Persistency of methane mitigation by dietary nitrate supplementation in dairy cows

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ABSTRACT

Feeding nitrate to dairy cows may lower ruminal methane production by competing for reducing equivalents with methanogenesis. Twenty lactating Holstein-Friesian dairy cows (33.2 ± 6.0 kg of milk/d; 104 ± 58 d in milk at the start of the experiment) were fed a total mixed ration (corn silage-based; forage to concentrate ratio 66:34), containing either a dietary urea or a dietary nitrate source [21 g of nitrate/kg of dry matter (DM)] during 4 successive 24-d periods, to assess the methane-mitigating potential of dietary nitrate and its persistency. The study was conducted as paired comparisons in a randomized design with repeated measurements. Cows were blocked by parity, lactation stage, and milk production at the start of the experiment. A 4-wk adaptation period allowed the rumen microbes to adapt to dietary urea and nitrate. Diets were isoenergetic and isonitrogenous. Methane production, energy balance, and diet digestibility were measured in open-circuit indirect calorimetry chambers. Cows were limit-fed during measurements. Nitrate persistently decreased methane production by 16%, whether expressed in grams per day, grams per kilogram of dry matter intake (DMI), or as percentage of gross energy intake, which was sustained for the full experimental period (mean 368 vs. 310 ± 12.5 g/d; 19.4 vs. 16.2 ± 0.47 g/kg of DMI; 5.9 vs. $4.9 \pm 0.15\%$ of gross energy intake for urea vs. nitrate, respectively). This decrease was smaller than the stoichiometrical methane mitigation potential of nitrate (full potential = 28% methane reduction). The decreased energy loss from methane resulted in an improved conversion of dietary energy intake into metabolizable energy (57.3 vs. $58.6 \pm 0.70\%$, urea vs. nitrate, respectively). Despite this, milk energy output or energy retention was not affected by dietary nitrate. Nitrate did not affect milk yield or apparent digestibility of crude fat, neutral detergent fiber, and starch. Milk protein content (3.21 vs. $3.05 \pm 0.058\%$, urea vs. nitrate respectively) but not protein yield was

lower for dietary nitrate. Hydrogen production between morning and afternoon milking was measured during the last experimental period. Cows fed nitrate emitted more hydrogen. Cows fed nitrate displayed higher blood methemoglobin levels (0.5 vs. $4.0 \pm 1.07\%$ of hemoglobin, urea vs. nitrate respectively) and lower hemoglobin levels (7.1 vs. 6.3 ± 0.11 mmol/L, urea vs. nitrate respectively). Dietary nitrate persistently decreased methane production from lactating dairy cows fed restricted amounts of feed, but the reduction in energy losses did not improve milk production or energy balance.

Key words: methane, nitrate, dairy cow

INTRODUCTION

The production of ruminant meat and milk is associated with a relatively high production of greenhouse gases (GHG) compared with other food commodities (Williams et al., 2008). In a recent publication, the contribution of the dairy sector to the global production of greenhouse gases was estimated to be 2.7% (FAO, 2010). This high level of GHG production is mainly related to the anaerobic fermentation of fiber-rich feed-stuffs in the gastrointestinal tract of ruminants. Methane production from enteric fermentation accounts for 52% of the carbon footprint of milk, expressed in CO₂-equivalents, at the farm gate (FAO, 2010). A decrease in the amount of enteric methane could substantially decrease the amount of GHG associated with milk production. Although many dietary strategies have been proposed to decrease methane production from ruminants (Martin et al., 2010), few have shown a persistent decrease of methane production in vivo. Persistency of the methane-decreasing effect is an absolute requirement for any dietary strategy to be successful in abating GHG emissions from ruminants.

Most methane production during rumen fermentation is the result of the reduction of CO₂ with H₂ by methanogenic *Archaea* residing in the rumen. This process enables the removal of excess H₂ from the rumen and allows NADH to be reconstituted to NAD⁺, a process essential to the continuation of anaerobic rumen fermentation and microbial growth (Wolin, 1975).

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Anaerobic nitrate reduction is energetically more favorable than CO₂ reduction, and the presence of nitrate in the rumen redirects H₂ from methanogenesis to nitrate reduction, thereby decreasing methane production (Allison and Reddy, 1984). However, the sudden introduction of nitrate into ruminant diets may lead to the occurrence of methemoglobinemia, a condition caused by the oxidation of the ferric iron in hemoglobin, rendering the molecule incapable of oxygen transport. The oxidation of hemoglobin is caused by the presence of nitrite, an intermediate in nitrate reduction, in blood. Gradual introduction of nitrate into the diet can allow the rumen microbes to adapt and increase their capability to reduce nitrite (Alaboudi and Jones, 1985). van Zijderveld et al. (2010), using a 4-wk adaptation period to dietary nitrate, demonstrated decreased methane emissions (−32%) in sheep fed nitrate, while methemoglobinemia was not observed. Similarly Nolan et al. (2010) used an 18-d adaptation period and observed a 23% decrease in methane emissions in sheep fed nitrate, again without the occurrence of methemoglobinemia. The effect of feeding nitrate to dairy cows on enteric methane production has not yet been investigated. In addition, it is unknown if the methane-depressing effect of nitrate persists over time.

