CP/d; $P = 0.10$) with increasing dietary nitrate levels was observed (Table 2). The microbial efficiency in CP synthesis per kilogram of true rumen digested OM was unaffected by the dietary treatments.

Apparent rumen, small intestinal, hindgut, and total-tract digestibility coefficients for DM, OM, CP, and NDF were unaffected by dietary nitrate (Table 3). Dry matter and NDF digestibility in the rumen were on average 34.8 and 57.9%, respectively. Total-tract DM digestibility was on average 67.8% and small intestinal CP digestibility was 68.9%.

Milk and Urine

Urinary pH was unaffected by sampling time and treatment (Table 2). The total urinary nitrogen concentration was also unaffected by treatment (Table 2), but a tendency for a quadratic response ($P = 0.06$) to nitrate was observed, with lowest total nitrogen contents in urine at low and medium nitrate levels. Nitrate concentrations in milk and urine both increased linearly ($P < 0.001$) with increasing nitrate levels ($P < 0.001$). Milk nitrate concentration increased from 0.13 to 1.56 mg/L (Figure 2a), whereas urinary nitrate concentration increased from 1.96 to 14.3 mg/L (Figure 2b). The nitrite concentration in urine was unaffected by treatment (Table 2). Nitrite concentrations in milk were below the detection limit (<30 $\mu\text{g/L}$) of the analysis. The treatments did not affect fat, protein, and lactose concentrations in milk (Table 4). Also, milk yield and ECM yield were unaffected by treatment.

Hemoglobin and Methemoglobin

Methemoglobin levels increased linearly (1.3 to 1.6% of Hb; $P < 0.01$) with increasing nitrate levels (Figure 2c). Measured MetHb levels in individual blood samples taken throughout the experimental period ranged from 0.5 to 4.8% of Hb levels. The highest value was observed for a cow on the high nitrate diet on d 6 after the diet change. Hemoglobin levels were unaffected by feeding nitrate (Table 2).

Gas Exchange

Daily DMI during gas measurements was unaffected by feeding nitrate (Table 5). Methane production in liters per day, liters per kilogram of DMI, fraction of GEI, and liters per kilogram of ECM (Table 5) decreased linearly with increasing dietary nitrate levels. Methane production per kg of DMI was lowered by 6% for the low, 13% for the medium, and 23% for the high nitrate diet compared with the control. Methane production was lowered with efficiencies of 82.3, 71.9, and 79.4% of the stoichiometric potential for the low, medium, and high nitrate diets, respectively. The level of nitrate in the diet did not affect the methane efficiency. The hourly methane production for each treatment is shown in Figure 1b. The decreasing effect of nitrate on methane production was apparent up to 5 h (1130 and 1930 h) after feeding. The control and low treatments showed highest increments from 0530 to 0930 h

(control: from 14.1 to 24.9 L of CH₄/h; low: from 14.7 to 24.5 L of CH₄/h), compared with the medium (19.7 L of CH₄/h) and high (18.0 L of CH₄/h) nitrate treatments at 0930 h. No differences in methane production were observed between 1230 to 1630 h and 2030 to 0630 h, although methane production on the high nitrate diet seemed to be lowest and more stable throughout the day compared with the other treatments. Carbon dioxide and oxygen consumption did not differ between treatments. The CH₄:CO₂ ratio declined linearly ($P < 0.001$), whereas the H₂:CH₄ ratio increased quadratically ($P = 0.03$) with increased nitrate level (Table 5). Hydrogen emissions (L per kg of DMI) increased quadratically ($P = 0.02$) with a factor 2.5, 3.4, and 3.0 for the low, medium, and high treatments compared with the control. Hydrogen emissions increased from 0.14 and 0.24 L/h at 0530 h to 4.9 and 5.1 L/h 1 h after morning feeding for both the low and medium treatments (Figure 1c) respectively, which was higher than for the control and high treatment (around 3.0 L of H₂/h). Differences in hydrogen emissions between treatments were apparent between 0930 and 1130 h and at 0030 and 0330 h. Hydrogen emissions were not different between 1230 and 1830 h.

