Effects of calcium ammonium nitrate fed to dairy cows on nutrient intake and digestibility, milk quality, microbial protein synthesis, and ruminal fermentation parameters

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ABSTRACT

We evaluated the effects of supplemental calcium ammonium nitrate (CAN) fed to dairy cows on dry matter (DM) intake, nutrient digestibility, milk quality, microbial protein synthesis, and ruminal fermentation. Six multiparous Holstein cows at 106 ± 14.8 d in milk, with 551 ± 21.8 kg of body weight were used in a replicated 3×3 Latin square design. Experimental period lasted 21 d, with 14 d for an adaptation phase and 7 d for sampling and data collection. Cows were randomly assigned to receive the following treatments: URE, 12 g of urea/kg of DM as a control group; CAN15, 15 g of CAN/kg of DM; and CAN30, 30 g of CAN/kg of DM. Supplemental CAN reduced DM intake (URE 19.0 vs. CAN15 18.9 vs. CAN30 16.5 kg/d). No treatment effects were observed for apparent digestibility of DM, organic matter, crude protein, ether extract, and neutral detergent fiber; however, CAN supplementation linearly increased nonfiber carbohydrate digestibility. Milk yield was not affected by treatments (average = 23.1 kg/d), whereas energy-corrected milk (ECM) and 3.5% fat-corrected milk (FCM) decreased as the levels of CAN increased. Nitrate residue in milk increased linearly (URE 0.30 vs. CAN15 0.33 vs. CAN30 0.38 mg/L); however, treatments did not affect nitrite concentration (average: 0.042 mg/L). Milk fat concentration was decreased (URE 3.39 vs. CAN15 3.35 vs. CAN30 2.94%), and the proportion of saturated fatty acids was suppressed by CAN supplementation. No treatment effects were observed on the reducing power and thiobarbituric acid reactive substances of milk, whereas conjugated dienes increased linearly (URE

47.6 vs. CAN15 52.7 vs. CAN30 63.4 mmol/g of fat) with CAN supplementation. Treatments had no effect on microbial protein synthesis; however, molar proportion of ruminal acetate and acetate-to-propionate ratio increased with CAN supplementation. Based on the results observed, supplementing CAN at 30 g/kg of DM should not be recommended as an optimal dose because it lowered DM intake along with ECM and 3.5% FCM, although no major changes were observed on milk quality and ruminal fermentation.

Key words: antioxidant capacity, nonprotein nitrogen, methemoglobin, milk fatty acid

INTRODUCTION

Nitrate (NO_3^-) compounds have been explored as feed additives to decrease methane (CH_4) production and as dietary NPN sources in ruminants. Recently, Feng et al. (2020), using a meta-analytical approach, reported the efficacy of NO_3^- supplementation (mean = 16.7 g/kg of DM) at reducing CH_4 yield (g/DMI) in both dairy cows ($15.5 \pm 1.15\%$) and beef cattle ($8.95 \pm 1.76\%$). Methane mitigation with NO_3^- occurs because of its role as an alternative metabolic hydrogen ($[\text{H}]$) sink in the rumen, because the reduction from NO_3^- to NH_3 is more thermodynamically favorable than the use of CO_2 to synthesize CH_4 . In addition, nitrite (NO_2^-), an intermediate formed during reduction from NO_3^- to NH_3 , is toxic to rumen methanogens (Latham et al., 2016; Popova et al., 2019). Although the efficacy of NO_3^- at reducing the metabolic activity of methanogens (Patra and Yu, 2013; Popova et al., 2019) is consistent, effects of dietary NO_3^- on ruminal fermentation parameters remain inconsistent. According to Olijhoek et al. (2016) and Wang et al. (2018), feeding NO_3^- to dairy cows at 21 and 14.6 g/kg of DM, respectively, did not affect total VFA concentration and molar proportion of acetate. However, other studies have reported

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greater acetate proportion and attributed it to the stimulation of a thermodynamically favorable pathway for [H] disposal during the conversion of NO_3^- to NH_3 (Ungerfeld and Kohn, 2006; Nolan et al., 2010; Latham et al., 2016).

In addition to the role of NO_3^- as an alternative electron acceptor, NO_3^- supplementation provides NPN and may favor microbial protein synthesis in dairy cows because of its capacity to provide NH_3 to ruminal microbes (Wang et al., 2018). Although it is believed that for diets sufficient in RDP, additional NH_3 may not increase microbial protein synthesis (Olijhoek et al., 2016), the absence of effects may also be due to lack of synergy between the rate of fermentable carbohydrate degradation and available ruminal N (Sinclair et al., 1993). Because NH_3 synthesis from NO_3^- is slower compared with urea, we speculated better synergy and more efficient NH_3 incorporation for synthesis of microbial protein with NO_3^- supplementation (Nolan et al., 2010; Li et al., 2012).

Gradual adaptation to dietary NO_3^- is essential to avoid toxicity to rumen microorganisms and prevent greater levels of blood methemoglobin (**MetHb**) that can lower the O_2 carrying capacity of hemoglobin (Leng, 2008). In addition, gradual inclusion of dietary NO_3^- allows ruminal microbes to adapt and increase their capability to reduce NO_2^- (Alaboudi and Jones, 1985), thereby lowering the chances of accumulation of NO_3^- and NO_2^- residues under ruminal conditions and subsequently in animal products. However, incomplete ruminal metabolism of dietary NO_3^- to NH_3 may result in ruminal accumulation of NO_3^- and NO_2^- , which can be absorbed in blood and recovered in urine and milk (Olijhoek et al., 2016). Dietary NO_3^- supplemented at 21 g/kg of DM in lactating dairy cow diets resulted in negligible concentrations of NO_3^- in milk and was therefore considered safe for human consumption (Olijhoek et al., 2016; Beauchemin et al., 2020). Nevertheless, we are lacking studies investigating transfer of dietary NO_3^- and its intermediates NO_2^- in milk when NO_3^- supplementation exceeds 21 g/kg of DM, and, considering WHO (2011) guidelines of NO_3^- concentration below 50 mg/L in the drinking water supply, additional studies are required to validate low transfer of dietary NO_3^- , regardless of the dose supplemented.

