Potential roles of nitrate and live yeast culture in suppressing methane emission and influencing ruminal fermentation, digestibility, and milk production in lactating Jersey cows

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ABSTRACT

Concern over the carbon footprint of the dairy industry has led to various dietary approaches to mitigate enteric CH₄ production. One approach is feeding the electron acceptor NO₃[−], thus outcompeting methanogens for aqueous H₂. We hypothesized that a live yeast culture (LYC; *Saccharomyces cerevisiae* from Yea-Sacc 1026, Alltech Inc., Nicholasville, KY) would stimulate the complete reduction of NO₃[−] to NH₃ by selenomonads, thus decreasing the quantity of CH₄ emissions per unit of energy-corrected milk production while decreasing blood methemoglobin concentration resulting from the absorbed intermediate, NO₂[−]. Twelve lactating Jersey cows (8 multiparous and noncannulated; 4 primiparous and ruminally cannulated) were used in a replicated 4 × 4 Latin square design with a 2 × 2 factorial arrangement of treatments. Cattle were fed diets containing 1.5% NO₃[−] (from calcium ammonium nitrate) or an isonitrogenous control diet (containing additional urea) and given a top-dress of ground corn without or with LYC, with the fourth week used for data collection. Noncannulated cows were spot measured for CH₄ emission by mouth using GreenFeed (C-Lock Inc., Rapid City, SD). The main effect of NO₃[−] decreased CH₄ by 17% but decreased dry matter intake by 10% (from 19.8 to 17.8 kg/d) such that CH₄:dry matter intake numerically decreased by 8% and CH₄:milk net energy for lactation production was unaffected by treatment. Milk and milk fat production were not affected, but NO₃[−] decreased milk protein from 758 to 689 g/d. Ruminal pH decreased more sharply after feeding for cows fed diets without NO₃[−]. Acetate:propionate was greater for cows fed NO₃[−], particularly when combined with LYC (interaction effect). Blood methemoglobin was higher

for cattle fed NO₃[−] than for those fed the control diet but was low for both treatments (1.5 vs. 0.5%, respectively; only one measurement exceeded 5%), indicating minimal risk for NO₂[−] accumulation at our feeding level of NO₃[−]. Although neither apparent organic matter nor neutral detergent fiber digestibilities were affected, apparent N digestibility had an interaction for NO₃[−] × LYC such that apparent N digestibility was numerically lowest for diets containing both NO₃[−] and LYC compared with the other 3 diets. Under the conditions of this study, NO₃[−] mitigated ruminal methanogenesis but also depressed dry matter intake and milk protein yield. Based on the fact that few interactions were detected, LYC had a minimal role in attenuating negative cow responses to NO₃[−] supplementation.

Key words: nitrate, yeast culture, methane emission

INTRODUCTION

Methane contributes significantly to global warming, with dairy cattle estimated to produce 128 kg of CH₄/head per year (Hristov et al., 2013). In addition to dietary additives, managerial aspects must be considered more strategically before mitigation practices become more practicable (Knapp et al., 2014). The rumen is populated by bacteria, protozoa, and fungi that degrade feedstuffs and ferment resultant sugars to VFA used by their host. Then, archaea convert formate and CO₂ plus H₂ from sugar fermentation into methane, which is lost in the metabolizable energy system. Pyruvate decarboxylation to acetyl CoA often conserves energy from reducing equivalents (referred to as [2H]) for electron transport phosphorylation (Hackmann and Firkins, 2015). Buildup of [2H] has been hypothesized to thermodynamically limit rumen fermentation pathways to limit the production of H₂ used in methanogenesis (Janssen, 2010). Rather than strategies to inhibit H₂ production to suppress methanogenesis, alternative electron receptors such as NO₃[−] salts have been added so that NO₃[−]-reducing bacteria compete with methano-

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gens for aqueous H_2 (Morgavi et al., 2010). Therefore, our aim was to assess whether dietary NO_3^- salts would mitigate methanogenesis while potentially shifting fermentation pathways and bacterial populations, as we detected in continuous culture (Welty et al., 2019).