DISCUSSION

Dry Matter Intake

In the present study DMI during the gas measurement period was unaffected by feeding nitrate, which

Table 3. Apparent digestibility (%) for DM, OM, CP, and NDF in different sections of the digestive tract per treatment (control: 0 g of NO₃[−]/kg of DM; low: 5.3 g of NO₃[−]/kg of DM; medium: 13.6 g of NO₃[−]/kg of DM; and high: 21.1 g of NO₃[−]/kg of DM)

Item	Treatment				SEM	P-value		
	Control	Low	Medium	High		Treatment	Linear	Quadratic
DM digestibility								
Rumen	35.1	33.3	35.3	35.6	1.68	0.47	0.42	0.48
Small intestine	38.7	44.0	41.2	40.1	2.55	0.42	1.00	0.23
Hindgut	18.5	15.2	16.3	14.6	3.64	0.84	0.53	0.84
Total tract	67.7	68.3	68.2	67.1	1.43	0.85	0.67	0.44
OM digestibility								
Rumen	44.5	43.4	44.3	44.6	1.67	0.81	0.71	0.57
Small intestine	37.5	43.6	40.5	39.6	2.52	0.31	0.91	0.19
Hindgut	12.3	7.65	10.1	8.21	4.15	0.81	0.62	0.76
Total tract	69.6	70.3	70.2	69.2	1.47	0.87	0.74	0.45
CP digestibility								
Rumen	−16.8	−18.4	−14.6	−17.5	2.55	0.67	0.83	0.66
Small intestine	67.7	70.4	68.4	68.5	1.31	0.38	0.94	0.35
Hindgut	12.8	8.89	11.9	10.8	3.55	0.81	0.90	0.74
Total tract	67.4	67.9	68.4	67.0	1.13	0.56	0.79	0.19
NDF digestibility								
Rumen	59.5	57.6	56.9	57.6	2.72	0.73	0.47	0.45
Small intestinal	−27.7	−12.4	−18.5	−23.3	5.70	0.22	0.91	0.10
Hindgut	11.2	4.72	6.77	6.53	4.92	0.73	0.62	0.52
Total tract	53.8	54.5	53.4	51.2	2.81	0.73	0.35	0.54

Figure 2. Least squares means for nitrate (NO_3^-) concentrations in milk; treatment $P < 0.001$; linear $P < 0.001$; quadratic $P = 0.001$ (a), nitrate concentration in urine; treatment $P < 0.001$; linear $P < 0.001$; quadratic $P = 0.17$ (b), and methemoglobin (MetHb) as a proportion of hemoglobin (Hb) in blood; treatment $P = 0.01$; sampling time $P < 0.001$; treatment $\times$ sampling time $P = 0.27$; linear $P < 0.01$; quadratic $P = 0.86$ (c) for dairy cows fed a control diet (control: 0 g of NO_3^- /kg of DM) or nitrate diet (low: 5.3 g of NO_3^- /kg of DM; medium: 13.6 g of NO_3^- /kg of DM; and high: 21.1 g of NO_3^- /kg of DM). Error bars are defined as SE in panels a and b, and as SEM in panel c.

is in agreement with previous studies in cattle (van Zijderveld et al., 2011; Newbold et al., 2014; Guyader et al., 2015; Troy et al., 2015), and tended to increase during the digestibility period. Dry matter intake did not differ between treatments in contrast to other studies that reported lower DMI when nitrate was fed (Hulshof et al., 2012; de Raphélis-Soissan et al., 2014; Lee et al., 2015a,b; Klop et al., 2016). These differences between studies could be related to the feeding strategy (i.e., nitrate given as a single dose or as part of the TMR, and restricted vs. ad libitum feeding). Overall, nitrate does not seem to have a consistent effect on DMI, but nitrate could make the animal more prone to a potential decrease in DMI.