As mentioned earlier, ruminal metabolism of NO_3^- results in the formation of intermediates such as NO_2^- . Nitrates and NO_2^- absorbed in blood and tissues are physiologically recycled to form nitric oxide (NO) and other bioactive nitrogen oxides (Lundberg et al., 2008). The rapid oxidation of NO in milk to nitro and nitroso compounds might alter its antioxidant capacity; however, its mechanistic details are not well established

(Titov et al., 2010). We are lacking data to establish whether supplementing NO_3^- to dairy cows would alter milk antioxidant properties. This is much-needed work, considering the importance of food safety in human health.

We hypothesized that gradual inclusion of calcium ammonium nitrate (**CAN**) in diets of lactating dairy cows would improve microbial protein synthesis, influence ruminal fermentation, and subsequently affect production, without increasing MetHb levels and inducing toxicity, regardless of the dose used. In addition, we hypothesized that regardless of the dose used, minimal NO_3^- and NO_2^- residues would be observed in milk and subsequently no effects will be observed on antioxidant properties of milk. The objectives of this study were to determine the effects of dietary supplementation of different levels of CAN on milk composition, milk antioxidant capacity and fatty acid profiles, nutrient digestibility, ruminal fermentation, and microbial protein synthesis.

MATERIALS AND METHODS

All procedures involving animals in the experiment were approved by the State University of Maringa (Maringa, Brazil) Animal Care Ethics Committee under protocol number 9512221018. The experiment and laboratorial analyses were conducted at the dairy unit and ruminant nutrition laboratory of the State University of Maringa.

Cows, Experimental Design, and Diets

Six multiparous Holstein dairy cows (106.3 ± 14.8 DIM; 550.7 ± 21.8 kg of BW; mean $\pm$ SD), adapted to experimental facilities, were used in a replicated 3×3 Latin square design balanced for carryover effects. Each experimental period was 21 d each, with 14 d for adaptation to the experimental diets and 7 d for sampling and data collection. Animals were housed in tiestalls and fed individually for measuring DM intake. Experimental diets were formulated to meet the NRC (2001) requirements for a Holstein cow at mid-lactation with 600 kg of BW, 110 DIM, and producing 25 kg of milk per day, with 3.5% of milk fat, and 3% of milk protein (Table 1).

Feed ingredients (corn silage, ground corn, and soybean meal) were analyzed for chemical composition before the diet formulation. The source of NO_3^- was the double salt of CAN decahydrate [$5\text{Ca}(\text{NO}_3)_2 \cdot \text{NH}_4\text{NO}_3 \cdot 10\text{H}_2\text{O}$], with 85.0% DM, 16.5% N, 19.6% Ca, and 76.5% NO_3^- on a DM basis (Yara International). To ensure that all treatments had the same amount of N and Ca, urea

Table 1. Ingredient and nutritional composition of the experimental diets containing urea or nitrate as nonprotein nitrogen source

Item	Treatment ¹		
	URE	CAN15	CAN30
Ingredient proportion, g/kg of DM			
Corn silage	530	530	530
Ground corn	305	304	304
Soybean meal	121	121	121
Urea ²	11.9	5.94	0.00
Calcium ammonium nitrate ³	0.00	15.0	30.0
Limestone ⁴	17.3	8.65	0.00
Mineral and vitamin supplement ⁵	15.0	15.0	15.0
Nutritional composition, g/kg of DM ⁶			
DM, as-fed basis	469	468	468
OM	932	928	924
CP	164	164	164
RDP	113	113	113
Ether extract	29.8	29.8	29.8
NDF	342	342	342
Forage NDF	289	289	289
Nonfibrous carbohydrate	421	420	420
Nitrate	0.00	11.5	22.9
Ca	9.71	9.71	9.71
P	3.67	3.66	3.66
Ca:P	2.65	2.65	2.65

¹URE = control, without nitrate, containing 12 g of urea/kg of DM; CAN15 = 15 g of calcium ammonium nitrate (CAN)/kg of DM; CAN30 = 30 g of CAN/kg of DM.

²Prote-N, 99.5% DM and 41.7% N on a DM basis; GRASP Ind. & Com. Ltda.

³Double salt of CAN decahydrate [5Ca(NO₃)₂·NH₄NO₃·10H₂O] (85.0% DM; 16.5% N, 19.6% Ca, and 76.5% NO₃⁻ on a DM basis; Yara).

⁴340 g/kg of Ca and 40 g/kg of Mg; Granisul Ind. & Com. Ltda.

⁵Bovigold; composition per kg: 120 g of Ca, 35 g of P, 15 g of S, 38 g of Mg, 50 g of K, 106 g of Na, 15 mg of Co, 417 mg of Cu, 16.5 mg of Cr, 875 mg of Fe, 17.5 mg of I, 1,165 mg of Mn, 15 mg of Se, 2,300 mg of Zn, 350 mg of F, 170,000 IU of vitamin A, 60,000 IU of vitamin D, 1,000 IU of vitamin E; DSM Tortuga.

⁶Unless otherwise stated.

(Prote-N, GRASP Ind. & Com. Ltda.; 99.5% DM, and 41.7% N), and limestone (Granisul Ind. & Com. Ltda.; 340 g of Ca and 40 g of Mg per kg of product) were used for diet formulation.

Cows were assigned randomly to receive 3 different diets: **URE**, 12 g of urea/kg of DM as a control group (without NO₃⁻); **CAN15**: 15 g of CAN/kg of DM (11.5 g/kg of NO₃⁻ on a DM basis); and **CAN30**: 30 g of CAN/kg of DM (23 g/kg of NO₃⁻ on a DM basis). Animals were pre-adapted to the treatments during the first 4 d of each experimental period, where CAN was gradually added (increasing 25% per day) until achieving final concentration for each diet. Experimental diets were offered as a TMR, twice a day at 0630 and 1530 h in proportions of 70 and 30% of the total DM intake, respectively. Diets were adjusted daily to keep 5% of refusals and avoid sorting. Animals were weighed at the end of each period before the morning feeding, and the voluntary DM intake was calculated daily during the week of data collection by the difference between the total DM offered and refused.

