Nitrate improves ammonia incorporation into rumen microbial protein in lactating dairy cows fed a low-protein diet

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ABSTRACT

Generation of ammonia from nitrate reduction is slower compared with urea hydrolysis and may be more efficiently incorporated into ruminal microbial protein. We hypothesized that nitrate supplementation could increase ammonia incorporation into microbial protein in the rumen compared with urea supplementation of a low-protein diet fed to lactating dairy cows. Eight multiparous Chinese Holstein dairy cows were used in a crossover design to investigate the effect of nitrate or an isonitrogenous urea inclusion in the basal low-protein diet on rumen fermentation, milk yield, and ruminal microbial community in dairy cows fed a low-protein diet in comparison with an isonitrogenous urea control. Eight lactating cows were blocked in 4 pairs according to days in milk, parity, and milk yield and allocated to urea (7.0 g urea/kg of dry matter of basal diet) or nitrate (14.6 g of NO_3^- /kg of dry matter of basal diet, supplemented as sodium nitrate) treatments, which were formulated on 75% of metabolizable protein requirements. Nitrate supplementation decreased ammonia concentration in the rumen liquids (−33.1%) and plasma (−30.6%) as well as methane emissions (−15.0%) and increased dissolved hydrogen concentration (102%), microbial N (22.8%), propionate molar percentage, milk yield, and 16S rRNA gene copies of *Selenomonas ruminantium*. Ruminal dissolved hydrogen was positively correlated with the molar proportion of propionate ($r = 0.57$), and negatively correlated with acetate-to-propionate ratio ($r = -0.57$) and estimated net metabolic hydrogen production relative to total VFA produced ($r = -0.58$). Nitrate reduction

to ammonia redirected metabolic hydrogen away from methanogenesis, enhanced ammonia incorporation into rumen microbial protein, and shifted fermentation from acetate to propionate, along with increasing *S. ruminantium* 16S rRNA gene copies, likely leading to the increased milk yield.

Key words: nitrate, dissolved hydrogen, rumen fermentation, microbial protein

INTRODUCTION

Molecular hydrogen (H_2) is produced from metabolic hydrogen ([H]) during carbohydrate fermentation in the rumen and then used by methanogens to produce methane (Hungate, 1967), which is a potent greenhouse gas (Reisinger and Clark, 2018) and represents a loss of between 2 and 12% of ingested gross energy in ruminants (Johnson and Johnson, 1995). Redirecting [H] from methanogenesis to alternative fermentation end-products could have potential nutritional benefits to ruminants. Nitrate (NO_3^-) is a strong inorganic anion with a high redox potential. In the rumen, nitrate is first reduced to nitrite (NO_2^- ; $\text{NO}_3^- + [\text{H}] \rightarrow \text{NO}_2^- + \text{H}_2\text{O}$), and then further reduced to ammonia ($\text{NO}_2^- + 3 [\text{H}] + 2\text{H}^+ \rightarrow \text{NH}_4^+ + 2\text{H}_2\text{O}$; Latham et al., 2016). Nitrate reduction to ammonia is energetically more favorable [Gibbs energy changes (ΔG^0) = −599.6 kJ/mol] than the reduction of carbon dioxide to methane (ΔG^0 = −131 kJ/mol; Latham et al., 2016). Ammonia resulting from nitrate reduction can be incorporated into carbon skeletons to form microbial AA and protein. Nutritionally, microbial protein is the most important, and the most economical, source of AA for ruminants (Hristov and Broderick, 1996).

Previous studies have reported that nitrate supplementation decreased methane production in ruminants in comparison to isonitrogenous urea supplementation

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(van Zijderveld et al., 2010; Guyader et al., 2015; Troy et al., 2015); however, replacement of urea with nitrate has not benefited the productivity of cattle (Lee and Beauchemin, 2014; Troy et al., 2015). It is thought that for diets sufficient in N, additional ammonia formed from nitrate reduction may not be nutritionally beneficial (Olijhoek et al., 2016). The lack of benefit may also be affected by other factors, such as the rate of dietary carbohydrate degradation being synchronized with N release in the rumen (Sinclair et al., 1993). The generation of ammonia from nitrate reduction is slower than urea hydrolysis (Marais et al., 1988; Lee et al., 2017), which may benefit ammonia incorporation into carbon skeletons to form microbial AA (Nolan et al., 2010; Li et al., 2012). Herein, we hypothesized that nitrate supplementation could increase ammonia incorporation into microbial protein in the rumen, which might exert a nutritional benefit to rumen function, and hence productivity of lactating dairy cows fed a low-protein diet, and lead to an improvement in the productivity. To test this hypothesis, we replaced dietary urea with nitrate in lactating dairy cows fed low-protein diets and then measured nutrient digestibility, milk yield, methane emissions, blood metabolites, ruminal fermentation, and selected groups of microorganisms in the rumen.

MATERIALS AND METHODS

Experimental Methods

All procedures involving animals were approved by the Animal Care Committee, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China.