Decline in Methane Production by Nitrate Addition

Methane production decreased linearly as dietary nitrate concentrations increased. A linear decrease in methane production was also reported by Newbold et al. (2014) where methane production from Holstein steers decreased linearly across 6 treatments (0, 6, 12, 18, 24, and 30 g of NO_3^- /kg of DM) from 13.6 g CH_4 /kg of DMI for the urea treatment, to 9.6 g CH_4 /kg of DMI for the 2 highest nitrate treatments, corresponding to a decline of 29%. In the current study, methane production (L/kg of DMI) was lowered by 10.9% per 1% nitrate inclusion in diet DM and is in agreement with earlier studies performed in dairy cattle. van Zijderveld et al. (2011) and Klop et al. (2016) observed declines in methane production (g/kg of DMI) of 7.6 and 10.0% for nitrate inclusion at 1% of DM, respectively.

Effects on methane production are apparent up to 5 h after feeding (Figure 1b), which is in line with van Zijderveld et al. (2011). When it is reduced in the rumen, nitrate diverts available hydrogen away from methanogenesis. Methane production showed less fluctuation throughout the day with the high nitrate treatment compared with the control treatment. The disappearance of differences in methane production around 5 h after feeding may indicate that nitrate has been metabolized, absorbed, or left the rumen by passage.

Nitrate reduction to ammonia is energetically more favorable than carbon dioxide reduction (Ungerfeld and Kohn, 2006), which is a mechanism by which nitrate lowers methane production. The observed efficiencies were 82.3, 71.9, and 79.4% for the low, medium, and high nitrate diets, respectively, and are somewhat higher than efficiencies of 49% (beef cattle; Newbold et al., 2014) and 59% (dairy cattle; van Zijderveld et al., 2011), and somewhat lower than efficiencies of 87% (beef cattle; Hulshof et al., 2012), 86% (dairy cattle; Lund et al., 2014), and 85% (dairy cattle; Klop et al., 2016). The observed efficiencies in the current study

Table 4. Milk yield, milk composition, and ECM yield per treatment (control: 0 g of NO_3^- /kg of DM; low: 5.3 g of NO_3^- /kg of DM; medium: 13.6 g of NO_3^- /kg of DM; and high: 21.1 g of NO_3^- /kg of DM)

Item	Treatment				SEM	P-value		
	Control	Low	Medium	High		Treatment	Linear	Quadratic
Milk yield (kg/d)	23.1	23.3	24.0	23.9	1.01	0.69	0.33	0.76
Fat (%)	4.30	4.12	4.05	4.19	0.26	0.68	0.63	0.31
Protein (%)	3.55	3.58	3.54	3.60	0.09	0.67	0.57	0.57
Lactose (%)	4.72	4.75	4.71	4.77	0.09	0.13	0.29	0.23
ECM ¹ (kg/d)	24.1	23.7	24.0	24.9	1.56	0.70	0.39	0.42

¹Energy-corrected milk yield (Sjaunja et al., 1991).

did not differ due to a large variation for individual cows within each treatment (low: 57 to 142%; medium: 25 to 125%; high: 35 to 101%). Although animals were fed the same diet, efficiencies of individual cows were lower or higher than 100% irrespective of the nitrate level fed. Assuming other metabolic processes are not changed, then efficiencies below 100% indicate that nitrate reduction to ammonia was incomplete. Efficiencies above 100% suggest that the methane lowering effect of nitrate cannot only be attributed to competition for hydrogen. In the latter case, the efficiency expression will not be an appropriate tool to use. Plotting (data not shown) the efficiency with DMI ($R^2 = 0.16$), methane production per kilogram of DMI ($R^2 = 0.09$), and NDF digestibility ($R^2 = 0.004$) separately did not explain the variation in efficiencies.

Effects of Nitrate on Rumen Fermentation

Hydrogen Emission. The increased hydrogen levels emitted when cows were fed a ration with ad-

ditional nitrate reflects an accumulation of hydrogen in the rumen due to hydrogen being neither converted to methane nor used in nitrate reduction.