Sampling and Proximate Analyses

Sampling and data collection were performed during the last 7 d of each experimental period. Fecal grab samples (~100 g) were collected from d 15 to 21 at different times throughout the week (d 15 at 0600, d 16 at 0800, d 17 at 1000, d 18 at 1200, d 19 at 1400, d 20 at 1600, and d 21 at 1800 h) and frozen at -20°C. Grain mix, corn silage, and refusals were collected daily from d 15 to 20 and frozen at -20°C for analyses. Total mixed ration, refusals, and fecal samples were dried at 60°C for 48 h in a forced-air oven (Heratherm OMS180, Thermo Fisher Scientific) to determine DM content and then ground to pass through 4-mm and 1-mm sieves in a Wiley mill (Thomas Scientific) for chemical analyses.

Feed ingredients were pooled to obtain one sample per period, and fecal and refusals samples were proportionally pooled based on DM to achieve one sample per animal per period. All samples were analyzed according to AOAC (AOAC International, 2012) for total DM content (method 934.01), CP (method 990.03), NDF

(method 2002.04), ash (method 942.05), and ether extract (method 920.39). Organic matter was calculated by the difference between DM and ash. Nonfibrous carbohydrates were calculated based on Van Soest et al. (1991).

Indigestible NDF (**iNDF**) was used as an internal marker to estimate total fecal output and subsequently nutrient digestibility (Cochran et al., 1986). Briefly, samples of feed, refusals, and feces were weighed (0.50 g per bag) into Ankom F57 bags (25- μ m porosity, Ankom Technology) and incubated for 288 h in 2 rumen-cannulated dairy cows. Cows were fed a diet composed of 60% corn silage and 40% grain mix (DM basis). After removal from the rumen, bags were rinsed in tap water until clear and analyzed for NDF in an Ankom 200 Fiber Analyzer for determination of iNDF. Fecal output was calculated as: $\text{iNDF intake (kg/d)}/\text{iNDF\% feces}$, where $\text{iNDF intake (kg/d)} = [\text{iNDF\% TMR} \times \text{TMR offered (kg/d)}] - [\text{iNDF\% orts} \times \text{orts (kg/d)}]$. Total-tract apparent digestibility (%) was calculated for all nutrients analyzed as: $100 - \{[\text{nutrient\% feces} \times \text{fecal output (kg/d)}]/[\text{nutrient intake (kg/d)}]\}$, where $\text{nutrient intake (kg/d)} = [\text{nutrient\% TMR} \times \text{TMR offered (kg/d)}] - [\text{nutrient\% orts} \times \text{orts (kg/d)}]$.

Milk Collection and Laboratory Assays

Milking was performed twice daily at 0600 and 1500 h, and milk production was recorded during the last 7 d of each experimental period. Both a.m. and p.m. milk samples were collected on d 15 and d 16 and pooled for each day proportional to the total production for each milking (morning and afternoon). A 50-mL aliquot was collected and preserved using 2-bromo-2-nitropropane-1,3-diol to analyze fat, protein, and lactose contents by infrared spectrophotometry (Bentley 2000, Bentley Instrument Inc.), and MUN by Berthelot methodology (Chemspec 150, Bentley Instrument Inc.). The FCM was obtained by correcting milk yield per 3.5% of fat according to Sklan et al. (1992), and ECM was calculated using the equations proposed by Sjaunja et al. (1990). Another 5 aliquots of 50 mL, including backup samples, were collected and frozen at -20°C for analyses of NO_3^- and NO_2^- residues, fatty acid (**FA**) profile, and antioxidant capacity in milk.

Milk reducing power was analyzed based on the method from Zhu et al. (2002). Absorbance was measured at 700 nm by a UV-vis spectrophotometer (Spectrum SP2000, Castelnovo), and results were expressed as gallic acid equivalent per kilogram of milk. Conjugated dienes (**CD**) were measured at 232 nm by a UV-vis spectrophotometer (Spectrum SP2000, Castelnovo), calculated, and expressed as millimoles per kilogram of fat (Kiokias et al., 2006). Thiobarbituric acid reac-

tive substances in milk were analyzed as described by Vyncke (1970) at 532 nm by a UV-vis spectrophotometer (Spectrum SP2000, Castelnovo), and results were expressed as millimoles of malonaldehyde per kilogram of fat.

Concentration of NO_3^- was obtained by alkaline catalytic oxidation, which converts nitrogenous compounds into NO_3^- . Subsequently, through cadmium metal, NO_3^- was reduced to NO_2^- and determined by diazotization with sulfanilamide and N-naphthyl (1-naphthyl-ethylenediaminodihydrochloride) as described by Cortas and Wakid (1990).

Milk FA were analyzed via fat extraction by centrifugation, as proposed by Murphy et al. (1995) and esterification according to ISO 5509 method (ISO, 1978) using KOH and methanol. *N*-Heptane and the methyl esters were quantified in a gas chromatograph (Trace GC 52 Ultra, Thermo Scientific), equipped with a flame ionization detector at 240°C and a fused silica capillary column (100 m in length, 0.25-mm internal diameter, and 0.20 μm , Restek 2560). Gas flow rate was 45 mL/min of H_2 (carrier gas), 45 mL/min for N_2 (auxiliary gas), and 45 to 400 mL/min of synthetic air (flame gas). Column temperature was initially set at 50°C (10 min) and raised gradually up to 200°C (15 min), and finally raised to reach 240°C (8 min) as final temperature. Fatty acids were quantified by comparing retention time of FAME from standards (Sigma Aldrich) and milk samples.

Urine, Blood, and Ruminal Fluid Analyses

Urine samples were collected on d 17 and d 18 within 4 h after morning feeding, filtered through 2 layers of cheesecloth, and placed into 50-mL plastic bottles. An aliquot of 10 mL of urine was acidified with 40 mL of sulfuric acid (0.036 *N*) for further analyses. Creatinine was used as a marker to estimate daily urinary volume, assuming a factor for urinary creatinine excretion of 29 mg per kilogram of BW per day (Valadares et al., 1999). Uric acid was measured using a colorimetric assay kit (Gold Analisa), and allantoin was estimated using a method based on Chen and Gomes (1992), adapted to a 96-well plate reader. Total purine derivatives (**PD**) were calculated as the sum of uric acid and allantoin excreted in urine. Endogenous PD excretion was calculated from BW as $0.385 \text{ mmol/BW}^{0.75}$. Daily absorption of microbial purines was calculated as total PD excretion – endogenous PD. Microbial N flow (g/d) was estimated using daily absorption of microbial purines as $(\text{microbial purine absorption} \times 70)/(0.116 \times 0.83 \times 1,000)$, according to Chen and Gomes (1992).