Mixed responses to various dietary strategies (Hristov et al., 2013) and energetic efficiency (Ungerfeld, 2018) necessitate further in vivo research to abate methane production with less potential to lose productivity under different dietary conditions. In the rumen, NO_3^- reduction to NO_2^- is faster than NO_2^- reduction to NH_3 , leading to absorption of NO_2^- and formation of methemoglobin (Yang et al., 2016). Signs of NO_3^- poisoning are noted 3 to 6 h after feeding (Lee and Beauchemin, 2014), suggesting a bottleneck in NO_2^- reduction and a greater need to understand microbial species and processes involved in the rumen. The current study was designed to help explain variability in production responses associated with feeding NO_3^- .

Wolinella succinogenes and *Selenomonas ruminantium* are bacteria capable of fully reducing NO_3^- to NO_2^- to NH_3 (Latham et al., 2016; Yang et al., 2016). However, *W. succinogenes* is a minor member of the rumen, and *S. ruminantium* can increase (Wang et al., 2018) or can remain in relatively low concentration when NO_3^- is fed (Popova et al., 2017). Such variability might be a result of sequencing approaches compared with quantitative PCR (Jiang et al., 2017). However, protozoa and uncharacterized bacteria other than *S. ruminantium* appear to have important roles in NO_3^- and NO_2^- reduction (Yang et al., 2016), and protozoa appear to reduce NO_3^- but also are inhibited by NO_2^- (Roman-Garcia et al., 2019). In a previous study, Welty et al. (2019) suggested that live yeast culture (LYC) could help mediate NO_2^- accumulation in continuous cultures. Therefore, the LYC Yea-Sacc *Saccharomyces cerevisiae* 1026 (Alltech Inc., Nicholasville, KY) was fed to lactating dairy cattle in the current study.

Many strains or extracts of *S. cerevisiae* have been used, with mixed responses on methanogenesis (Jeyanathan et al., 2014). However, one mechanism might be that *S. cerevisiae* could stimulate growth of the lactolytic and propionigenic strains of *S. ruminantium* (Nisbet and Martin, 1991), which also could improve NO_2^- reduction indirectly. In addition to a potential role in preventing lower ruminal pH (Desnoyers et al., 2009), *S. cerevisiae* also can help quench O_2 (Jeyanathan et al., 2014). Increased cellulolytic bacterial abundance and NDF digestibility (Pinloche et al., 2013; AlZahal et al., 2014) could increase acetate and, therefore, CH_4 production but also increase DMI and milk production such that more research is needed to ascertain

whether the ratio of CH_4 produced per unit of milk NE_L will consistently decrease. Variable factors include the potential for LYC to increase frequency of feeding (DeVries and Chevaux, 2014; Yuan et al., 2015), whereas nitrates have suppressed DMI, likely through palatability issues that also could influence feeding behavior (Lee and Beauchemin, 2014; Yang et al., 2016). Therefore, we questioned whether LYC would interact with dietary NO_3^- to influence DMI and the ratio of CH_4 :milk NE_L production.

Our objectives were to feed diets without or with NO_3^- and without or with LYC to assess potential interactions on methane production, lactation performance, total-tract digestibility, ruminal fermentation characteristics, and ruminal microbial populations. We hypothesized that NO_3^- would decrease CH_4 emission by lactating Jersey cows without affecting DMI or digestibility if LYC also was fed, LYC would improve NDF digestibility regardless of whether or not NO_3^- was fed, and LYC would stimulate the complete reduction of NO_3^- to NH_3 by selenomonads or related bacterial populations.

MATERIALS AND METHODS

Experimental Design

Twelve lactating Jersey cows (4 were primiparous and ruminally cannulated) were assigned to 1 of 4 diets in a replicated 4×4 Latin square design that was balanced for carryover effects. Treatments were arranged in a 2×2 factorial arrangement of diets containing either urea or 1.5% NO_3^- (DM basis; isonitrogenous to control) and without or with Yea-Sacc 1026 (Alltech Inc.). Each of the 4-wk periods had 1 wk for diet adjustment, 2 subsequent weeks for rumen adaptation, and 1 wk for sampling. Cows were adapted to the NO_3^- diet by feeding 0.5% NO_3^- on a DM basis for 3 d, followed by 1.0% NO_3^- on a DM basis for 4 d for the first week of each period before feeding 1.5% NO_3^- on a DM basis at the start of the second week of each period. The dose of 1.5% NO_3^- was chosen because a previous study feeding 2.2% NO_3^- had a tendency to increase methemoglobin over 5% (van Zijderveld et al., 2011a), which we wanted to avoid. In NO_3^- treatment diets, we fed 2.0% of DM as calcium ammonium nitrate (Calcinit, Yara North America Inc., Tampa, FL; 75% NO_3^-). To ensure both diets were isonitrogenous, the control diet was balanced with urea. Both rations also were balanced for calcium. Fresh feed was mixed once daily with part fed at 0630 h, and the remainder was delivered at 1700 h. Feed offered andorts were recorded daily. Refusals were limited to

3% of feed offered to prevent sorting of nitrate-based concentrate in the TMR.