Experimental Design and Animals

Eight Chinese Holstein dairy cows (200 ± 10 DIM; parity = 2.56 ± 0.88 ; milk yield = 15.9 ± 0.86 kg/d; BW = 508 ± 20.2 kg; mean $\pm$ SD) were blocked in 4 pairs according to DIM, parity, and milk yield. Within a pair, cows were randomly allocated to 1 of 2 treatments, with either urea (control) or sodium nitrate (NaNO_3 , Hunan Huihong Reagent Co. Ltd., Changsha, China) as an N supplement. The study consisted of 2 consecutive experimental periods in a crossover design. Each period consisted of 20 d of adaptation to the diets and 15 d of sampling and data collection. The 15 d of sampling and data collection included 5 d for total fecal sample collection, 8 d for measurements of methane emissions (2 d for each of 4 cows), and 2 d for collection of rumen fluid and blood at 2.5 and 6 h after fresh feed offered in the morning. We further randomly separated the 4 cows of each treatment into 4 groups of 2 cows

each on different treatments, which were staggered by 8 d in the application of the treatments.

Diet and Feeding

The diets were supplied by a basal diet (Table 1) and supplementation of urea or nitrate and formulated to deliver 75% of MP requirements according to the software of Cornell-Penn-Miner Dairy System (CPM-Dairy; Boston et al., 2000). The basal diet contained 87.9 g of CP/kg of DM. Urea (7.0 g of urea/kg of DM of basal diet) or sodium nitrate (14.6 g of NO_3^- /kg of DM of basal diet), which equals 20.9 g of CP/kg of DM of basal diet, were added to the basal diet for urea or nitrate treatment, respectively. During each period, both urea and nitrate were gradually introduced in the diet at incremental portions of 25% from d 2 to 5 until 100% of the experimental dose was fed at d 6.

Cows were housed in a tiestall barn, fed individually at 0530 and 1500 h, and milked twice daily at 0630 and 1600 h; individual milk yields were recorded. All cows had free access to fresh water. Diets were offered *ad libitum* during the first 7 d of each period to let the cows

Table 1. Ingredient and chemical composition (g/kg of DM) of basal diet

Item	Basal diet
Ingredient (g/kg of DM)	
Grass silage ¹	520
Corn grain	272
Soybean meal	50
Wheat middlings	10
Wheat bran	80
Oil	40
Sodium chloride	5.0
Calcium carbonate	8.0
Calcium bicarbonate	5.0
Premix ²	10
Chemical composition ³ (g/kg of DM)	
OM	880
CP	87.9
Starch	278
Forage NDF	295
NDF	426
ADF	210
NDF/Starch	1.48
NE_L ⁴ (Mcal/kg of DM)	1.49
ME^4 (Mcal/kg of DM)	2.31
MP ⁴ (g/kg of DM)	71.3

¹The grass silage contained 24.7% DM, and 932 g/kg of OM, 58.9 g/kg of CP, 65.6 g/kg of starch, 591 g/kg of NDF, and 376 g/kg of ADF on a DM basis.

²The premix (vitamins and microelements) provided (per kilogram of DM) 1,000,000 IU of vitamin A, 200,000 IU of vitamin D, 1,250 IU of vitamin E, 8,000 mg of Zn, 80 mg of Se, 120 mg of I, 2,000 mg of Fe, 40 mg of Co, 2,500 mg of Mn, and 2,000 mg of Cu.

³Mean values from the analysis of 3 samples.

⁴Calculated based on Cornell-Penn-Miner Dairy System (CPM-Dairy; Boston et al., 2000).

adapt to the treatment diets and measure individual feed intake. From d 8 to 35, DMI within a block was restricted to 95% of the ad libitum DMI of the animal to avoid diet selection. Feed refusals, if present, were collected and analyzed to determine the actual nutrient intake.

Nutrient Digestibility and Milk Yield

Nutrient digestibility was measured for 5 d. Total feces were collected, weighed, and mixed daily. A subsample (~1%) was frozen immediately at -20°C and another subsample (~1%) was acidified using 10% (wt/wt) H_2SO_4 to prevent N loss and then frozen immediately at -20°C . Fecal samples were dried at 65°C for 48 h in a forced-air oven and ground through a 1-mm screen. The acidified oven-dried samples were used for total N analysis, whereas nonacidified oven-dried samples were used for the rest of chemical analyses.

Methane and Carbon Dioxide Production

For each cow, methane emissions were measured for 48 h in a respiration chamber using the protocol by Wang et al. (2015) slightly modified. Each block of 2 cows containing 1 cow of each treatment was passed sequentially through the chamber, and within each block 1 cow passed at a time. Thus, the 8 cows were passed through the chamber during 16 d (8 d of measurement plus 8 d in which each group of 2 blocks of cows was staggered). Airflow was maintained under negative chamber pressure (flow rate = $190 \pm 2 \text{ m}^3/\text{h}$), controlled by the air pump. The mean ambient temperature in the chamber was $17 \pm 2^{\circ}\text{C}$. The outlet gases from the chamber and ambient gas were connected to a multiport inlet unit of the gas analyzer (GGA-30p, Los Gatos Research, San Jose, CA) using 1/8-inch (3.18 mm) polyurethane tubing. Methane concentration was measured and recorded every 30 min. Staff entered each chamber twice daily at 0530 and 1500 h for approximately 20 min for feeding and milking. Within the chamber, cows were restrained with free access to the bin and drinking water.