Blood was sampled via jugular vein on d 20 of each experimental period before (0 h) and 4 h after the

morning feeding and transported to a commercial laboratory within 1 h for MetHb analysis. Other samples were collected into serum separator evacuated tubes, centrifuged at $3,200 \times g$ for 15 min at 4°C and stored at -20°C for subsequent analyses. Plasma urea nitrogen (PUN) was analyzed colorimetrically by commercial kits (Gold Analisa) using a spectrophotometer (Bioplus 2000).

Ruminal fluid was collected on d 21 of each period using an esophageal tube attached to a vacuum pump 2 and 8 h after the morning feeding. An aliquot of 50 mL was collected, and the pH was immediately measured by a pH meter (Tecnal). Another aliquot of 50 mL was filtered through 4 layers of cheesecloth, acidified with 1 mL of sulfuric acid (1:1), and stored at -20°C. The concentration of VFA was determined in a gas chromatograph (Shimadzu GC-2010 Plus), equipped with an AOC-20i automatic injector, Stabilwax-DA capillary column (30 m, 0.25-mm ID, 0.25 µm, Restek), and a flame ionization detector after acidifying with 1 M phosphoric acid and fortifying with a WSFA-2 standard (Sigma Aldrich). A 1-µL aliquot of each sample was injected with a 40:1 split rate using He as the carrier gas. Injector and detector temperatures were 250°C and 300°C, respectively. Column temperature ramp started at 40°C, was raised to 120°C at a rate of 40°C/min, followed by a gradient of 120°C to 180°C at a rate of 10°C/min and a rate of 120°C/min for 180°C to 240°C, maintaining the temperature at 240°C for an additional 3 min. Ammonia-N (NH₃-N) concentration was measured via colorimetric quantification of N content using the phenol-hypochlorite reaction as described by Broderick and Kang (1980).

Statistical Analyses

Normality of residuals and homogeneity of variance were examined for each variable analyzed, using the Shapiro-Wilk test. Responses that violated the assumption

of normality were subjected to power transformation using the TRANSREG procedure of SAS (version 9.4, SAS Institute Inc.) as described by Box and Cox (1964). The LSM and SEM were back transformed for presentation of results (Jorgensen and Pedersen, 1998).

Data were analyzed using the MIXED procedure of SAS, using treatment (URE, CAN15, and CAN30) as a fixed effect, and cow within square and period as random effects. Responses with repeated measures were analyzed using the REPEATED statement with statistical models that included the fixed effects of treatment, time, and their interactions, and random effects of cow within Latin square and period. The covariance structure was modeled based on the spatial exponential covariance pattern by using the option TYPE.

Orthogonal polynomial contrasts were used to determine linear and quadratic effects of treatments on the responses analyzed. Treatment significances and trends were declared at $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively. Data were assessed for carryover effects between periods using the ORTHOREG procedure of SAS.

RESULTS

This study was designed as replicated Latin squares balanced for carryover effects; however, considering the short adaptation period, we analyzed data for carryover effects, and no significant differences (Supplemental Tables S1 and S2, <https://doi.org/10.6084/m9.figshare.17159195.v2>, <https://doi.org/10.6084/m9.figshare.17159225.v2>; Almeida, 2021a,b) were observed, except tendencies for allantoin ($P = 0.06$; Table S1) and microbial protein synthesis ($P = 0.07$; Table S1).

DMI and Nutrient Digestibility

Feeding CAN to dairy cows linearly decreased DMI ($P < 0.01$; Table 2). Apparent digestibility of DM, OM, CP, ether extract, and NDF were not affected by treat-

Table 2. Effects of calcium ammonium nitrate (CAN) supplementation on DMI and nutrient digestibility in lactating Holstein dairy cows

Item	Treatment ¹			SEM	P-value ²	
	URE	CAN15	CAN30		Linear	Quadratic
DMI, kg/d	19.1	18.9	16.5	0.68	<0.01	0.09
Apparent digestibility, g/kg of DM						
DM	633	627	639	10.1	0.55	0.34
OM	649	645	658	10.1	0.44	0.37
CP	594	607	588	20.3	0.77	0.35
Ether extract	661	671	670	11.0	0.27	0.45
NDF	474	473	456	21.9	0.45	0.71
NFC	819	825	869	17.0	0.01	0.22

¹URE = control group (without nitrate); CAN15 = 15 g of CAN per kg of DM; CAN30 = 30 g of CAN per kg of DM.

²Linear and quadratic effects of CAN.

Table 3. Effects of calcium ammonium nitrate (CAN) supplementation on milk production, content, yield, and antioxidant capacity in lactating Holstein dairy cows

Item	Treatment ¹			SEM	P-value ²	
	URE	CAN15	CAN30		Linear	Quadratic
Production, kg/d						
Milk	23.3	23.2	23.0	1.31	0.55	0.93
3.5% FCM ³	22.8	22.5	20.7	1.03	0.05	0.38
ECM ⁴	22.6	22.4	20.7	0.99	0.05	0.32
ECM/DMI	1.19	1.19	1.26	0.05	0.03	0.27
Content, %						
Fat	3.39	3.35	2.94	0.23	0.03	0.25
True protein	3.03	3.10	2.91	0.07	0.09	0.05
Lactose	4.64	4.61	4.58	0.07	0.35	1.00
Yield, kg/d						
Fat	0.78	0.77	0.66	0.04	0.03	0.30
True protein	0.71	0.72	0.67	0.04	0.19	0.29
Lactose	1.08	1.07	1.05	0.06	0.35	0.90
Antioxidant capacity						
Reducing power ⁵	12.7	13.7	12.3	1.91	0.89	0.61
Conjugated dienes ⁶	47.6	52.6	63.4	6.56	0.02	0.59
TBARS ⁷	6.42	6.04	7.03	1.23	0.60	0.49
MUN, mg/dL	13.4	14.0	13.9	0.96	0.53	0.50

¹URE = control group (without nitrate); CAN15 = 15 g of CAN per kg of DM = CAN30: 30 g of CAN per kg of DM.

²Linear and quadratic effects of CAN.

³Sklan et al., 1992.

⁴Sjaunja et al., 1990.

⁵Milligrams of gallic acid equivalents per liter.

⁶Millimoles per kilogram of fat.