This experiment was designed to investigate to what extent dietary nitrate can decrease enteric methane production in lactating dairy cows and if this would affect animal productivity, feed digestibility, or energy balance. After a 4-wk adaptation period, methane production was measured in 4 consecutive periods with 24-d intervals to investigate if the methane decrease caused by dietary nitrate was persistent.

MATERIALS AND METHODS

Experimental Design

The Animal Care and Use Committee of Wageningen University (Wageningen, the Netherlands) approved the experimental protocol. The experiment was designed as a completely randomized block with repeated measurements. Twenty Holstein-Friesian dairy cows were blocked according to parity, lactation stage, and milk production at the start of the experiment and subsequently within a block randomly allocated to 1 of 2 diets. One diet (hereafter, the nitrate diet) contained a nitrate source (Calcinit, Yara, Oslo, Norway) which was replaced by urea, on an isonitrogenous basis, in the other diet (the urea diet).

Animals and Housing

The experiment was conducted at the experimental dairy farm of Wageningen University and Research

(Wageningen, the Netherlands). The experimental group included 20 lactating Holstein-Friesian dairy cows (initial milk production 33.2 ± 6.0 kg; 104 ± 58.0 DIM). During a 4-wk adaptation period, cows were housed in a freestall dairy barn in 2 separate treatment groups. The adaptation period served to introduce the experimental concentrates gradually into the diet.

After adaptation to the diet, cows were subjected to a 17-d experimental period. During this period, cows were housed in tie-stalls for 12 d and subsequently moved to respiration chambers for 5 d. In the tie-stalls, animals were accustomed to be restricted in movement, and individual feed intake was measured. In the respiration chambers, gaseous exchange, individual feed intake, and feed digestibility were determined. The 17-d experimental period was replicated 4 times for each pair of animals with 24-d intervals between measurements to evaluate persistency of effects. During the 7 d that cows were not in the tie-stalls, they were housed in a freestall dairy barn, but were maintained in their treatment groups and fed their respective treatment diets. In this publication, d 1 refers to the first day cows were housed in the tie-stalls. Only 2 respiration chambers were available for implementation of this experiment, which limited the number of animals that could be measured at the same time to 4 (2 on each treatment; 2 cows per chamber), and the groups of cows had to be allocated to the chambers in a staggered manner. Because of this allocation, the first group of cows completed the experiment after 89 d on the full nitrate diet, whereas the last group of cows had been on the full nitrate diet for 107 d.

Diets and Feeding

Both diets consisted of 53% corn silage, 9% dried alfalfa, 4% barley straw, and 34% concentrates on a DM basis. The diets were offered as TMR and were prepared on a daily basis using a mixer wagon (Vertimix 500, Strautmann, Bad Laer, Germany). The concentrates for the urea and nitrate diets were balanced for N and Ca by the isonitrogenous exchange of urea for nitrate and the addition of limestone to the urea diet concentrate, respectively. Before the start of the adaptation period, the concentrate portion of the diet consisted of adaptation concentrates (Table 1). At the start of the adaptation period, 25% of the adaptation concentrates were replaced by 1 of the 2 experimental concentrates (urea or nitrate) for each treatment.

This ration was fed for 1 wk, after which the proportion of experimental concentrates was increased to 50% of the total amount of concentrates. Dietary proportions of experimental concentrates were increased by 25% in each additional week of the adaptation period,

Table 1. Ingredient composition (% of DM) of concentrates containing either urea or nitrate as NPN source

Item	Adaptation	Urea	Nitrate
Formaldehyde-treated soybean meal	—	31.8	31.8
Soybean meal	51.3	0.0	0.0
Wheat	6.3	19.0	19.0
Corn	12.8	12.7	12.7
Dried beet pulp	20.1	13.3	13.3
Fractionated palm oil	3.7	7.3	7.3
Trace mineral and vitamin premix	2.2	2.2	2.2
Monocalcium phosphate	1.5	2.0	2.0
MgSO ₄	—	1.5	1.5
NaCl	0.7	1.5	1.5
Urea	1.5	3.5	—
Wood cellulose	—	0.3	—
CaCO ₃	—	5.1	—
Nitrate source ¹	—	—	8.8

¹5Ca(NO₃)₂·NH₄NO₃·10H₂O; 75% NO₃ in DM.

until the concentrate proportion of the diets consisted fully of experimental concentrates after 3 wk. A fourth week was added to the adaptation period when the full amount of experimental concentrates was fed. The 4-wk period served to allow adaptation of the rumen microflora and to allow sufficient time for increased activity of bacterial nitrate and nitrite reductases (Alaboudi and Jones, 1985).