Lund et al. (2014) observed an increase from 1 L of H_2 /d for the control to 49.0 L of H_2 /d when unadapted cows were fed 20 g of NO_3^- /kg of DM during 24 h. Increased hydrogen emissions from cattle have also been reported by van Zijderveld et al. (2011; 15.0 L/d) and Troy et al. (2015; 12.7–15.0 L/d). In the present study, higher daily hydrogen emission (35.0 L/d) was observed for similar nitrate levels (21–22 g of NO_3^- /kg of DM) as fed by van Zijderveld et al. (2011) and Troy et al. (2015), but it should be noted that hydrogen emission on the control diet (11.4 L/d) was also higher than those (5.0 L/d and 4.8–5.4 L/d) for the control diets in the studies of van Zijderveld et al. (2011) and Troy et al. (2015), respectively.

The diurnal pattern in hydrogen emissions (Figure 1c) followed the pattern of DMI (Figure 1a) and had higher peaks of hydrogen emissions after feeding for the low and medium treatment compared with the con-

Table 5. Gas exchange and DMI during the 4-d gas measurements¹ per treatment (control: 0 g of NO_3^- /kg of DM; low: 5.3 g of NO_3^- /kg of DM; medium: 13.6 g of NO_3^- /kg of DM; and high: 21.1 g of NO_3^- /kg of DM)

Item	Treatments				SEM	P-value		
	Control	Low	Medium	High		Treatment	Linear	Quadratic
DMI during gas measurements (kg/d)	18.5	18.9	18.5	19.6	0.68	0.64	0.36	0.54
CH_4 (L/d)	491	468	424	396	37.7	0.04	0.01	0.87
CH_4 (L/kg of DMI)	26.5	24.9	23.0	20.3	1.79	<0.001	<0.001	0.57
CH_4 (% of GEI ²)	5.73	5.39	4.93	4.33	0.39	<0.001	<0.001	0.60
CH_4 (L/kg of ECM ³)	20.5	20.0	17.7	15.8	0.65	<0.001	<0.001	0.38
H_2 (L/d)	11.4	27.4	37.8	35.0	7.10	0.01	<0.01	0.03
H_2 (L/kg of DMI)	0.60	1.47	2.02	1.81	0.38	<0.01	<0.01	0.02
CO_2 production (L/d)	5,797	5,910	5,904	6,146	243	0.52	0.18	0.71
O_2 consumption (L/d)	5,142	5,240	5,220	5,366	210	0.63	0.26	0.84
CH_4 : CO_2 ratio	0.085	0.080	0.072	0.065	0.006	0.001	<0.001	0.99
H_2 : CH_4 ratio	0.022	0.058	0.085	0.087	0.012	0.001	<0.001	0.03
Methane efficiency ⁴	—	82.3	71.9	79.4	15.3	0.83	0.87	0.59

¹Gas measurements were taken on d 24 to 28 of each period.

²GEI = gross energy intake.

³Energy-corrected milk yield (Sjaunja et al., 1991).

⁴Methane efficiency = observed reduction in methane production/potential reduction in methane production based on stoichiometry * 100%.

trol. Guyader et al. (2015) observed increased dissolved hydrogen concentrations in the rumen 1 h after the morning feeding and declined after 3 h, which coincided with a drop in methane production. Surprisingly, in the present study at high dietary nitrate concentration the peaks after both feedings were comparable to the control. This cannot be explained by differences in feed intake (Figure 1a), but could indicate that the rumen was saturated with hydrogen, which results in consistently higher level of hydrogen emission in between feedings and less pronounced peaks after feeding.