Two top-dresses were provided to cows twice daily at a rate of 50 g. The control top-dress was 100% ground corn. The other consisted of 5% Yea-Sacc 1026 and 95% ground corn, which was thoroughly mixed before feeding. The top-dress was formulated to contain 1×10^8 cfu/g. The yeast product and its top-dress were stored separately in a shed maintained above freezing to maintain yeast efficacy.

All procedures were approved by The Ohio State University Institutional Animal Care and Use Committee. Cows were individually housed in a tiestall barn at the Waterman Dairy Center and milked twice per day. Cows were weighed every Saturday after the morning milking. Each BW was averaged per period or was regressed over time using Microsoft Excel from which the linear slope represented ADG during that period.

Sample Collection and Laboratory Analyses

During the fourth week, feed offered andorts were collected for 4 consecutive days. Samples of TMR were composited by weight per animal per period, and refusals were composited as percentage of total refusals. Samples were dried at 55°C in a forced-air oven for 48 h to determine DM and then ground through a 2-mm screen with a Wiley mill (Arthur H. Thomas, Philadelphia, PA). Samples were analyzed for N in duplicate by Kjeldahl with a Foss Tecator unit (1988, Foss, Hoganas, Sweden). Kjeldahl does not completely recover $\text{NO}_3\text{-N}$ (Bremner and Mulvaney, 1982). Hence, we spiked the control concentrate with 4 increasing dosages of NaNO_3 and repeated Kjeldahl analysis. Nitrate in feeds was assessed by Cumberland Valley Analytical Services (Waynesboro, PA) using method 986.31 (AOAC, 1990). Using sodium sulfite and heat-stable α -amylase, NDF was ashed at 450°C overnight (Van Soest et al., 1991).

Milk production was recorded electronically for all cows twice daily using Afimilk (Kibbutz Afikim, Israel). Milk samples were collected from 4 consecutive milkings each week and were composited by cow and analyzed by DHI Cooperative Inc. (Columbus, OH) for fat and true protein (B2000 Infrared Analyzer; Bentley Instruments, Chaska, MN) and MUN (Skalar SAN Plus segmented flow analyzer; Skalar Inc., Norcross, GA). Following the procedure by Woollard and Indyk (2014), milk was clarified using solutions of Carrez I and II (Sigma-Aldrich, St. Louis, MO) before NO_3^- and NO_2^- were assayed using EPA method 353.2 (O'Dell, 1993).

Rumen measurements of pH and samples for determination of ammonia, VFA, protozoa counts, and bacterial relative abundance were taken from the

rumen-cannulated cows during the sampling week at 0, 3, 6, and 9 h after feeding. Rumen samples were taken from 3 different locations; a subsample of particulate contents and fluid was blended in a Waring blender (Stamford, CT) for 1 min with buffer modified from Weller and Pilgrim (1974) to exclude urea and frozen at -80°C . After thawing, we pooled 3 mL of each of the 4 sampling times using sterilized pipette tips cut to sample particulate matter before DNA extraction following methods described by Yu and Morrison (2004). The extracted DNA was sequenced using the Illumina MiSeq platform (Illumina Inc., San Diego, CA) at the Molecular and Cellular Imaging Center (Ohio Agricultural and Research and Development Center, The Ohio State University, Wooster, OH). Primer pairs 515F and 806R amplified sequences from the V4-V5 hypervariable region of bacteria and archaea using procedures described by Park and Yu (2018). Computational analyses, richness (number of operational taxonomic units), Shannon-Wiener diversity index, and Bray-Curtis dissimilarity index were derived as described by Faulkner et al. (2017). The remainder of the original ruminal sample was squeezed through 2 layers of cheesecloth; pH was immediately taken from the sample with a portable pH meter before 3 mL of 6 N HCl was added to 50 mL to prevent microbial growth, after which the sample was stored at -20°C . Samples were later thawed and centrifuged at $15,000 \times g$ for 20 min at 4°C . The supernatant was filtered through Whatman no. 1 filter paper (Whatman International, Maidstone, UK). One aliquot of supernatant was analyzed for $\text{NH}_3\text{-N}$ (Chaney and Marbach, 1962), and the other was analyzed for VFA (Harvatine et al., 2002). From the original rumen fluid, 6 mL was added to 6 mL of 50% formalin and kept at room temperature; after pooling across days within cow by period, protozoa were enumerated according to Dehority (1993).