Cows were group-fed during the adaptation period. Cows were fed once daily during the period in the freestall barn (0900 h) and twice daily (0630 and 1630 h; equal portions) during each 17-d experimental period. Water was freely available during the entire experiment.

In the tie-stalls and respiration chambers cows were individually fed. Orts were collected daily. Cows were fed ad libitum for the first 8 d of the experimental period and subsequently restricted in feed intake for the remainder of the 17-d experimental period. Within a block, feed intake was restricted to 95% of the ad libitum intake of the animal consuming the lowest amount of feed during d 5 to 8. This approach was chosen to ensure similar feed intake between treatments, thus avoiding confounding effects of DMI on methane production.

Sampling and Chemical Analysis

Representative samples of TMR (± 500 g) were collected at each preparation of fresh TMR and stored frozen (-20°C) pending analysis. Samples were thawed, pooled per period and treatment, subsampled (± 500 g), freeze-dried, and ground to pass a 1-mm screen before analysis. Individual roughage and concentrate samples were taken on d 10 of each period and stored frozen. After the experiment, corn silage, straw, and alfalfa samples were analyzed by near infrared spectroscopy

(Blgg, Oosterbeek, the Netherlands). Samples of dried alfalfa and straw were pooled by roughage type over the entire experiment and subsampled before analysis (1 sample for each roughage type); corn silage samples were analyzed individually per measuring period. The total production of feces and urine was collected after completion of each measurement period, mixed thoroughly, and subsampled for analysis. Dry matter, CP, crude fat, sugar, starch, and NDF content of TMR, concentrate, and manure samples were determined according to the methods described in detail in Abrahamse et al. (2008). Gross energy (**GE**) content was determined using bomb calorimetry (IKA-C700, Janke & Kunkel, Heitersheim, Germany). Nitrate was determined as described previously by van Zijderfeld et al. (2010).

Gaseous Exchange and Diet Digestibility

The respiration indirect calorimetric chambers used in this study have been described in detail by Verstegen et al. (1987). Measurements of gaseous exchange and diet digestibility were performed as described previously by van Zijderfeld et al. (2011). Heat production was calculated according to the methods of Brouwer (1965). Cows were housed in pairs on the same treatment in the respiration chambers.

Determination of Hydrogen Production

Hydrogen production was determined in period 4 of this experiment between 0630 and 1530 h. Air samples were taken manually from the in- and outgoing air from the respiration chambers by means of a syringe (2–3 samples/h). The syringe was injected into a gas chromatograph (Quintron Breathtracker DP, Quintron Instrument Company, Milwaukee, WI) within approxi-

mately 10 min after the sample was taken. Hydrogen production was subsequently calculated by multiplication of the H_2 concentrations by the measured airflow rate through the chambers.

Milk Yield and Composition

Cows were milked twice daily during the entire experiment (0630 and 1630 h). Milk yield was determined daily. In the respiration chamber, milk was sampled during each milking and stored in a refrigerator at 4°C in tubes containing sodium azide. These samples were analyzed for fat, CP, lactose, urea, and SCC as described previously by van Zijderveld et al. (2011) at the end of each experimental period. Average milk component concentrations during the measurement period were calculated from the weighted average of all samples taken. At each milking, additional samples (3 g/kg of milk) were taken for GE determination.

Blood Sampling

In the respiration chambers, blood was sampled on the third day of each measurement period. Blood was sampled from the tail vein at 3 h postfeeding in heparinized collection tubes (Becton Dickinson, Breda, the Netherlands). Blood was analyzed for hemoglobin (**Hb**) and methemoglobin (**MetHb**) contents within 1 h after collection with a hemoximeter (OSM 3, Radiometer, Copenhagen, Denmark).

Statistical Analyses

Data collected during the measurement period only were used for statistical analyses. Data on gaseous emissions from the last 72 h of each measurement period were averaged per period before analysis. Data measured on individual animals (milk production, milk

composition, blood parameters, and DMI) were subjected to repeated-measures ANOVA including block and treatment as fixed factors. Data collected during the 4 experimental periods were treated as repeated measures per animal or pair of animals. Data collected on pairs of cows (gaseous exchange and diet digestibility parameters) were subjected to repeated-measures ANOVA with treatment as fixed factor. Significance of effects was declared at $P < 0.05$. The statistical program Genstat (11th ed., Lawes Agricultural Trust, Rothamsted, UK) was used to analyze the data.

RESULTS

Diet Composition

Chemical composition of dietary concentrates was established as formulated (Table 2); complete diets were isoenergetic (on a GE basis) and isonitrogenous.