Rumen and Blood Sampling

Rumen and blood sampling were performed at 2.5 and 6 h after morning feeding for consecutive 2 d. Rumen contents were collected by oral stomach tubing according to the method described by Wang et al. (2016a). Briefly, samples of rumen contents were collected by inserting an oral stomach tube (Anscitech Co. Ltd., Wuhan, China) to a depth of about 180 cm, so that the probe head could reach the central rumen. Rumen content was collected in a bottle, which was

placed on the ground. A suction pump was applied for a few seconds to free any blockage and facilitate the collection of rumen samples, if rumen contents did not automatically flow out. The first initial $>150 \text{ mL}$ of rumen contents were discarded to avoid saliva contamination, and the second $>300 \text{ mL}$ of rumen contents were used for subsequent measurement. One subsample (100 mL) was immediately frozen at -80°C for DNA extraction and subsequent quantification of microbial groups. Two other subsamples (35 mL each) were immediately transferred into 50-mL plastic syringes for measuring dissolved H_2 (dH_2) and dissolved methane (dCH_4) concentration. About 20 mL of rumen content was used for immediate measurement of rumen pH using a portable pH meter (Starter 300; Ohaus Instruments Co. Ltd., Shanghai, China). Samples of 2 mL of rumen fluid were centrifuged at $12,000 \times g$ for 10 min at 4°C . Aliquots of the supernatants (1.5 mL) were transferred into plastic tubes each containing 0.15 mL of 25% (wt/vol) metaphosphoric acid and stored at -20°C overnight. Frozen rumen fluid samples were thawed and centrifuged at $12,000 \times g$ for 10 min at 4°C and the supernatants were used for subsequent analysis of ammonia concentration and VFA profile. The remaining samples (50 mL) were stored at -20°C for determination of concentrations of nitrate, nitrite, and lactate.

Blood samples were obtained from the tail vein using sodium heparinized 5-mL Vacutainer tubes (Shan Dong Ao Sai Te Medical Devices Co. Ltd., Shandong, China). All tubes, for plasma, were kept on ice or in the refrigerator before being centrifuged at $3,500 \times g$ at 4°C for 10 min. About 1.5 mL of supernatant was collected and stored at -20°C for subsequent analyses.

Sample Analysis

All samples of feeds, refusals, and feces were dried and ground to pass through a 1-mm sieve and analyzed for DM (105°C , 24 h). Ash was determined in a muffle furnace at 550°C for 8 h. Organic matter was calculated by difference between DM and ash content. Crude protein ($\text{N} \times 6.25$) was analyzed using the Kjeldahl method (AOAC International, 2000). Gross energy content was determined using an isothermal automatic calorimeter (5E-AC8018, Changsha Kaiyuan Instruments Co. Ltd., Changsha, China). Neutral detergent fiber and ADF contents were determined and expressed inclusive of residual ash according to Van Soest et al. (1991), and NDF was assayed with the addition of α -amylase and without sodium sulfite. The starch content was determined after pre-extraction with ethanol (80%), and glucose liberated from starch by enzyme hydrolysis was measured with the use of amyloglucosidase (Karthner and Theurer, 1981).

Dissolved gases (dH_2 and dCH_4) were extracted from the liquid phase of rumen contents to gas phase using the procedure by Wang et al. (2014) with slight modification. In brief, a 20-mL syringe containing 10 mL of N_2 gas (>99.99%) was injected into a 50-mL plastic syringe containing 35 mL of rumen content via polyurethane tubing. The gases dissolved in the liquid phase were exhaled into the gas phase by shaking at 200 revolutions per second for 5 min in an orbital shaker (WSZ-100A, Shanghai Yiheng Scientific Instruments Co. Ltd., Shanghai, China). Gas samples were collected at evacuated tubes for subsequent analysis using GLC (Agilent 7890A, Agilent Inc., Palo Alto, CA; Wang et al., 2016a). Concentrations of dH_2 and dCH_4 in the original liquid fraction were calculated using the equations by Wang et al. (2016a).