Blood was collected during of the sampling week (same day for all cows) at 0 and 3 h after feeding in heparinized blood tubes. Methemoglobin was analyzed spectroscopically by a StatProfile pHox Ultra blood gas analyzer (Nova Biomedical division of Tecom Analytical Systems, Vienna, Austria) at The Ohio State University Veterinary Medical Center (Columbus, OH).

Pellets consisting of 95% soy hulls and 5% Cr_2O_3 were dosed into the rumen via the cannula of the 4 cannulated cows at morning and evening feed delivery. The amount given to each cow equaled 1% of the estimated DMI. After 5 d of predosing Cr_2O_3 , fecal collection times were staggered over the 4 d to represent every 1.5 h of a 12-h period between a.m. and p.m. feedings. The fecal samples were dried (55°C) and ground through a 2-mm screen with a Wiley mill. Samples were then composited

within cow by weight for each period. Fecal samples were ashed in duplicate overnight at 600°C. Chromium was extracted from the residue using the procedure of Williams et al. (1962) except that CaCl_2 was not added because Cr was analyzed by inductively coupled plasma spectrometry (STAR laboratory, Wooster, OH) rather than atomic absorption spectroscopy, as in the original procedure. Similarly, Cr was analyzed in the Cr_2O_3 pellets to compute Cr dose. Concentrations of NDF, OM, and N of the TMR, refusals, and fecal samples were determined as described previously. Digestibilities of NDF, OM, and N were calculated from these results.

For each period, samples of corn silage and alfalfa being fed during each period were dried at 55°C and ground through a 2-mm screen using a Wiley mill. Corn silage and alfalfa were composited according to their relative proportion in the diet (3 g total per Dacron bag; $50 \pm 10 \mu\text{m}$; Ankom Technology, Macedon, NY). Bags were incubated in situ for 0, 2, 4, 8, 12, 24, 48, 72, and 96 h. After removal, bags were placed in cold water and then rinsed in running cold tap water until the rinse was clear. Each bag was dried at 55°C in a forced-air oven for at least 48 h. Residual NDF and N were analyzed as described previously.

Methane and CO_2 measurements were collected using an automated system (GreenFeed, C-Lock Inc., Rapid City, SD) as per Hristov et al. (2015). In brief, 8 samples were collected over a 3-d period to have a representative collection throughout a day while minimizing the disturbance of cows' lying and feeding times. Only noncannulated cows were used because ruminal gasses can escape through the fistula. Calibration and recovery should be verified in methane measurements (Hammond et al., 2016). Therefore, before sampling for each period, calibration and usage procedures followed Hristov et al. (2015). GreenFeed was warmed for 30 min before being calibrated using a zero gas, 100% N_2 and span gas (1,059 mg/m^3 of CH_4 and 0.98% CO_2 based on gas certificates; Praxair Inc., Columbus, OH). Recovery of CO_2 was conducted in triplicate after calibration and before each sampling time. Cattle were baited into the GreenFeed system using a portion of their specific grain mix for that day, so nutrient composition and intakes were not biased among treatments. The grain was continuously dropped into the GreenFeed feed pan to keep each cow's muzzle within manufacturer's recommended range to properly sample all expired CH_4 and CO_2 . At least 5 min of uninterrupted gas recording (averaging 5 min and 57 s) was stored from each cow (i.e., if a cow moved away, the clock restarted) after which the system was backed away from the cow. It was flushed with air with a box fan for 2 min before being placed in front of another cow. It took approximately 1.2 h to sample 8 cows at each sampling time. Blinded to treatment, CH_4

and CO_2 recordings were confirmed and converted to flux rates courtesy of the manufacturer after accounting for tracer gas recovery.