Animal Performance and Methane Production

Methane production was reduced by 16% on the nitrate diet (Table 3) when expressed on a daily basis, as grams per kilogram of DMI or as percentage of gross energy intake (**GEI**). When expressed per kilogram of milk, methane was decreased by 14%. Daily methane production increased with time during the experiment for both treatments. No significant interaction was observed between treatment and time.

Dry matter intake was not affected by treatment, but increased with time for both treatments. Milk yield was unaffected by treatment, but decreased in time as cows advanced in lactation. Protein content of milk was lower for the nitrate diet (3.21 vs. $3.05 \pm 0.058\%$, urea vs. nitrate respectively), but milk protein yield was unaffected by treatment. Other milk constituents were unaffected by treatment or time.

Table 2. Analyzed chemical composition of TMR ingredients and complete TMR containing either a urea or nitrate source

Item	TMR ingredient						TMR Urea	TMR Nitrate
	Corn silage	Alfalfa	Barley straw	Concentrates				
				Urea	Nitrate			
Inclusion (% of DM)	53	9	4	34	34			
Gross energy (MJ/kg of DM)	NA ¹	NA	NA	NA	NA	18.7	18.7	
DM (g/kg)	365	881	894	899	884	520	523	
Crude ash (g/kg of DM)	42	67	67	133	134	77	76	
CP (g/kg of DM)	74	114	28	318	317	156	156	
Crude fat (g/kg of DM)	32	NA	NA	96	92	44	43	
NDF (g/kg of DM)	402	531	783	112	116	329	325	
Starch (g/kg of DM)	327	NA	NA	259	259	234	236	
Nitrate (g/kg of DM)	0	0	0	0	63	0	21	

¹NA = not analyzed.

Energy Balance, Nitrogen Balance, and Digestibility

Gross energy intake was not affected by treatment, but increased as the experiment progressed (Table 4). Energy lost in methane production was lower for the nitrate diet and increased during the experiment for both treatments. The energy lost in urine and feces and ME intake (MEI) were unaffected by treatment. Although MEI was not affected by treatment, the MEI:GEI ratio was elevated as a consequence of nitrate feeding (57.3 vs. 58.6 ± 0.70%, urea vs. nitrate, respectively). Calculated heat production was unaffected by treatment, but increased over time. Milk energy yield was unaffected by treatment or time. Despite the higher MEI:GEI ratio, energy retention (ER) was not increased for the nitrate treatment. Apparent total-tract digestibility of NDF, starch, and crude fat were unaffected by treatment.

Nitrogen balance (Table 5) was mostly positive for both treatments and no differences between treatments were observed. Nitrogen intake increased as the experiment progressed. The conversion efficiency of feed N into milk N was not affected by treatment but decreased with time for both treatments.

Hydrogen Production

Cumulative hydrogen production was higher for the nitrate diet during the 9-h measurement period in experimental period 4 (1.9 L/cow per 9 h for urea vs. 5.8 L/cow per 9 h for nitrate; P = 0.003). Hydrogen production was higher up to 7 h after feeding for the nitrate diet (Figure 1). The variability in hydrogen production was much larger for pairs of animals on the nitrate treatment than for those on the urea treatment. Methane production was suppressed for a period of 5 h after feeding with nitrate, and this depression in methane production largely coincided with the increase in hydrogen production for this treatment.

Blood Parameters

Methemoglobin content of blood was consistently higher for cows on the nitrate diet (Table 6) and was not affected by time on treatment. Hemoglobin content was lower for the nitrate-fed animals and increased with time for both treatments.

DISCUSSION

Methane Abatement by Dietary Nitrate

In this research, we demonstrated for the first time that nitrate decreases methane production persistently

Table 3. Dry matter intake, milk production, milk composition, and methane production of limit-fed dairy cows fed either a urea source or a nitrate source