The question remains: why was methane production lowered in the presence of accumulated levels of hydrogen available for methanogenesis? The direct competition of nitrate reduction with methanogenesis for available hydrogen does not run efficiently at increased hydrogen emission. This suggests that methanogenesis might be inhibited in another way, which is also suggested by literature (Lund et al., 2014; Guyader et al., 2015; Latham et al., 2016). Nitrite, which is an intermediate in nitrate reduction, may accumulate in rumen fluid because the initial reduction of nitrate to nitrite occurs at a higher rate than the subsequent reduction of nitrite to ammonia (Allison and Reddy, 1984). Nitrite was found to be toxic to methanogens (*Methanosarcina barkeri* and *Methanobacterium bryantii*) in rice field soils (Klüber and Conrad, 1998), and in vitro studies suggest that nitrite is toxic for rumen methanogens and therefore can contribute to the lower methane production (Iwamoto et al., 2002; Sar et al., 2005; Božić et al., 2009; Zhou et al., 2011). van Zijderfeld et al. (2010) observed a significant decline in total methanogen numbers (from 9.0 to 8.3 log/mL of rumen content for the control and nitrate diet, respectively), but not in total bacteria or protozoa numbers, when growing lambs were fed nitrate. As part of the current experiment, Petersen et al. (2015) measured a pronounced but temporary accumulation of nitrite in rumen liquid shortly after feeding the medium and high nitrate diets. A concentration of 630 μM was detected 1.5 h after feeding, which declined to 5.3 μM at 3.5 h after the onset of feeding for the high nitrate diet. It should be noted that nitrite was analyzed in rumen liquid only in period 4; thus, results should be interpreted with care. Nevertheless, these observations are in support of our hypothesis that nitrite might exert toxic effects on methanogens, especially immediately after consuming a meal, which might contribute to the decrease in methane production and increase in hydrogen emissions in the first 4 h after a meal. Besides nitrite accumulation, increased N_2O emissions were observed (Petersen et al., 2015).

Besides methane production, the marked increase in hydrogen emission is another source of feed energy

loss. However, the relative energetic benefit of lowering methane is alleviated to only a minor degree by hydrogen emission (i.e., 1.9, 2.8, and 2.9% of the methane loss expressed as a percentage of GEI for the low, medium, and high nitrate diets, respectively). Hydrogen itself is an indirect greenhouse gas with a global warming potential of 5.8 over a 100-yr time horizon (Derwent, 2004).

VFA Concentration. Changes in rumen fermentation and microbial activity are usually reflected in shifting VFA profiles. Linear tendencies to increased total VFA concentrations and butyrate proportions and decreased propionate proportions were observed, despite the lack of an overall treatment effect, and might only explain the increased hydrogen emissions to a small extent. An increase in acetate and decrease in propionate molar proportions upon feeding nitrate were previously observed in cattle (Hulshof et al., 2012; Troy et al., 2015). Further, the amount of hydrogen originating from VFA fermentation that potentially can be emitted (VFA hydrogen ratio) tended to increase linearly with increasing levels of nitrate (Table 2). In contrast, increased hydrogen emissions did not lead to an overall change in molar proportions of acetate and propionate in the ventral rumen, although an interaction between treatment and sampling time for the acetate:propionate ratio indicates an effect of nitrate at specific time points after a meal. This suggests that the excess of hydrogen was not completely incorporated into propiogenesis. Propionate proportions actually tended to decrease linearly with increased nitrate intake, although propiogenesis is considered the thermodynamically favored pathway under high hydrogen pressures (Ellis et al., 2008; Janssen, 2010). Accumulated hydrogen should preferably be directed into alternative sinks, especially those of nutritional importance for the animal such as reductive acetogenesis or propiogenesis (Ungerfeld, 2013). However, based on modeling, Ungerfeld (2013) found that both sinks were unable to incorporate all released hydrogen from inhibited methanogenesis, perhaps because ΔG values of reductive acetogenesis and propiogenesis under high hydrogen pressures are similar to ΔG for ATP generation, making both reactions energetically unfavorable to occur. Therefore, other hydrogen sinks, such as formate and microbial protein or fatty acid synthesis, become more important with increasing methanogenesis inhibition (Ungerfeld, 2015).