Individual VFA concentrations were analyzed by GC (Agilent 7890A, Agilent Inc.) according to the method described by Wang et al. (2014). The estimated net $[\text{2H}]$ production relative to the amount of total VFA produced (R_{N2H}) was calculated according to the stoichiometric equation developed by Wang et al. (2016a), under the assumption of equal fractional rates of absorption of individual VFA. Lactate and ammonia concentrations were measured according to the methods by Taylor (1996) and Weatherburn (1967), respectively. Ruminal fluid-associated microbes were isolated by differential centrifugation. First, samples of rumen fluid were centrifuged twice times at $500 \times g$ for 5 min at 3°C . Then the supernatant was transferred into new plastic tubes and centrifuged at $17,300 \times g$ for 20 min at 3°C , according to the method of Olijhoek et al. (2016). Feed particles were separated by centrifuging at $500 \times g$ at 4°C for 10 min, and the supernatant was further centrifuged at $20,000 \times g$ at 4°C for 20 min to obtain the pellet of fluid-associated microbes, which was further analyzed to obtain total N by using trichloroacetic acid (Klita et al., 1996). Total microbial N in the rumen contents was estimated using purines method with the ratio of purines to N of yeast as the standard (Zinn and Owens, 1986), and microbial N concentration was expressed as the amount of microbial N relative to the dry matter in the rumen contents (g/100 g of DM in rumen content).

Nitrite concentration was measured using the Griess reagents sulfanilamide and *N*-(1-naphthyl) ethylenediamine to form a diazo-compound, which can be analyzed by flow injection analysis (Smart Chem 140, Ams-Systea, Rome, Italy; Wang et al., 2017). Total nitrate plus nitrite concentration was measured by converting nitrate to nitrite using the Griess reaction after pretreatment with hydrazine sulfate (Pons et al., 2008). Nitrate concentration was calculated by the difference between the total concentration of nitrate plus nitrite

and the concentration of nitrite. Dissolved nitric oxide concentration was measured by reacting with chromogenic agent using the nitric oxide Colorimetric Assay Kit (A013, Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Total DNA was extracted from a pooled sample of rumen contents obtained through mixing 2 equal samples taken at 2.5 and 6 h after the morning feeding, using the QIAampDNA Stool Mini Kits (Qiagen GmbH, Hilden, Germany) following the manufacturer's procedures (Jiao et al., 2014). The quality of the DNA extracts were assessed using agarose gel (0.8%) electrophoresis. Total DNA extracted was quantified with Nano Drop ND1000 (NanoDrop Technologies, Thermo Fisher Scientific, Waltham, MA), and then stored at -80°C until further analyses. Quantitative real-time PCR analysis was performed on total bacteria, protozoa, fungi and methanogens, and selected groups of bacteria (*Ruminococcus albus*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, *Selenomonas ruminantium*, and *Prevotella rumenicola*), as well as *Prevotella* spp. The 16S rRNA gene-targeted primers employed are shown in Table 2. Quantitative PCR results are expressed as copies of 16S rRNA genes targeted per gram of rumen contents.

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 software (SPSS Inc., Chicago, IL). All data were analyzed using a Mixed linear model including dietary treatment as fixed effects, and animal, period, and block as a random effect. When sampling time was considered, the model included dietary treatment, interaction between treatment and time as fixed effect, time as a repeated measurement, and period, animal, and block as random effect, with covariance being compound symmetry structure. Statistical significance of effects was declared at $P \leq 0.05$, and a tendency toward significance was declared $0.05 < P \leq 0.10$.

Associations between propionate molar proportion, the acetate-to-propionate ratio, and R_{N2H} (y) with dH_2 concentration were studied using the RStudio software (Horton and Kleinman, 2015). The regression model was

$$y = \text{intercept} + \text{dH}_2 + \text{treatment} + \text{time} \\ + \text{dH}_2 \times \text{treatment} + \text{dH}_2 \times \text{time} + \text{residual}.$$

RESULTS

Dietary nitrate did not alter ($P > 0.50$) total-tract digestibility of nutrients studied but it did increase milk yield ($P < 0.01$) and ECM production ($P < 0.01$) (Table

Table 2. Primers used for quantitative PCR

Target species	Primer	Primer sequences (5' to 3')	Size (bp)	Reference	Primer efficiency (%)
Bacteria	Forward	CGGCAACGAGCGCAACCC	146	Denman and McSweeney (2006)	100.4
	Reverse	CCATTGTAGCACGTGTGTAGCC			
Protozoa	Forward	GCTTTCGWTGGTAGTGTATT	223	Sylvester et al. (2004)	96.3
	Reverse	CTTGCCCTCYAATCGTWCT			
Methanogens	Forward	GGATTAGATACCCSGGTAGT	192	Hook et al. (2009)	101.9
	Reverse	GTTGARTCCAATTAACCGCA			
Fungi	Forward	GAGGAAAGTAAAGTCGTAACAAGGTTTC	120	Denman and McSweeney (2006)	97.2
	Reverse	CAAATTCACAAAGGGTAGGATGATT			
<i>Prevotella</i> spp.	Forward	GTTCTGAGAGGAAGGTCCCC	121	Stevenson and Weimer (2007)	95.3
	Reverse	TCTGCACGCTACTTGGCTG			
<i>Prevotella ruminicola</i>	Forward	GAAAGTCGGATTAATGCTCTATGTTG	74	Stevenson and Weimer (2007)	99.8
	Reverse	CATCCTATAGCGGTAAACCTTTGG			
<i>Selenomonas ruminantium</i>	Forward	CAATAAGCATTCCGCCTGGG	138	Stevenson and Weimer (2007)	102.2
	Reverse	TTCCTCAATGTCAAGCCCTGG			
<i>Ruminococcus albus</i>	Forward	CCCTAAAAGCAGTCTTAGTTTCG	176	Koike and Kobayashi (2001)	101.3
	Reverse	CCTCCTTGCGGTTAGAACA			
<i>Ruminococcus flavefaciens</i>	Forward	CGAACGGAGATAATTTGAGTTTACTTAGG	132	Denman and McSweeney (2006)	102.2
	Reverse	CGGTCTCTGTATGTTATGAGGTATTACC			
<i>Fibrobacter succinogenes</i>	Forward	GTTTCGGAATTACTGGGCGTAAA	121	Denman and McSweeney (2006)	100.7
	Reverse	CGCCTGCCCCTGAACTATC			