Item	d 13 – d 17				d 37 – d 41				d 61 – d 65				d 85 – d 89				Pooled		P-value ¹	
	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate	SEM	Trt	Time	Trt × time
DMI (kg/d)	17.9	18.0	19.1	19.7	19.4	19.2	19.4	19.2	19.4	19.2	19.7	19.7	19.7	19.7	19.7	19.7	0.76	0.877	<0.001	0.402
Milk production (kg/d)	27.9	28.3	27.4	28.3	26.2	26.6	26.2	26.6	26.2	26.6	25.5	26.6	25.5	26.6	25.5	26.6	0.87	0.452	0.009	0.877
FPCM ² (kg/d)	28.1	27.7	27.9	27.9	26.6	26.4	26.6	26.4	26.6	26.4	25.7	26.4	25.7	26.4	25.7	26.4	0.91	0.990	0.020	0.814
Fat (%)	4.27	4.02	4.28	4.02	4.24	4.07	4.24	4.07	4.24	4.07	4.19	4.04	4.19	4.04	4.19	4.04	0.172	0.353	0.947	0.900
Protein (%)	3.09	2.95	3.24	3.05	3.25	3.09	3.25	3.09	3.25	3.09	3.24	3.09	3.24	3.09	3.24	3.09	0.058	0.041	0.001	0.902
Fat (g/d)	1,165	1,133	1,152	1,132	1,097	1,079	1,097	1,079	1,097	1,079	1,052	1,077	1,052	1,077	1,052	1,077	71.6	0.852	0.050	0.782
Protein (g/d)	860	834	884	864	848	821	848	821	848	821	821	823	821	823	821	823	27.4	0.547	0.138	0.861
Lactose (%)	4.47	4.48	4.47	4.53	4.46	4.53	4.46	4.53	4.46	4.53	4.46	4.49	4.46	4.49	4.46	4.49	0.058	0.623	0.285	0.311
SCC (×1,000 cells/mL)	82	120	231	108	94	108	94	108	94	108	75	111	75	111	75	111	40.7	0.799	0.202	0.172
MUN (mg/dL)	12.1	12.4	12.0	11.9	13.2	12.3	13.2	12.3	13.2	12.3	12.2	12.3	12.2	12.3	12.2	12.3	0.49	0.735	0.176	0.334
CH ₄ (g/cow per day)	341	283	371	313	378	318	378	318	378	318	383	326	383	326	383	326	12.5	0.009	<0.001	0.961
CH ₄ (g/kg of DMI)	19.1	15.8	19.6	15.9	19.5	16.6	19.5	16.6	19.5	16.6	19.5	16.5	19.5	16.5	19.5	16.5	0.47	<0.001	0.041	0.313
CH ₄ (g/kg of milk)	11.8	10.4	13.3	11.5	13.9	11.9	13.9	11.9	13.9	11.9	15.0	12.4	15.0	12.4	15.0	12.4	0.52	0.010	<0.001	0.449
CH ₄ (% of GEI ³)	5.7	4.7	5.7	4.7	6.1	5.1	6.1	5.1	6.1	5.1	6.1	5.1	6.1	5.1	6.1	5.1	0.15	<0.001	<0.001	0.817

¹Trt = treatment.
²Fat- and protein-corrected milk.
³Gross energy intake.

Table 4. Energy balance and apparent total-tract diet digestibility of limit-fed dairy cows fed either a urea source or a nitrate source

Item	d 13 – d 17		d 37 – d 41		d 61 – d 65		d 85 – d 89		Pooled SEM	<i>P</i> -value ¹		
	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate		Trt	Time	Trt × time
Metabolic BW (kg/cow)	117	120	118	121	122	118	121	122	3.2	0.912	0.273	0.132
GEI ² (kJ/kg of BW ^{0.75} per day)	2,831	2,789	3,033	3,060	2,834	2,930	2,904	2,887	75.5	0.863	0.002	0.433
Energy feces + urine (kJ/kg of BW ^{0.75} per day)	1,023	1,012	1,108	1,112	1,086	1,077	1,058	1,053	35.2	0.904	0.003	0.983
Methane production (kJ/kg of BW ^{0.75} per day)	161	131	174	143	172	149	176	148	5.0	0.002	<0.001	0.425
MEI ³ (kJ/kg of BW ^{0.75} per day)	1,647	1,645	1,750	1,805	1,577	1,704	1,670	1,686	49.0	0.375	0.015	0.332
MEI:GEI ratio (%)	58.2	59.0	57.7	59.0	55.6	58.2	57.5	58.3	0.70	0.036	0.118	0.498
Heat production (kJ/kg of BW ^{0.75} per day)	951	962	989	998	975	987	987	991	17.9	0.713	<0.001	0.929
Energy in milk (kJ/kg of BW ^{0.75} per day)	704	710	702	700	715	691	656	697	18.1	0.699	0.269	0.290
ER ⁴ total (kJ/kg of BW ^{0.75} per day)	–9	–27	60	107	–114	26	29	–3	49.9	0.433	0.105	0.275
ER protein ⁴ (kJ/kg of BW ^{0.75} per day)	0	22	8	17	11	22	28	33	11.6	0.247	0.287	0.799
ER fat ⁴ (kJ/kg of BW ^{0.75} per day)	–9	–48	52	90	–124	4	1	–36	43.1	0.558	0.051	0.180
NDF digestibility (%)	52.1	52.7	52.6	53.9	52.1	53.9	53.3	52.7	1.09	0.364	0.846	0.672
Starch digestibility (%)	97.3	96.6	98.0	98.5	97.5	98.7	98.4	98.5	0.28	0.217	0.002	0.053
Fat digestibility (%)	70.2	71.8	60.4	66.3	63.1	57.9	64.8	59.6	3.19	0.791	0.027	0.217