⁷Thiobarbituric acid reactive substances, expressed as millimoles of malondialdehyde per kilogram of fat.

ments; however, NFC digestibility linearly increased with CAN supplementation ($P = 0.01$; Table 2).

Yield, Composition, Antioxidant Capacity, and Nitrate and Nitrite Concentrations in Milk

Supplemental CAN did not affect milk yield (Table 3); however, 3.5% FCM and ECM were decreased ($P = 0.05$; linear effect), whereas feed efficiency (ECM/DMI) was linearly increased ($P = 0.03$; linear effect). Dietary CAN linearly reduced ($P = 0.03$; linear effect) fat concentration, whereas true protein concentration was quadratically affected ($P = 0.05$; quadratic effect) with greater effects observed with CAN15 compared with URE and CAN30. No effects were observed on milk lactose and MUN concentration (Table 3).

Milk reducing power was not affected by CAN supplementation (Table 3). Similarly, no CAN effect was observed on concentration of thiobarbituric acid reactive substances, whereas the concentration of CD in milk linearly increased ($P = 0.02$; linear effect) as the levels of CAN increased (Table 3).

Nitrate concentration in milk linearly increased with dietary inclusion of CAN ($P = 0.02$; linear effect; Figure 1a), but no effects were observed on milk NO_2^- residues (average: 0.042 mg/L; Figure 1b).

Milk Fatty Acids Profile

No significant treatment differences were observed on milk FA proportions of 6:0, 8:0, 10:0, and 11:0 (Table 4); however, feeding CAN linearly decreased the proportions of 12:0 ($P = 0.01$; linear effect) and 13:0 ($P < 0.01$; linear effect). The proportion of 14:0, 14:1, and 15:1 were unaffected, whereas 15:0 linearly decreased ($P < 0.01$; linear effect) with CAN supplementation. The proportion of 16:0 was linearly decreased ($P = 0.02$; linear effect) with CAN supplementation; however, no effects were observed on the proportion of 16:1, 17:0, 17:1, and 18:0. Similarly, we observed no effects of CAN supplementation on *trans*-9 18:1, *cis*-6 18:2, *cis*-9 18:1, and 18:3n-6, whereas *trans*-6 18:2 ($P = 0.06$) and 18:3n-3 ($P = 0.09$) tended to increase linearly with CAN supplementation. Proportions of *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA were not affected by treatment, but 20:0 ($P = 0.04$; linear effect) and 21:0 ($P = 0.03$; linear effect) were linearly increased, and no effects were observed on 20:1 and 20:2 with CAN supplementation (Table 4).

Total SFA linearly decreased ($P = 0.05$; linear effect), whereas MUFA ($P = 0.06$; linear effect; Table 4) and PUFA ($P = 0.10$; linear effect; Table 4) tended to increase linearly with CAN supplementation. Total

Figure 1. Effects of calcium ammonium nitrate (CAN) supplementation on (a) milk nitrate concentration (NO₃⁻; *P*-values: linear = 0.02; quadratic = 0.87; SEM = 0.02) and (b) milk nitrite concentration (NO₂⁻; *P*-values: linear = 0.26; quadratic = 0.69; SEM = 0.009). URE = control group (without nitrate); CAN15 = 15 g of CAN per kg of DM; CAN30 = 30 g of CAN per kg of DM.

Table 4. Effects of calcium ammonium nitrate (CAN) supplementation on milk fatty acid (FA) profile in lactating Holstein dairy cows

Item ¹	Treatment ²			SEM	<i>P</i> -value ³	
	URE	CAN15	CAN30		Linear	Quadratic
FA proportions						
6:0	0.40	0.41	0.47	0.05	0.36	0.69
8:0	0.63	0.62	0.65	0.05	0.68	0.79
10:0	2.66	2.60	2.52	0.20	0.44	0.92
11:0	0.25	0.24	0.21	0.02	0.10	0.59
12:0	4.60	4.32	4.02	0.32	0.01	0.98
13:0	0.29	0.27	0.22	0.02	<0.01	0.39
14:0	15.7	15.7	15.2	0.57	0.50	0.69
14:1	1.45	1.43	1.49	0.06	0.64	0.55
15:0	2.87	2.83	2.57	0.18	<0.01	0.16
15:1	0.28	0.25	0.25	0.04	0.37	0.67
16:0	37.9	36.9	35.2	1.24	0.02	0.67
16:1	2.00	2.00	2.08	0.13	0.52	0.69
17:0	1.00	0.97	1.01	0.05	0.73	0.43
17:1	0.17	0.17	0.20	0.03	0.27	0.70
18:0	8.49	8.51	9.16	0.58	0.35	0.60
<i>trans</i> -9 18:1	5.39	5.39	5.89	0.53	0.40	0.62
<i>cis</i> -9 18:1	13.6	14.6	15.8	1.22	0.14	0.91
<i>trans</i> -6 18:2	0.59	0.72	0.77	0.08	0.06	0.60
<i>cis</i> -6 18:2	1.00	1.13	1.19	0.09	0.13	0.77
18:3n-6	0.05	0.07	0.05	0.01	0.75	0.31
18:3n-3	0.04	0.05	0.06	0.00	0.09	0.42
<i>cis</i> -9, <i>trans</i> -11 CLA	0.00	0.17	0.11	0.09	0.40	0.31
<i>trans</i> -10, <i>cis</i> -12 CLA	0.01	0.01	0.01	0.01	0.96	0.93
20:0	0.11	0.13	0.14	0.00	0.04	0.84
20:1	0.02	0.04	0.05	0.02	0.31	0.98
20:2	0.06	0.05	0.09	0.01	0.53	0.31
21:0	0.23	0.28	0.42	0.06	0.03	0.50
FA groups						
SFA	75.2	73.8	71.8	1.77	0.05	0.83
MUFA	23.0	23.9	25.8	1.55	0.06	0.68
PUFA	1.71	2.18	2.21	0.22	0.10	0.38
n-3	0.04	0.05	0.06	0.01	0.09	0.42
n-6	1.66	2.12	2.16	0.21	0.10	0.38
n-6:n-3 ratio	38.0	39.3	39.1	2.95	0.65	0.72
Total CLA ⁴	0.02	0.20	0.13	0.10	0.46	0.37

¹G per 100 g of total FA.

²URE = control group (without nitrate); CAN15 = 15 g of CAN per kg of DM; CAN30 = 30 g of CAN per kg of DM.