3). Cows fed nitrate had different kinetics of methane emissions compared with those fed urea, with higher ($P \leq 0.10$) rate of methane emissions within 3 h after morning feeding (Figure 1). Nitrate supplementation decreased methane emissions in terms of grams per day (-15.0% ; $P = 0.02$), grams per kilogram of OM intake ($P = 0.01$), and grams per kilogram of OM digested ($P = 0.01$). Nitrate supplementation did not alter carbon dioxide emissions ($P = 0.68$; Table 3).

Nitrate treatment resulted in greater ruminal nitrite ($P < 0.01$) and nitric oxide concentration ($P < 0.01$), and lower ammonia concentration ($P < 0.01$; Table 4). In plasma, nitrate treatment increased ruminal nitrite ($P < 0.01$) and nitric oxide concentration ($P < 0.01$)

and decreased ammonia concentration ($P < 0.01$; Table 4). We observed no interactions between treatment and time of sampling on metabolites concentration in the rumen and plasma ($P \geq 0.14$).

Nitrate treatment resulted in greater ruminal dH_2 concentration (102% ; $P < 0.01$) and propionate molar percentage ($P < 0.01$) as well as decreased acetate-to-propionate ratio ($P < 0.01$) and $\text{R}_{\text{N}_2\text{H}}$ ($P < 0.01$) in comparison with urea treatment (Table 5). Ruminal dH_2 was positively correlated with propionate molar percentage ($r = 0.57$; $P < 0.01$) and negatively correlated with acetate-to-propionate ratio ($r = -0.57$; $P < 0.01$) and $\text{R}_{\text{N}_2\text{H}}$ ($r = -0.58$; $P < 0.01$; Figure 2). We found no significant effects of class (treatment and

Table 3. Feed intake, digestibility, and methane and carbon dioxide emissions in lactating dairy cows fed on urea or nitrate diet ($n = 8$)

Item	Treatment		SEM	P-value
	Urea	Nitrate		
DMI (kg/d)	10.2	10.2	—	—
Apparent total-tract digestibility (%)				
OM	74.2	74.8	1.30	0.62
CP	62.2	62.1	1.83	0.96
NDF	65.1	66.0	1.48	0.58
ADF	56.4	57.2	1.38	0.58
Starch	97.1	97.1	0.13	0.83
Milk yield (kg/d)	15.9	16.5	0.11	<0.01
ECM production (kg/d)	14.4	15.1	0.12	<0.01
Methane emissions				
g/d	206	175	10.4	0.02
g/kg of OM intake	22.6	19.3	0.95	0.01
g/kg of OM digested	30.5	25.9	1.38	0.01
Carbon dioxide emissions (kg/d)	8.99	8.87	0.247	0.66

Table 4. Nitrate, nitrite, and ammonia in the rumen and plasma of dairy cows fed on urea or nitrate diet (n = 8)

Item	Treatment		Time after feeding		SEM	P-value		
	Urea	Nitrate	2.5 h	6 h		Treatment	Time	Treatment × Time
Rumen fluid								
Nitrate (μM)	11.3	11.1	11.7	10.7	0.31	0.43	<0.01	0.60
Nitrite (μM)	16.8	23.0	18.3	21.5	1.43	<0.01	0.04	0.78
Nitric oxide (μM)	3.03	3.57	3.58	3.02	0.156	<0.01	<0.01	0.21
Ammonia (mM)	7.05	4.63	7.55	4.13	0.612	<0.01	<0.01	0.52
Plasma								
Nitrite (μM)	1.18	1.65	1.15	1.68	0.162	<0.01	<0.01	0.65
Nitric oxide (μM)	1.44	1.90	1.45	1.89	0.151	<0.01	<0.01	0.14
Ammonia (μM)	112.8	77.1	88.6	101.3	11.12	<0.01	0.27	0.52

time) and its interactions with the continuous dH₂. The nitrate treatment resulted in greater fluid-associated microbial N concentration (3.74%; *P* < 0.01) and microbial N (22.8%; *P* < 0.01) in rumen content. We observed no interaction between the effect of treatment and time of sampling on dissolved gases concentration (*P* ≥ 0.10). The nitrate treatment had greater the 16S rRNA gene copies of *S. ruminantium* (*P* = 0.01) and *Prevotella* spp. (*P* = 0.07) compared with the urea treatment (Table 6).