¹Trt = treatment.²GEI = gross energy intake.³MEI = ME intake; MEI = GEI – energy feces + urine – methane production.⁴ER = energy retention. ER total = MEI – heat production – energy in milk; ER protein = protein gain × 23.6 kJ/g of protein; ER fat = ER total – ER protein.**Table 5.** Nitrogen balance (mg/kg of BW^{0.75} per day) and N efficiency (%) of limit-fed dairy cows fed either a urea source or a nitrate source

Item	d 13 – d 17		d 37 – d 41		d 61 – d 65		d 85 – d 89		Pooled SEM	<i>P</i> -value ¹		
	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate		Trt	Time	Trt × time
N intake	3,814	3,829	3,974	4,037	4,032	3,981	4,050	4,064	103.2	0.935	0.024	0.819
N urine + feces	2,573	2,509	2,707	2,734	2,746	2,680	2,749	2,680	80.2	0.660	0.009	0.729
N milk	1,148	1,080	1,154	1,122	1,145	1,078	1,052	1,091	28	0.107	0.189	0.242
N condens ²	92	95	60	66	70	74	61	65	21.6	0.794	0.360	0.978
N balance	1	146	53	114	72	149	189	226	78.7	0.247	0.287	0.799
N efficiency (N milk/N feed)	30.8	28.9	29.6	28.5	29.2	27.7	26.6	27.4	0.98	0.267	0.073	0.450

¹Trt = treatment.²N in condensate that was collected from the heat exchanger in the respiration chamber.

Figure 1. Ruminal hydrogen and methane production over a 9-h period from lactating dairy cows fed either a urea or a nitrate source. Data represent mean values obtained during experimental period 4 of the main experiment. Error bars indicate SEM. Arrow indicates time of feeding. Symbols indicate whether the treatment effect was significant for the respective time point: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; + $P < 0.10$; ns = not significant.

in dairy cows. The methane decrease confirms earlier observations in nitrate-fed sheep (Nolan et al., 2010; van Zijderfeld et al., 2010) or sheep ruminally infused with nitrate solutions (Takahashi and Young, 1991; Sar et al., 2004). We hypothesized that reduction of nitrate to nitrite and then ammonia would provide a sink for metabolic hydrogen, thus decreasing its availability for methanogenesis. Nitrate reduction is energetically more favorable than methanogenesis (Ungerfeld and Kohn, 2006). Cows in this experiment consumed, on average, 21 g of nitrate/kg of DM. Complete reduction of this nitrate to ammonia would consume sufficient hydrogen to decrease methane emissions by 5.4 g of methane/kg of DM (25.8 g of CH_4 reduction/100 g of nitrate fed; van Zijderfeld et al., 2010). The decrease in methane production was only 3.2 g of methane/kg of DM or 59% of this theoretical potential. This could indicate that nitrate was not fully reduced to ammonia, using only part of the theoretical amount of hydrogen required compared with a situation in which full reduction would have occurred. Considering that hydrogen losses also increased with the nitrate diet, the actual efficiency of nitrate reduction in abating methanogenesis would be even lower.

Cows in both treatment groups were fed a diet containing 156 g of CP/kg of DM. Twenty-two percent of this CP consisted of NPN, originating from either urea or nitrate. Rumen microbes require a minimum level of rumen ammonia to maintain optimal growth conditions (Dijkstra et al., 1998). If 44% of the nitrate would not

have been reduced to ammonia, this would theoretically have lowered the available CP content of the nitrate diet by 15 g/kg of DM, mainly limiting availability of rumen-available N. When calculated according to the Dutch protein evaluation (DVE) system, the decreased availability of N from nitrate would have resulted in a negative rumen available N balance, which could be expected to have a negative effect on microbial protein synthesis and dairy cow productivity. Milk CP concentration was lower for the nitrate diet; however, daily milk protein production was unaffected by treatment and the lower protein concentration may have been a consequence of dilution. In addition, the efficiency with which N was used for milk protein synthesis was unaffected by treatment. Moreover, MUN did not differ between treatments; if ammonia levels in the rumen were lower because of incomplete reduction of nitrate, this probably would have been reflected in decreased MUN levels (Gustafsson and Palmquist, 1993). It therefore seems unlikely that incomplete nitrate reduction was responsible for the lower relative effectiveness of nitrate in dairy cows.