Formate can be used by methanogens for methanogenesis (Hungate et al., 1970). It may also function as an electron donor for nitrate reduction to ammonium (Leng, 2008). Addition of nitrate was accompanied by a linear increase in molar proportions of rumen formate in the current study (Table 2), which indicates either an increased formate production or decreased formate

utilization. Formate can be synthesized from hydrogen (Wu et al., 1993; Bleicher and Winter, 1994) or can be formed as an end product of fermentation (Ellis et al., 2008). Hydrogen concentrations increased and formate accumulated in vitro when methanogenesis was inhibited by different methane inhibitors (Wu et al., 1993; Asanuma and Iwamoto, 1998; Asanuma et al., 1999; Ungerfeld et al., 2003). Moreover, formate synthesis by methanogens in the biofilm is stimulated by CH₄ analogs (Bleicher and Winter, 1994), and as argued by Leng (2014), the produced formate may be a mechanism to locally lower the hydrogen pressure in the biofilm as it diffuses from the biofilm to rumen fluid where it is converted to hydrogen and carbon dioxide. In in vitro experiments with *Methanobacterium formicicum*, Wu et al. (1993) observed that high hydrogen pressures suppress the conversion of formate to hydrogen and carbon dioxide and its subsequent conversion to methane. The observed increase of formate in rumen liquid in the present study is most likely related to the combination of increased hydrogen emissions and inhibited methanogens.

Ammonia Concentration. Nitrate and urea are both reduced to ammonia by rumen microbes. Diets were iso-nitrogenous, however, ammonia concentrations in rumen liquid increased, which is in agreement with recent studies (Hulshof et al., 2012; Guyader et al., 2015; Lee et al., 2015b). Lee et al. (2015b) reported a linear increase in ammonia concentrations from 4.39 to 7.32 mmol/L across 7 different levels of encapsulated nitrate intake. The 32% increase in the current study suggests that (assuming rumen volume did not change) either less ammonia disappeared (by passage, absorption through the rumen wall, or assimilation by rumen microorganisms) or more ammonia was produced with increasing dietary nitrate intake. True protein digestibility in the rumen was not affected by nitrate addition and is unlikely to have contributed to the rise in ammonia concentration. Microbial CP production was numerically increased upon feeding nitrate, which would be expected to coincide with decreased rather than increased ammonia concentrations in rumen fluid.

Digestibility, Milk, and Urine

Zhou et al. (2011) reported sodium nitrate to decrease numbers of the major rumen cellulolytic bacteria species (*Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens*), which could cause a decline in NDF digestibility. In lactating dairy cattle, Klop et al. (2016) observed a decreased apparent total-tract digestibility of OM, NDF, and GE when nitrate (21 g/kg of DM) was included in the diet, but digestibility was not affected when nitrate was combined

with docosaehaenoic acid (3 g/kg of DM) in the diet. However, in line with an absence of an effect of nitrate on NDF total-tract digestion found by van Zijderveld et al. (2011), we also found no evidence for decreased NDF digestibility in the rumen, hindgut, and total tract. Nitrate did not seem to interfere with digestion processes, since digestibility coefficients of DM, OM, NDF, and CP in various part of the digestive tract were all unaffected.

Negative NDF digestibility values were observed in the small intestine due to the well-known problems of the use of markers and obtaining representative samples from intestinal cannulas. Chromic oxide may behave differently for fluids and particles and therefore Brask et al. (2013) used chromic oxide and indigestible NDF to determine NDF digestibility. However, similar negative values for NDF digestibility in the small intestine were found for indigestible NDF as a marker.

van Zijderveld et al. (2011) and Klop et al. (2016) did not observe an effect of nitrate supplementation on milk yield and composition, except for decreased milk protein content. In the present study, milk yield and composition were not affected by nitrate supplementation, which is in line with the absence of effects of nitrate on digestibility and total VFA concentration. Responses in milk yield in Latin square designed studies should, however, be interpreted with care due to lack of replication of squares.

Nitrate concentrations in milk and urine were elevated when nitrate was fed (Figure 2a and 2b). Nitrate in urine was determined using a method based on the Griess assay validated for use in milk, and this method has been shown to underestimate the nitrate concentration in human urine due to interfering substances (Tsikas et al., 1997). This underestimation seems to be more pronounced at higher nitrate levels (Tsikas et al., 1997). Thus absolute values should be interpreted with care, but the observed differences between treatments could be regarded as valid.