³Linear and quadratic effects of CAN.

⁴Total CLA = *cis*-9,*trans*-11 CLA + *trans*-10,*cis*-12 CLA.

Table 5. Effects of calcium ammonium nitrate (CAN) supplementation on allantoin, uric acid, and microbial protein synthesis in lactating Holstein dairy cows

Item	Treatment ¹			SEM	P-value ²	
	URE	CAN15	CAN30		Linear	Quadratic
Allantoin, mmol/d	343	354	345	25.1	0.90	0.50
Uric acid, mmol/d	50.9	55.9	67.2	8.56	0.06	0.63
Microbial protein synthesis, ³ g/d	1,871	1,955	1,970	173	0.37	0.71

¹URE = control group (without nitrate); CAN15 = 15 g of CAN per kg of DM; CAN30 = 30 g of CAN per kg of DM.

²Linear = linear effect of CAN; Quadratic = quadratic effect of CAN.

³Chen and Gomes (1992) = $\{[\text{purine derivatives production} - (0.385 \times \text{BW}^{0.75})]/0.85\} \times 0.727$.

CLA and n-6:n-3 ratio were not affected by treatments, but n-3 ($P = 0.09$; linear effect) and n-6 ($P = 0.10$; linear effect) FA tended to increase linearly with CAN supplementation (Table 4).

Blood Parameters and Microbial Protein Synthesis

Levels of PUN were not affected by supplemental CAN (Supplemental Figure S1, <https://doi.org/10.6084/m9.figshare.17159240.v2>; Almeida, 2021c); however, MetHb (% of hemoglobin) increased linearly with CAN supplementation ($P < 0.01$; linear effect) regardless of the sampling time (Supplemental Figure S2, <https://doi.org/10.6084/m9.figshare.17159252.v2>; Almeida, 2021d). Supplemental CAN did not affect allantoin excretion and microbial protein synthesis but tended to increase uric acid concentration ($P = 0.06$; linear effect; Table 5).

Ruminal Fermentation Parameters

No interactions between time (2 and 8 h after feeding) and treatment were observed on ruminal pH, NH_3 -

N concentration, and VFA profile (data not shown). Total VFA, molar proportion of propionate, and butyrate were not affected by treatments (Table 6). However, the molar proportion of acetate linearly increased ($P < 0.01$; linear effect) and acetate-to-propionate ratio tended to increase ($P = 0.07$; linear effect) with CAN supplementation. Isovalerate ($P = 0.01$; linear effect) and valerate proportions ($P = 0.01$; linear effect) were linearly decreased, and isobutyrate tended to decrease with CAN supplementation ($P = 0.08$; linear effect; Table 6).

DISCUSSION

Supplemental NO_3^- has been established as an effective feed additive for mitigating CH_4 emissions in ruminants (Lee and Beauchemin, 2014; Beauchemin et al., 2020; Feng et al., 2020). However, studies aiming to evaluate milk characteristics such as antioxidant capacity and residues of NO_3^- and NO_2^- in response to NO_3^- supplementation are still needed, due to the importance of milk for human consumption. We evaluated milk composition of lactating dairy cows along with

Table 6. Effects of calcium ammonium nitrate (CAN) supplementation on ruminal pH, NH_3 -N concentration, and VFA profile in lactating Holstein dairy cows

Item	Treatment ¹			SEM	P-value ²	
	URE	CAN15	CAN30		Lin	Quad
pH	6.80	6.75	6.78	0.13	0.88	0.71
NH_3 -N, mM	13.3	10.9	10.5	1.22	0.27	0.64
Total VFA, mM	73.0	72.5	71.7	6.17	0.85	0.97
Individual VFA, mol/100 mol						
Acetate	59.2	60.7	63.0	1.24	<0.01	0.54
Propionate	23.6	23.9	21.7	1.64	0.20	0.34
Isobutyrate	0.83	0.75	0.77	0.05	0.08	0.10
Butyrate	12.7	11.4	11.8	0.68	0.27	0.24
Isovalerate	1.81	1.61	1.33	0.17	0.01	0.81
Valerate	1.77	1.71	1.44	0.16	0.01	0.33
Acetate:propionate	2.59	2.65	2.95	0.20	0.07	0.42

¹URE = control group (without nitrate); CAN15 = 15 g of CAN per kg of DM; CAN30 = 30 g of CAN per kg of DM.

²Linear and quadratic effects of CAN.

antioxidant properties of milk and rumen fermentation parameters in response to CAN supplementation. The 14-d adaptation period used in this study is admittedly short and may not be long enough to allow for adaptation of microbes; hence, data should be interpreted cautiously. Long-term studies are needed, to confirm the findings observed in the present study.

Feed Intake and Nutrient Digestibility

Nitrate supplementation reduced feed intake by 13% in the present study. In agreement with our findings, Lund et al. (2014) reported lower DMI with 20 g/kg of DM of CAN supplementation in dairy cows. Similarly, supplemental CAN (up to 23 g/kg NO_3^- on a DM basis) reduced intake of both pasture and concentrate in grazing cows, subsequently lowering milk production (van Wyngaard et al., 2018). Recently, Meller et al. (2019) also observed lower DMI with CAN fed to Jersey cows (20 g/kg NO_3^- on DM basis). The effects of CAN supplementation on lowering feed intake may be attributable to lower palatability of the diets due to the bitter taste of NO_3^- salts (Lee and Beauchemin, 2014). Lower DMI in response to CAN supplementation may have resulted in the lower ECM and FCM observed in the present study. Encapsulating NO_3^- salts can alleviate negative effects of NO_3^- supplementation on DMI (Lee et al., 2015b), as its slow release would ensure faster conversion of NO_3^- to NH_3 . However, encapsulation may lower the efficacy of NO_3^- at inhibiting methanogens, thereby reducing the antimethanogenic potential of NO_3^- supplementation. Nevertheless, previous studies using an encapsulated NO_3^- source with vegetable oil (GRASP Ind. & Com. Ltda.; EW Nutrition GmbH) did not reduce negative effects on feed intake (Lee et al., 2015a; Rebelo et al., 2019). Future studies are required to evaluate supplementing NO_3^- salts combined with other feed additives that could enhance feed palatability; for instance, molasses, which, according to DeVries and Gill (2012), increases feed palatability and minimizes negative effects on DMI and milk yield.