Statistical Analyses

Data were analyzed using PROC MIXED of SAS (version 9.4, SAS Institute Inc., Cary, NC) using ANOVA. In a Latin square design, cow and period were random effects, and treatments (fixed effect) were arranged as a 2×2 factorial that was analyzed with ANOVA. Disappearance kinetics from Dacron bags were analyzed with PROC NLIN with the resultant parameter estimates being analyzed with the same model using PROC MIXED. Digestibility data were assessed in single Latin square of cannulated cows, CH_4 data were assessed in the 2 squares of intact cows, and performance data were assessed in all 3 squares. Ruminal pH and VFA were analyzed using repeated measures using the covariance structure with the lowest Akaike information criterion, which was the first-order moving average model. When the interaction with time was $P > 0.10$, main effect means were evaluated using the same ANOVA; when $P < 0.10$, ANOVA was done by hour. Microbial relative abundance data from the rumen-cannulated cows were analyzed as a single Latin square, as above; however, results for almost all of the taxa had an estimated G matrix that was not positive definite (i.e., variance was bounded at zero). Therefore, period was changed from a random to a fixed effect. One cow was removed in period 3 from 1 of the 2 squares of noncannulated cows for reasons unrelated to her treatment ($-\text{NO}_3^-/\text{+LYC}$) when milk production dropped 45% in 2 wk to be below 11 kg/d and she was dried off during period 4 (she would have been fed the $+\text{NO}_3^-/\text{+LYC}$ treatment). Significance was declared at $P \leq 0.05$; and trends at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Diet Compositions

Diet ingredients are shown in Table 1. Chemical composition and NO_3^- concentration of the forages and grain mixes are shown in Table 2. The as-is feed offered was adjusted weekly for varying DM concentration in feed ingredients. Composite samples per period of concentrate had an average 3.41% DM as NO_3^- , which deviated slightly from the 3.57% formulated value as a result of sampling or measurement errors. Nelson and Sommers (1973) noted a recovery of 16 to 55% of NO_3^- -N, and our analyses of the control concentrate that was spiked with 4 increasing amounts of NO_3^- -N resulted in a mean of 29% (SD 21%). Some indication existed

Table 1. Ingredient composition (% of DM) for cows fed diets without (–) or with (+) NO_3^-

Item	Diet	
	– NO_3^-	+ NO_3^-
Corn silage	33.687	33.687
Alfalfa baleage	16.518	16.518
Whole cottonseed	6.520	6.520
Soybean hull pellets ¹	1.000	1.000
Shelled corn, ground ²	0.479	0.479
Concentrate		
Soybean hulls	6.172	6.172
Shelled corn, ground	21.811	21.691
Urea	0.689	0
Calcinit ³	0	1.986
Amino Plus ⁴	8.259	8.259
Copper sulfate	0.003	0.004
Dicalcium phosphate	0.543	0.543
Gypsum	0.217	0.217
Limestone	1.224	0
Magnesium oxide ⁵	0.076	0.120
Selenium, 200 mg/454 g	0.150	0.150
Trace-mineralized salt ⁶	0.500	0.500
Zinc sulfate	0.004	0.004
Biotin-100 ⁷	0.348	0.348
Vitamin A, 30,000 IU/g	0.022	0.022
Vitamin D, 3,000 IU/g	0.039	0.039
Vitamin E, 20,000 IU/454 g	0.067	0.067
Dried molasses	0.836	0.836
Animal fat	0.836	0.836

¹Soybean hulls were used as a carrier for chromic oxide for digestibility measurements.

²Ground corn was used as a top-dress for live yeast culture.

³ $\text{Ca}(\text{NO}_3)_2 \cdot \text{NH}_4\text{NO}_3 \cdot 10\text{H}_2\text{O}$, Yara North America Inc. (Tampa, FL).

⁴Ag Processing Inc. (Omaha, NE).

⁵Premier Magnesia LLC (Conshohocken, PA).

⁶Guaranteed analysis: Na, 36.6%; Cl, 56.4%; Zn, 3,500 mg/kg; Mn, 2,800 mg/kg; Fe, 1,750 mg/kg; Cu, 350 mg/kg; I, 70 mg/kg; and Co, 70 mg/kg.

⁷DSM Nutritional Products (Parsippany, NJ).

of a quadratic response, but results were too variable to yield a confident regression. Therefore, 29.4% was used as a $\text{NO}_3\text{-N}$ recovery factor in all NO_3^- diets for calculating apparent N digestibility, which made them isonitrogenous with the control diet; the same factor was used for orts for cows fed NO_3^- . A composite sample of all 4 periods had 0.02% NO_3^- in the control. Plate counts confirmed that cows actually received 3.65×10^{10} to 5.30×10^{10} cfu/d during the trial, which was greater than the target (i.e., minimum to protect against storage losses) LYC dose of 1×10^{10} cfu/d per cow. The control top-dress analysis indicated that <100 cfu/g of viable wild-type yeast was present.