Davison et al. (1964) observed nitrate concentrations in milk of 5, 9, and 15 mg/L when Holstein cows were fed 0, 440, or 660 mg of NO₃⁻/kg of BW per d. In the present study, on average only 0.012, 0.013, and 0.011% of the daily dietary nitrate intake was excreted in milk as either nitrate or nitrite for the low, medium, and high nitrate treatments, respectively. Thus, negligible amounts of nitrate and nitrite were excreted in milk compared with nitrate intake. The excreted concentrations were well below the drink water guidelines (nitrate <50 mg/L) from WHO (2011) and thus can be regarded as safe for human consumption. Higher nitrate concentrations were found in urine compared with milk, which is in agreement with Sebaugh et al. (1970) who reported that cows adapted to 5% potassium nitrate in the diet

and fed slightly above energy requirement excreted 2.5 mg of NO_3^-/L in milk and 75.2 mg of NO_3^-/L in urine. In the present study, nitrate concentrations in urine were elevated, whereas urinary nitrite and total urinary nitrogen concentrations were unaffected. Lee et al. (2015a) reported that urinary nitrate-nitrogen was only 0.21% of total urinary nitrogen when beef cattle were fed with encapsulated nitrate at 2% of DM, which is in agreement with our findings (data not shown). The total urine excretion of nitrate and nitrite could not be calculated because total collection of urine was not performed, but Setchell and Williams (1962) found that on average 5.1, 3.3, and 8.3% of the 5, 10, and 15 g of sodium nitrate given to ewes per os, respectively, was recovered as nitrate in urine. Recovery of nitrate in milk and urine indicates incomplete metabolism of nitrate to ammonia in the rumen and part of the nitrate intake was absorbed into the blood contributing to the below 100% efficiencies of lowering methane production.

Methemoglobin Levels

Methemoglobin levels in blood increased with increasing dietary nitrate intake (Figure 2c) and varied throughout the day with highest values observed 1.5 h after morning feeding. The maximum value from all individual observations for MetHb was 4.8% of Hb. Despite an increase, MetHb levels in blood were below the level for clinical signs of methemoglobinemia (30 to 40% MetHb; Bruning-Fann and Kaneene, 1993). The increase in MetHb is caused by the absorption of nitrite from the rumen into blood because of incomplete reduction of nitrate to ammonia (Bruning-Fann and Kaneene, 1993). The reaction of nitrite and Hb in erythrocytes induces the conversion of ferrous iron (Fe^{2+}) into ferric iron (Fe^{3+}), thereby forming MetHb (Takahashi and Young, 1991) and decreases the oxygen-carrying capacity of erythrocytes (Takahashi et al., 1998). Petersen et al. (2015) observed increased nitrite concentrations in rumen liquid after feeding, which explains higher MetHb levels after feeding.

The results agree with other recent studies which also found that feeding nitrate can be possible without the occurrence of methemoglobinemia (van Zijderveld et al., 2010, 2011; Li et al., 2012; El-Zaiat et al., 2014; Klop et al., 2016). Sufficient time for adaptation of animals to nitrate enables rumen microbes to increase the rate of nitrate and nitrite reduction together with a quantitative increase in and selection toward nitrate reducing bacteria, which has been demonstrated in vitro (Allison and Reddy, 1984). Our 6-d increase in dietary nitrate concentration for the high treatment did not lead to methemoglobinemia and seems to be of appropriate duration.

Implications

Nitrate as a methane mitigation strategy is effective and promising. Further studies are required to determine to which extent methane is lowered by competition for hydrogen and to which extent by toxicity on rumen microbes. Efforts could also be made to characterize the rumen microbiota when nitrate is fed to possibly explain variation in methane efficiencies among cows. Also, the increase in hydrogen emissions when methane is mitigated needs further study.

CONCLUSIONS

Nitrate linearly decreased methane production and quadratically increased hydrogen emission from dairy cows without substantial effects on rumen fermentation and blood MetHb levels, and no effects on rumen and total-tract nutrient digestibility and rumen microbial protein synthesis. Overall, nitrate addition seems to be a promising methane mitigation strategy, although increased nitrogen pollution if the nitrate is added on top of a diet already sufficient in protein should be taken into account.

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