Supplemental CAN had no effect on nutrient digestibility in dairy cows, and the effects may be attributable to gradual adaptation of dairy cows to NO_3^- diets, possibly alleviating the effects of CAN on rumen microbiota and, subsequently, nutrient digestibility. Zhou et al. (2012) reported, using an in vitro study, that supplementing NO_3^- at 12 $\mu\text{mol/mL}$ (~ 12 g/kg of DM) decreased methanogens ($\sim 97\%$ less) and also decreased the abundance of cellulolytic bacteria (*Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens*). Increased redox potential and NO_2^- ac-

cumulation may negatively affect NDF digestibility (Zhou et al., 2012). Nevertheless, the lack of effects on nutrient digestibility observed in the present study is in agreement with earlier studies evaluating dietary NO_3^- in dairy cows (van Zijderveld et al., 2011; Olijhoek et al., 2016; Wang et al., 2018). The lack of NO_3^- effects on nutrient digestibility might be attributable to the dose used; however, we cannot rule out the confounding effect of lower intake with NO_3^- and subsequently slower passage rate on nutrient digestibility. The linear improvement in NFC digestibility observed in this study might be attributable to increased starch digestibility, since starch is a major constituent of NFC. However, the effects of CAN supplementation on increased starch digestibility are difficult to explain considering lack of NO_3^- effects on total VFA and ruminal propionate.

Milk Composition and Milk Antioxidant Capacity

Both ECM and FCM were lower with NO_3^- supplementation, and these effects could be attributed to lower DMI and fat content observed in the present study. Similar findings have been observed earlier, as van Wyngaard et al. (2018) reported negative effects of NO_3^- supplementation on FCM and ECM due to a reduction in fat and protein content, respectively. However, Olijhoek et al. (2016) did not find differences in ECM in response to supplementing CAN at 21 g/kg (DM basis), and the results were attributed to lack of differences in DMI, nutrient digestibility, and rumen fermentation parameters.

The effects of NO_3^- on decreasing milk fat yield may be explained by lower DMI, including NDF fractions. Similarly, milk protein concentration was quadratically affected, with greater values observed for CAN15 and lower values observed with CAN30. van Zijderveld et al. (2011) observed reduction in milk protein concentration with supplementation of 21 g/kg NO_3^- on a DM basis for 89 d and attributed this to a dilution effect. Milk protein responses might be attributable to the effects of NO_3^- supplementation on DMI, which may have affected ruminal and post-ruminal N availability and subsequently milk protein synthesis, although no effects were observed on microbial protein synthesis, which makes it difficult to explain. Similarly, Klop et al. (2016) evaluated NO_3^- versus docosaheptaenoic acid in a factorial arrangement of treatments and observed low protein content and yield when supplementing 21 g/kg DM of NO_3^- to dairy cows. The effects on milk protein responses were attributed to lower DMI and limited supply of gluconeogenic precursors with NO_3^- supplementation (Klop et al., 2016). Milk urea nitrogen was also unaffected by treatment, and similar findings

were observed earlier with supplemental NO_3^- (van Zijderveld et al., 2011; Klop et al., 2016; van Wyngaard et al., 2018).

To the best of our knowledge, we are lacking data on the effects of supplemental NO_3^- on milk antioxidant capacity. In addition to FA, vitamins, and minerals, the antioxidant capacity of milk has potential for improving human health by removing free radicals from the body (Khan et al., 2019). In our study, reducing power and thiobarbituric acid reactive substances were unaffected by treatment; however, CD linearly increased with CAN supplementation. Conjugated dienes are considered an indicator of lipid peroxidation (Guillén and Cabo, 2002). Although we are still lacking a concrete explanation for the increase in CD in response to NO_3^- supplementation, we can speculate that lower SFA, along with a tendency to increase MUFA and PUFA as a response to CAN supplementation, may have predisposed milk FA to oxidation, resulting in greater concentration of CD. Previous studies have shown that oxidation of UFA contributes to the formation of hydroperoxide and shifts of double bonds (Hamilton, 1983; Jadhav et al., 1996). Another speculation is that greater concentrations of NO_3^- and NO_2^- may form NO and other bioactive nitrogen oxides after being absorbed in the blood and tissues (Lundberg et al., 2008). The presence of NO in milk can induce nitrosative stress due to its reaction with oxygen, superoxide, or hydroperoxide (Silanikove et al., 2009, 2012). Thus, these reactions likely lowered milk antioxidant capacity and induced lipid peroxidation, resulting in greater production of CD.

Nitrate and Nitrite Residues in Milk

Nitrate as a feed additive for dairy cows may induce potential toxicity if animals are not well adapted to the treatments. Furthermore, presence of NO_3^- or NO_2^- residues in animal tissues and products such as meat and milk may cause health problems in humans (Bryan and van Grinsven, 2013; Lee et al., 2017a). Based on WHO (2011) guidelines, the maximum NO_3^- concentration in milk for safe consumption is 50 mg/L. We observed greater levels of milk NO_3^- (0.30 to 0.38 mg/L for URE and CAN30, respectively) with CAN supplementation, whereas NO_2^- residue was similar for all treatments, averaging 43 µg/L. Olijhoek et al. (2016) observed much greater NO_3^- residue (1.56 mg/L) in milk with CAN supplementation (21.1 g/kg on DM basis); however, NO_2^- residues were not detected. Overall, studies have shown low NO_3^- and NO_2^- residues in milk with NO_3^- supplementation in dairy cows, suggesting that consuming milk from treated dairy cows would not be a concern for food safety (Guyader et al., 2016a; Meller et al., 2019).