Total-Tract Digestibility and Rumen Disappearance In Situ

Treatments did not significantly affect digestibilities of NDF and OM ($P > 0.15$; Table 3). Apparent N digestibility was decreased ($P = 0.02$) when NO_3^- was fed, but the main effect lost significance ($P = 0.14$) after correction for incomplete $\text{NO}_3\text{-N}$ recovery by Kjeldahl analysis. Data from in situ analyses also did not show a difference ($P > 0.15$) among treatments for NDF degradabilities (Table 3). Values from this approach tend to be much lower than expected based on several factors, particularly inflated passage rates (Firkins, 1997). Williams et al. (1991) demonstrated that *S. cerevisiae* tended to decrease the lag time for NDF disappearance in hay and hay plus barley diets. However, the diet fed in the current experiment had a markedly lower percentage of NDF, so the effect of LYC could be more evident with increasing concentrations of NDF (Desnoyers et al., 2009).

Table 2. Nutrient composition (% of DM, unless otherwise noted) for feed ingredients for cows fed diets without (–) or with (+) NO_3^-

Item	– NO_3^- grain mix	+ NO_3^- grain mix	Corn silage	Alfalfa	WCS ¹	Soy hulls	– NO_3^+ diet	+ NO_3^+ diet
DM (% as is)	90.2	90.9	33.4	53.7	91.0	90.9	65.0	65.3
NDF	18.3	18.2	39.7	49.0	50.5	65.5	33.2	33.2
ADF	10.7	9.9	22.7	39.2	36.9	44.6	21.5	21.2
CP ²	20.7	17.7	6.97	19.4	22.4	13.9	16.2	15.4
Starch	34.6	32.8	38.1	2.0	0.3	0.0	27.8	27.0
NE _L ³ (Mcal/kg of DM)	1.96	1.99	1.44	1.25	1.82	1.36	1.65	1.66
Ca	1.83	1.77	0.20	1.07	0.13	0.63	1.03	1.00
P	0.52	0.51	0.20	0.32	0.65	0.17	0.38	0.38
Mg	0.26	0.31	0.16	0.19	0.38	0.25	0.22	0.24
K	0.85	0.86	0.81	2.32	1.18	1.51	1.11	1.11
Na	0.37	0.36	0.006	0.054	0.004	0.01	0.17	0.16
NO_3 ion	0.02	3.41	0.02	—	—	—	0.02	1.44

¹WCS = whole cottonseed.

²Crude protein is lower in the + NO_3^- grain mix because Kjeldahl analysis does not completely recover $\text{NO}_3\text{-N}$.

³Net energy for lactation is lower in the grain mix because Kjeldahl analysis does not completely recover $\text{NO}_3\text{-N}$. Values are from Spartan Dairy 3 (Michigan State University, East Lansing, MI).

DMI and Milk Production

The main effect of NO_3^- decreased ($P < 0.01$) DMI from 18.4 to 16.9 kg/d (8.2% reduction for $-\text{NO}_3^-$ vs. $+\text{NO}_3^-$) when data were from all 3 squares (Table 4). Lee and Beauchemin (2014) noted that NO_3^- tastes bitter, decreasing palatability and lowering consumption per meal and, therefore, DMI in some studies. Reduction in DMI was inconsistent in 2 studies within the same report (Veneman et al., 2015). The decrease in DMI in our study tended to decrease production of milk ($P = 0.10$), fat ($P = 0.06$), and lactose ($P = 0.07$) and corresponding ADG ($P = 0.06$). Numerical responses ($0.10 < P < 0.15$) for an interaction for the same milk responses in addition to milk lactose concentration ($P = 0.10$) were also reflected by an interaction for ADG ($P < 0.01$); the negative ADG (i.e., BW loss) corresponded with the numerically lowest DMI for the $+\text{NO}_3^-$ /+LYC treatment. Milk protein percentage and yield were decreased ($P < 0.01$) by 4.5% when cows were fed NO_3^- . Decreased DMI also would decrease nutrients to support milk protein synthesis. Feeding NO_3^- decreased ($P = 0.05$) milk NE_L output; however, milk NE_L /DMI and was not different ($P > 0.15$) among treatments.