DISCUSSION

Nitrate supplementation did not alter fiber digestibility, which was consistent with lack of changes in 16S rRNA gene copies of known major ruminal fiber-degrading fungi and fibrolytic bacteria *R. albus*, *R. flavefaciens*, and *F. succinogenes*; however, this is not in agreement with several published reports. Some studies indicated that nitrate inhibits microorganisms by degrading plant cell walls, leading to the decreased

Figure 1. Diurnal methane production pattern (g/h, means ± SEM) over a 24-h period in dairy cows fed by urea diet (Δ) or nitrate diet (▲) (n = 8). Arrows indicate the time of feeding. Symbols indicate whether the treatment effect was significant for the respective time point: **P* ≤ 0.05, +0.05 < *P* ≤ 0.10, ⁿ*P* > 0.10.

fiber digestion (Marais et al., 1988; Klop et al., 2016), whereas others reported that nitrate increases cellulolytic bacteria, resulting in increased fiber digestibility (Patra and Yu (2015)). These inconsistent effects of nitrate supplementation on fiber digestibility may be due to differences in dosage supplemented, efficiency of ruminal nitrate reduction, experiment methods (in vitro or in vivo), and ruminant species, among others.

Nitrite is an intermediate during the reduction of nitrate to ammonia (Guyader et al., 2015; Troy et al., 2015). Because the reduction of nitrate to nitrite is generally faster than the reduction of nitrite to ammonia, nitrite can accumulate in the rumen fluid and be absorbed through the rumen wall and enter blood circulation (Olijhoek et al., 2016). Once in the blood, nitrite oxidizes ferrous iron (Fe^{2+}) in hemoglobin to ferric iron (Fe^{3+}); as a consequence, the hemoglobin molecule is impaired to transport oxygen to the tissues (Ozmen et al., 2005; van Zijderveld et al., 2011). In our study, both nitrate and nitrite were detected in the rumen contents and plasma of cows fed the control diet with urea supplementation, which might have been caused by nitrate present in the corn silage component of the diet, as farmers used excess nitrogenous fertilizer containing nitrate to grow the corn (Takahashi et al., 1998; Wang et al., 2018). However, nitrate and nitrite concentration were very low, within ranges from 10.5 to 11.8 and 15.4 to 24.7 μM in the rumen with both treatments, indicating that the dietary nitrate was almost completely chemically reduced. Such low ruminal nitrate and nitrite concentrations cannot have been high enough to cause occurrence of methemoglobinemia, in

agreement with previous results reported by Veneman et al. (2015).

Nitrate reduction to ammonia is thermodynamically more favorable than carbon dioxide reduction to methane in the rumen, leading to redirection of [H] flow away from methanogenesis and toward ammonia production (Latham et al., 2016). In the rumen, dietary urea is hydrolyzed to ammonia, a neutral reaction in terms of release or incorporation of [H] release or incorporation. Previous studies have shown that replacing urea with nitrate decreases 10 to 35% of daily methane emissions (Li et al., 2012; de Raphlis-Soissan et al., 2014; Guyader et al., 2015; Lee et al., 2015; Klop et al., 2016). In our study, replacing urea with nitrate decreased daily methane emission in dairy cows by about 15% (i.e., 31 g/d), which is 82% of the theoretical maximum potential to reduce methane (i.e., 38 g/d) by 149 g/d of nitrate intake. Such a shift of [H] flow from methanogenesis to ammonia production may have caused the enhancement of rumen microbial activity and cow productivity with the low-protein diet evaluated in the current study. However, when nitrate is supplemented to a diet (in exchange with urea) already sufficient in protein, dietary N may not lead to a more efficient rumen fermentation, as evidenced by increased ruminal ammonia concentration (Hulshof et al., 2012; Guyader et al., 2015; Olijhoek et al., 2016). The inefficient nitrate-N utilization may be due to absorption of excess ammonia through the rumen wall and elimination of most N as urea in urine (Wallace et al., 1997) as well as incomplete reduction of nitrate to ammonia and microbial protein synthesis by rumen microorganisms

Table 5. Dissolved gases and fermentation end-products in the rumen of dairy cattle fed on urea or nitrate diet (n = 8)