Milk Fatty Acid Profile

Dietary manipulation can have significant effects on the milk FA profile in dairy cows (Lock and Bauman, 2004). Klop et al. (2016) investigated the effects of NO_3^- and docosahexaenoic acid on the milk FA profile and observed no effects on SFA and MUFA profiles with NO_3^- supplementation; however, the PUFA proportion was increased. In the current study, we observed lower SFA and greater proportions of MUFA and PUFA in milk. Nitrates and NO_2^- are alternative [H] sinks that divert H_2 away from methanogenesis and biohydrogenation, which would explain lower SFA in the present study; however, we are still lacking enough studies validating this hypothesis because of low H_2 dissipation at the expense of ruminal biohydrogenation (Yang et al., 2019). Indeed, similar to our results, Sharifi et al. (2021) observed no effects of NO_3^- supplementation to dairy cows on 18:0 proportion, as indicative of biohydrogenation in the rumen. According to Lourenço et al. (2010), biohydrogenation could be affected indirectly when other ruminal activities are changed (i.e., CH_4 inhibition), because FA metabolism is strictly dependent on the microbial species that are involved in multiple metabolic processes along with H_2 metabolism. In fact, NO_3^- intermediates have toxic effects on ruminal microorganisms, including the fibrolytic bacteria and methanogens (Latham et al., 2016). We can speculate that NO_2^- might have negatively affected ruminal abundance of a predominant biohydrogenating bacteria, *Butyrivibrio proteoclasticus*, as observed earlier in response to NaNO_2^- (2 g/L) supplementation in high-concentrate diets under in vitro conditions (Yang et al., 2019). Changes in ruminal bacteria populations may be associated with the effects observed on the milk FA profile in the current study.

Lower SFA may be considered positive for milk quality because of perceived effects of SFA on cardiac health (Lock and Bauman, 2004). Nevertheless, it is well established that milk with greater MUFA and PUFA proportions is more susceptible to lipid oxidation than milk with greater proportions of SFA (Zhao et al., 2013). Therefore, we can speculate that lower SFA and greater MUFA and PUFA may have predisposed milk FA to oxidation, resulting in increased concentration of CD.

Blood Methemoglobin and Plasma Urea Nitrogen

Nitrate poisoning is still a concern with its inclusion in the diets of dairy cows. However, a prior and gradual adaptation may lower the risk of NO_3^- toxicity, due to low levels of MetHb. In the present study, MetHb linearly increased with increased dietary NO_3^- levels,

and greater levels were observed 4 h after feeding (3.26% hemoglobin) for CAN30. However, the levels of MetHb observed were much lower than the 30 to 40% hemoglobin required to induce methemoglobinemia (Bruning-Fann and Kaneene, 1993). The levels of MetHb observed in this study were lower than previous studies evaluating dietary NO_3^- in changeover (4.8%; Olijhoek et al., 2016) and continuous studies (4.7%; van Zijderveld et al., 2011). Supplementing CAN up to 30 g/kg on DM basis with a gradual adaptation might have resulted in lower levels of MetHb, thereby lowering the chance of NO_3^- toxicity.

Treatment did not affect the concentration of PUN; however, PUN levels increased 4 h after feeding. We assume, with such an effect, that URE, CAN15, and CAN30 provided similar amounts of N to be absorbed through the rumen wall. Contrary to our findings, Wang et al. (2018) observed greater levels of NH_3 in the plasma of cows receiving urea compared with supplemental NO_3^- (14.6 g/kg of DM).

Microbial Protein Synthesis, Ammonia-N, and VFA Proportions

Besides acting as an [H] sink, NO_3^- salts are sources of NPN, essential for the rumen microbiota to synthesize microbial protein. Wang et al. (2018) speculated that sodium nitrate increased NH_3 incorporation into microbial protein in the rumen compared with urea in cows fed a low-protein diet. In our study, treatment did not affect microbial protein synthesis, suggesting that CAN seemed to have similar efficacy at providing NH_3 for bacteria growth, and such results agree with previous findings (Olijhoek et al., 2016) when NO_3^- was supplemented up to 21 g/kg of DM in the diet of lactating dairy cows. However, the estimation of microbial protein synthesis by PD must be interpreted with caution because of inaccurate relationship observed between microbial protein synthesis and urinary excretion of PD, as suggested recently (Hristov et al., 2019). Total PD used to evaluate microbial protein synthesis may not accurately reflect changes in microbial protein flow because duodenal digestibility of purines and recovery in urine are influenced by dietary factors as well as by the physiological state of animals (Dewhurst et al., 2000).

Nitrate supplementation had no effects on ruminal NH_3 -N concentration, corroborating previous studies that evaluated supplemental NO_3^- fed to dairy cows (Guyader et al., 2016a; Meller et al., 2019). Despite the absence of effects in our study, it is well established that NO_3^- has a slower conversion to NH_3 -N within the rumen compared with urea. In fact, NO_3^- reduction

occurs in 2 steps, in which NO_3^- is first converted into NO_2^- , and posteriorly NO_2^- is converted into NH_3 in a dissimilatory pathway (Leng, 2008; Lee et al., 2017b). In addition, NO_3^- could also be converted into N_2O by denitrification, which is an additional step of the dissimilatory pathway (Latham et al., 2016; Torres et al., 2016).

The lack of effects observed on total VFA is in agreement with previous studies (Olijhoek et al., 2016; Meller et al., 2019). However, total VFA concentrations (mM) in our study were lower than reported earlier, probably due to the use of stomach tubes for collecting rumen fluid (Shen et al., 2012). Supplemental CAN increased the molar proportion of acetate and the acetate-to-propionate ratio, and the results are in agreement with previous studies (Božić et al., 2009; Zhou et al., 2012; Latham et al., 2016). Similar responses in VFA proportions with NO_3^- supplementation were documented earlier (Patra and Yu, 2013; Guyader et al., 2016b; Wang et al., 2018). The greater acetate production observed may be attributable to the stimulation of thermodynamically favorable pathway for [H] disposal during NO_3^- to NH_3 conversion (Ungerfeld and Kohn, 2006). Nitrate reduction leads to a sharp decline in NADH and increased concentrations of NAD^+ , which favors greater production of acetate (Nolan et al., 2010).

CONCLUSIONS

Supplementing CAN at 30 g/kg of DM to dairy cows decreased DMI and lowered milk fat and milk protein concentration, as well as increased molar proportions of ruminal acetate; however, no effects were observed on microbial protein synthesis. Low levels of NO_3^- and NO_2^- residues in milk, along with low MetHb in blood, validate the importance of gradual adaptation of dietary NO_3^- in lactating dairy cows. The effects of CAN on milk FA and antioxidant capacity observed in this study may have induced faster milk lipid oxidation, as it lowered SFA proportion and increased CD concentration, respectively. Based on these results, dietary CAN supplemented at 30 g/kg of DM should not be recommended as an optimal dose for lactating dairy cows. However, data must be interpreted carefully due to the short period of animal exposure to NO_3^- .

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