Methanogenesis is not the only sink for hydrogen in the rumen. Hydrogen is also used for the production of the more reduced acids propionate and valerate (Czerkawski, 1972), and increases in molar proportions of propionate in rumen fluid are negatively associated with methanogenesis (Ellis et al., 2008; Janssen, 2010). Nitrate reduction is thermodynamically more favorable than propionogenesis (Ungerfeld and Kohn, 2006) and

Table 6. Hemoglobin (Hb) and methemoglobin (MetHb) levels of limit fed dairy cows fed either a urea source or a nitrate source

Item	d 13 – d 17			d 37 – d 41			d 61 – d 65			d 85 – d 89			P-value ¹		
	Urea	Nitrate		Urea	Nitrate		Urea	Nitrate		Urea	Nitrate		Pooled SEM	Trt	Time
Hb (mmol/L)	6.9	6.1		7.0	6.3		7.4	6.3		7.2	6.5		0.11	<0.001	0.003
MetHb (% of Hb)	0.4	4.2		0.4	3.6		0.5	3.5		0.6	4.7		1.07	0.008	0.814
MetHb minimum (% of Hb)	0.1	0.6		0.2	0.6		0.2	0.7		0.5	0.7		—	—	—
MetHb maximum (% of Hb)	0.8	14.5		0.6	19.0		0.7	7.9		0.8	15.3		—	—	—

¹Trt = treatment.

may successfully compete for hydrogen with propionogenesis. This would mean that nitrate reduction draws electrons from both methanogenesis and propionogenesis. Indeed, it has been demonstrated that feeding nitrate at 2% of DM to dairy cows increases the molar proportion of acetate, at the expense of propionate and butyrate (Farra and Satter, 1971), and such a shift will decrease the amount of hydrogen consumed in propionogenesis (Ellis et al., 2008). The efficacy of nitrate as a methane inhibitor may therefore depend on the relative importance of propionogenesis in the host animal.

In sheep, the apparent efficiency of nitrate use in methane mitigation was much higher (78%, Nolan et al., 2010; 89%, van Zijderveld et al., 2010) than in cattle in the present experiment (59%). Sheep have a much lower feed intake relative to their body weight and a higher rumen pH; in that situation, relatively less propionic acid and more acetic acid and butyric acid are produced (Bannink et al., 2008). Therefore, propionogenesis might play a smaller role in sheep compared with lactating cows and nitrate reduction might obtain more of its reducing equivalents in competition with propionogenesis in dairy cows and more in competition with methanogenesis in sheep. This would imply that nitrate is more effective in decreasing methane emissions in animals fed near maintenance, with a relatively minor role for propionogenesis.

It is likely that part of the ingested nitrate and part of the nitrite formed in the rumen were absorbed through the rumen wall into the blood and subsequently lost in urine (Takahashi et al., 1998) and thus unavailable for reduction to ammonia in the rumen. Clearance of nitrate and nitrite to blood might therefore be an additional explanation for the lower than expected inhibition of methanogenesis. In previous studies with sheep (Nolan et al., 2010; van Zijderveld et al., 2010), the dietary inclusion of nitrate was higher than that in the current study (24 and 26 g of nitrate/kg of DM in the respective sheep studies compared with 21 g of nitrate/kg of DM in the current study). However, when expressed relative to metabolic BW, the nitrate dose in the current experiment was considerably higher than in our previous study with sheep (3.4 g of nitrate/kg of BW^{0.75} per day vs. 1.6 g of nitrate/kg of BW^{0.75} per day for dairy cows and sheep, respectively). This higher dose may have led to a higher proportion of the dietary nitrate entering the blood through the rumen wall and being lost in urine. This could be another explanation for the relatively higher efficiency of methane abatement with dietary nitrate in sheep.

Cows on the nitrate diet had elevated blood MetHb levels. This indicates that a pool of nitrite, absorbed from the rumen, was present in the blood. In sheep studies (Nolan et al., 2010; van Zijderveld et al., 2010),

no significant elevations of MetHb were observed when similar levels of dietary nitrate on diet DM basis were fed. This supports the above-mentioned hypothesis that larger amounts of nitrate and nitrite are transferred from the rumen to the blood when higher doses, expressed per kilogram of metabolic weight, are fed.

Methane is a loss of dietary energy for the dairy cow (Johnson and Johnson, 1995), and the capture of lost hydrogen may be a way to enhance the energetic efficiency of the cow. In our experiment, the reducing equivalents that would normally have been lost in methane production are assumed to have been taken up during nitrate reduction to ammonia. This might have benefited animal productivity if ruminal ammonia concentrations had been limiting animal production. This is unlikely, however; dietary CP levels were adequate on both treatments, and MUN levels suggest that dietary N was not limiting productivity. Moreover, nitrate reduction may have even drawn reducing equivalents away from propionogenesis in our experiment. Propionate may be a nutrient limiting animal productivity (Huhtanen et al., 1998) and the redirection of reducing equivalents into ammonia rather than propionate may even be more limiting to animal productivity. In the present experiment, although methane production was decreased and the ratio of MEI to GEI increased, energy in milk or energy retention was not affected by nitrate supplementation.