Item	Treatment		Time after feeding			P-value		
	Urea	Nitrate	2.5 h	6 h	SEM	Treatment	Time	Treatment × Time
Dissolved gases								
Dissolved hydrogen (μM)	2.37	4.79	3.76	3.40	0.566	<0.01	0.54	0.45
Dissolved methane (mM)	0.41	0.45	0.44	0.42	0.038	0.37	0.78	0.83
pH	6.65	6.74	6.60	6.80	0.053	0.11	<0.01	0.36
Lactate (mM)	0.48	0.48	0.50	0.46	0.035	0.98	0.19	0.31
Fluid-associated microbial N (mg/mL)	1.87	1.94	1.93	1.89	0.015	<0.01	<0.01	0.70
Microbial N (g/100 g of DM rumen content)	6.68	8.10	7.39	7.38	0.411	<0.01	0.99	0.90
Total VFA concentration (mM)	90.8	89.3	94.0	86.1	2.88	0.61	0.01	0.049
Molar percentage of individual VFA (mol/100 mol)								
Acetate	65.2	64.4	64.7	64.9	0.53	0.12	0.65	0.94
Propionate	18.6	19.7	19.6	18.7	0.31	<0.01	0.01	0.10
Butyrate	12.5	12.4	12.1	12.8	0.51	0.78	0.15	0.34
Valerate	1.4	1.3	1.4	1.3	0.06	0.25	0.05	0.67
Isobutyrate	0.9	0.81	0.8	0.9	0.04	0.31	0.20	0.81
Isovalerate	1.5	1.5	1.5	1.5	0.12	0.12	0.96	0.62
Acetate-to-propionate ratio	3.57	3.33	3.35	3.55	0.052	<0.01	<0.01	0.07
Estimated net metabolic hydrogen production relative to the amount of total VFA produced (R_{N2H} , mol/mol)	1.36	1.33	1.33	1.36	0.008	<0.01	<0.01	0.12

Figure 2. Association of selected fermentation variables with dissolved hydrogen (dH₂) (n = 32). (A) Propionate molar percentage: $y = 18.1 (\pm 1.25; P < 0.01) + 0.41 (\pm 0.239; P = 0.09) dH_2 - 1.10 (\pm 1.25; P = 0.30) \text{ time} - 1.14 (\pm 1.537; P = 0.47) \text{ treatment} + 0.09 (\pm 0.288; P = 0.76) dH_2 \times \text{time} - 0.46 (\pm 0.486; P = 0.35) dH_2 \times \text{treatment}$. (B) Acetate-to-propionate ratio: $y = 3.83 (\pm 0.301; P < 0.01) - 0.12 (\pm 0.057; P = 0.04) dH_2 + 0.144 (\pm 0.301; P = 0.64) \text{ time} + 0.14 (\pm 0.369; P = 0.70) \text{ treatment} + 0.003 (\pm 0.069; P = 0.96) dH_2 \times \text{time} - 0.081 (\pm 0.117; P = 0.49) dH_2 \times \text{treatment}$. (C) Estimated net metabolic hydrogen production relative to the amount of total VFA produced (R_{N2H}): $y = 1.38 (\pm 0.034; P < 0.01) - 0.01 (\pm 0.007; P = 0.04) dH_2 + 0.003 (\pm 0.034; P = 0.43) \text{ time} + 0.01 (\pm 0.04; P = 0.73) \text{ treatment} - 0.0002 (\pm 0.008; P = 0.98) dH_2 \times \text{time} - 0.007 (\pm 0.013; P = 0.60) dH_2 \times \text{treatment}$. Δ = data collected at 2.5 h postfeeding of urea treatment; ∇ = data collected at 6 h postfeeding of urea treatment; $\blacktriangle$ = data collected at 2.5 h postfeeding of nitrate treatment; $\blacktriangledown$ = data collected at 6 h postfeeding of nitrate treatment. The solid line represents the regression line fitted using all data points (r_1 and P_1); the dashed line represents the regression line (r_2 and P_2) fitted after removing the 5 influential points in the circle.

(van Zijderveld et al., 2011; Olijhoek et al., 2016). In contrast, in the present study the diet had a relatively low CP content (11% including the supplementation), and the supplemented nitrate resulted in lower ammonia concentration in the rumen liquid and blood and a greater microbial protein content in the rumen compared with urea supplementation. These results suggest an enhancement of ammonia utilization in dairy cows supplemented with nitrate, in agreement with previous in vitro results (Guo et al., 2009). Enhancement of ammonia utilization may redirect more N, electrons,

and energy toward microbial protein synthesis at the expense of methane emissions.

On the other hand, nitrate may also have negative effects through the production of nitrite, which is probably toxic to microbial cells (Marais et al., 1988; Cammack et al., 1999). However, nitrate supplementation at the dose applied in the present study did not affect 16S rRNA copies of bacteria, protozoa, methanogens, or fungi. The toxic effect of nitrite can suppress growth and activity of methanogens (Iwamoto et al., 2001; van Zijderveld et al., 2010), decrease the rate of H₂ utilization by methanogenesis, and cause ruminal H₂ accumulation. Guyader et al. (2015) reported that nitrate supplementation induced a 12.5-fold peak of dH₂ concentration in the rumen up to 2 h after commencement of feeding, which coincides with a previously reported increase in gaseous H₂ emissions (van Zijderveld et al., 2011). In our study, nitrate supplementation resulted in greater ruminal dH₂ concentration than urea supplementation, but the increase was lower compared with the study by Guyader et al. (2015; 12.5 vs. 2.02 fold with respect to the urea controls), which is likely due to the difference in nitrate dose that was applied (22.7 vs. 14.6 g/kg of DM) and perhaps to the different techniques employed for measuring dH₂ concentration.

Nitrate acts as an electron acceptor in anaerobic environments, and the reduction of nitrate to nitrite or

Table 6. Selected groups microorganisms (log₁₀ copies/DM rumen contents) in the rumen of dairy cows fed on urea or nitrate diet (n = 8)

Item	Treatment		SEM	P-value
	Urea	Nitrate		
Bacteria	11.33	11.41	0.059	0.26
Protozoa	8.68	8.59	0.192	0.67
Methanogens	8.92	8.85	0.076	0.42
Fungi	7.36	7.44	0.145	0.62
Selected groups of bacteria				
<i>Prevotella</i> spp.	10.84	10.94	0.047	0.07
<i>Prevotella ruminicola</i>	10.02	10.00	0.052	0.68
<i>Selenomonas ruminantium</i>	8.49	8.69	0.061	0.01
<i>Ruminococcus albus</i>	7.94	8.03	0.050	0.13
<i>Ruminococcus flavefaciens</i>	7.12	7.08	0.153	0.77
<i>Fibrobacter succinogenes</i>	9.56	9.72	0.082	0.11

ammonia can alter the flows of [H] in the rumen. Studies have indicated that nitrate supplementation increased acetate molar percentage, because nitrate reduction favors the release of [H] with increased growth of acetate producers (Patra and Yu, 2015) and acetate molar percentage (Hulshof et al., 2012; Troy et al., 2015). This mechanism, however, is in contradiction with the elevated ruminal dH₂ and the increase in gaseous H₂ emission, also reported previously (van Zijderveld et al., 2011; Guyader et al., 2015), because nitrate reduction, being thermodynamically favorable, would be expected to decrease the availability of ruminal H₂. In our study, nitrate supplementation increased propionate molar percentage and decreased acetate-to-propionate ratio and the efficiency of [H] production derived from fermentation, presumably leading to an increase in the incorporation of [H] into propionate formation. Such a shift of fermentation pathways from acetate to propionate production agrees with increased dH₂ concentration, because propionogenesis is considered to be thermodynamically favored by increased H₂ partial pressures (Ellis et al., 2008; Janssen, 2010). Ungerfeld (2013) proposed that [H] can also be redirected into propionogenesis under higher H₂ partial pressure. Such a shift of [H] flow from methanogenesis to VFA would also contribute to redirecting electrons and energy in rumen metabolites, which is consistent with increased milk and ECM production.

It is well known that fiber digestion is associated with greater acetate-to-propionate concentration ratio compared with concentrates. *Ruminococcus flavefaciens* and *R. albus* produce a fermentation profile with a high acetate-to-propionate ratio. In fact, *F. succinogenes* grown in pure cultures produces a good deal of succinate (Bryant and Doetsch, 1954; Dehority, 1963; Weimer, 1993; Rychlik and May, 2000), a propionate precursor, and to a lesser extent so does *R. flavefaciens* (Dehority, 1963; Latham and Wolin, 1977; Shi and Weimer, 1992; Sawanon and Kobayashi, 2006). Furthermore, the proportion of *F. succinogenes*, *R. flavefaciens*, and *R. albus* in total bacterial 16S rRNA copies is relatively small; therefore, it is not clear that changes in the abundance of these fibrolytic bacteria are reflected in changes in the concentration of fermentation end-products. Exposure of animals to a gradual increment in dietary nitrate helps promote the growth of nitrite reducing bacteria in the rumen (Zhou et al., 2012; Asanuma et al., 2015). *Selenomonas ruminantium* is reported to be active in nitrate and nitrite reduction, and its abundance was increased by feeding a nitrate-containing diet (Asanuma et al., 2015). Reduction of nitrate by *S. ruminantium* is associated with phosphorylation of ADP to ATP via electron transport-linked phosphorylation (Hasan and Hall, 1975). In agreement, we found

that cows fed the nitrate diet had greater 16S rRNA gene copies of *S. ruminantium* than those fed with the urea diet. In addition, nitrate supplementation tended to increase population of *Prevotella* spp., which may partially contribute to the increased propionate molar proportion (Wang et al., 2016b).

CONCLUSIONS

When cows were fed a low-protein diet, replacing urea with nitrate seemed to improve ammonia incorporation into microbial protein and stimulate the disposition of electrons derived from fermentation into propionate production, which might have stimulated milk and ECM production. Increased 16S rRNA gene copies of *S. ruminantium* likely contributed to the reduction of nitrate to ammonia and the shift of fermentation toward propionate production. Overall, nitrate seemed to shift [H] flows from methanogenesis toward propionate and increase ammonia utilization for microbial CP synthesis, which benefited the host animals on this low-protein diet.

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