Occurrence of Methemoglobinemia

Methemoglobinemia occurs when nitrite is absorbed from the rumen into the blood of the animal. Ferrous iron (Fe^{2+}) in hemoglobin is transformed into ferric iron (Fe^{3+}), rendering the hemoglobin molecule (now called methemoglobin) incapable of transporting oxygen to the tissues (Ozmen et al., 2005). Nitrite is formed in the rumen as an intermediate in the reduction of nitrate to ammonia. In animals not previously adapted to nitrate in their diet, the reduction of nitrate to nitrite occurs at a higher rate than the reduction of nitrite to ammonia, resulting in an accumulation of nitrite in the rumen and subsequent absorption (Bruning-Fann and Kaneene, 1993). Adapting animals slowly to nitrate in their diet enables the population of nitrite-reducing bacteria to increase in size, increasing the capacity to reduce nitrite (Allison and Reddy, 1984). An adaptation period has been demonstrated to enable ruminants to cope with higher levels of nitrate in their diets (Alaboudi and Jones, 1985; van Zijderveld et al., 2010). In our study, an adaptation period to dietary nitrate was included, but MetHb levels were still higher ($P = 0.008$; Table 6) for the nitrate-fed cows. It has been proposed that animals respond to prolonged elevated MetHb lev-

els in their blood by producing more Hb, thereby compensating for the decreased oxygen-carrying capacity of the blood (Winter and Hokanson, 1964). No evidence of such a mechanism was observed in this study. Although average MetHb levels were no reason for concern on the nitrate diet, peak levels for individual animals were 4- to 5-fold the average level in the cows on the urea diet, but still below the level considered to cause subclinical methemoglobinemia (30–40%; Bruning-Fann and Kaneene, 1993).

Yield of Usable ME as a Consequence of Methane Reduction

If GEI and digestibility are not altered, a decrease in methane production will increase the amount of ME available to the animal, which could increase milk production or body tissue gain (Blaxter and Czerkawski, 1966). In this experiment, methane production was lowered ($-28 \text{ kJ/kg of BW}^{0.75}$ per day or 3.4 MJ/d) and apparent NDF, crude fat, and starch digestibility were unaffected by treatment. The conversion of GE into ME was also improved for the nitrate diet. If the energy spared from methanogenesis had been fully converted into milk with an efficiency of 0.64 (NRC, 2001), the increase in milk yield would have been 0.7 kg (milk energy was 3.1 MJ/kg for this experiment). However, no significant increase in milk energy output was observed in this experiment, although milk energy output was numerically higher for the nitrate diet ($+6 \text{ kJ/kg BW}^{0.75}$ per day or 0.7 MJ/d). The majority of the energy saved from decreased methane production appeared to accumulate in the calculated energy retention, which increased nonsignificantly for the nitrate diet ($+34 \text{ kJ/kg BW}^{0.75}$ per day or 4.1 MJ/d). Despite the significant reduction in methane production, no positive responses in animal performance parameters were observed.

During experimental period 4, hydrogen production was measured and was found to be higher for the nitrate diet. Our hypothesis was that nitrate would act as a hydrogen sink and the reason for the elevated hydrogen emissions for the nitrate diet is unknown. Hydrogen is an energy-dense gas and its emission by the animal could offset the energy benefit gained by the decrease in methane production. In experimental period 4, hydrogen production was measured from 0630 to 1530 h. The average hydrogen production over this 9-h period was 1.9 L/cow for the urea diet and 5.8 L/cow for the nitrate diet. If results from the 9-h period are extrapolated to a 24-h period, hydrogen production is estimated to amount to 5 L/cow per day for the urea diet and 15 L/cow per day for the nitrate diet. The additional 10 L/d of hydrogen produced for the nitrate

diet translate into 0.45 mol of hydrogen/d (22.4 L/mol) or 0.9 g of hydrogen/d. Hydrogen is energy dense (142 kJ/g of H₂; Afeefy et al. (2011), but the additional energy lost in hydrogen production for the nitrate diet was calculated to be relatively minor (approximately 1.0 kJ/kg BW^{0.75} per day or 3.6% of the observed methane decrease).

The simple statement that decreasing enteric methane emissions without affecting feed digestibility will increase the animals' productivity or energy retention therefore appears to be inadequate. A further requirement would be that the reducing equivalents spared from methane production are incorporated in molecules that are limiting either bacterial or animal metabolic processes. The direct environmental impact of methane production from the dairy cows in this experiment was decreased; daily methane production was lowered by 58 g/cow per day, or 1.5 kg of CO₂-equivalents/cow per day.

CONCLUSIONS

Nitrate addition to corn silage-based dairy cow diets for 89 d persistently decreased enteric methane emissions by 16% without negatively affecting diet digestibility and milk production. The energetic benefit from the decreased methane production did not appear to benefit the animal, as milk production and energy balance were not affected.

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