Methane Measurements

Methane production was decreased ($P < 0.01$) by 16.6% for cows fed NO_3^- , as expected (Table 4). van Zi-

jderveld et al. (2011a) reported a 16% decrease in CH_4 emission; however, these cows were limit fed. Veneman et al. (2015) reported that dietary NO_3^- decreased CH_4 production when expressed per day or per unit of DMI or fat- and protein-corrected milk. For our 2 squares of cows, DMI was decreased ($P < 0.01$) from 19.8 to 17.8 kg/d (10.0%; averaged over $-\text{NO}_3^-$ vs. $+\text{NO}_3^-$ treatments and data not shown). Feeding NO_3^- numerically decreased ($P = 0.14$) CH_4 /DMI by 7.5% but had no effect ($P > 0.15$) on emission of CH_4 /milk NE_L output. The addition of LYC did not affect CH_4 reduction or have any interactions, which is contrary to our hypothesis.

Nitrate in Feed, Milk, and Blood Methemoglobin

Milk NO_3^- increased ($P < 0.01$) for cows fed NO_3^- (Table 4). However, concentration of NO_3^- averaged 0.548 mg of N/L, which was below the 10 mg of NO_3^- (N basis) per liter maximum contaminant level for drinking water (US Environmental Protection Agency, 2018). Beverages or foods with similar NO_3^- levels have the potential to lower blood pressure in humans (Jonvik et al., 2016). Main effects of LYC and the interaction of NO_3^- and LYC were not significant for milk NO_3^- . Milk NO_2^- was below detection limits. Methemoglobin only exceeded 5% once in 46 analyses for cows fed NO_3^- , documenting low risk for feeding diets containing 1.5% NO_3^- after adequate adaptation. Although greater (P

Table 3. Apparent total-tract digestibilities of nutrients and in situ disappearance kinetics for NDF from a mixture of forages by cows fed diets without (–) or with (+) NO_3^- and without (–) or with (+) live yeast culture (LYC)¹

Item	Diet				SEM	Contrast (<i>P</i> -value)		
	−NO ₃ [−]		+NO ₃ [−]			NO ₃ [−]	LYC	NO ₃ [−] × LYC
	−LYC	+LYC	−LYC	+LYC				
Total-tract digestibility								
NDF (%)	39.0	36.6	36.4	34.1	3.1	NS ²	NS	NS
N (%)	62.9	64.0	58.6	52.3	3.4	0.02	NS	NS
N ³ (%)	62.9	64.0	62.3	56.7	3.2	0.14	NS	NS
OM (%)	64.2	64.0	64.9	60.4	2.0	NS	NS	NS
NDF disappearance in situ								
B fraction ⁴ (% of original NDF)	48.3	49.8	50.0	48.9	7.1	NS	NS	NS
kd of B pool ⁵ (/h)	0.0507	0.0429	0.0293	0.0548	0.0126	NS	NS	NS
Discrete lag time (h)	10.9	7.7	4.4	4.9	3.2	NS	NS	NS
C fraction ⁶ (% of original NDF)	50.9	49.8	46.1	47.7	6.5	NS	NS	NS
ERD ⁷ (% of original NDF)	18.5	17.3	14.7	23.0	2.4	NS	NS	NS

¹Data are from one square of cannulated cows. The LYC was from Yea-Sacc TS (Alltech Inc., Nicholasville, KY).

²NS = $P > 0.15$.

³Calculated to include a recovery of 29.4% for dietary NO_3^- , which is not quantitatively recovered by Kjeldahl analysis.

⁴B = potentially degradable NDF.

⁵kd = degradation rate of B pool.

⁶C = undegradable NDF.

⁷ERD = effective rumen degradability. Individual ERD were calculated according to Firkins (1997) using individual B, kd, lag, and C plus passage rates estimated according to Seo et al. (2006) plus individual cow inputs for each period.

Table 4. Intake, milk production, and methane production by cows fed diets without (–) or with (+) NO_3^- and without (–) or with (+) live yeast culture (LYC)¹

Item	Diet				SEM	Contrast (<i>P</i> -value)		
	−NO ₃ [−]		+NO ₃ [−]			NO ₃ [−]	LYC	NO ₃ [−] × LYC
	−LYC	+LYC	−LYC	+LYC				
DMI (kg/d)	18.2	18.6	17.2	16.6	0.7	<0.01	NS	NS
Milk (kg/d)	19.8	20.8	19.6	19.3	1.5	0.10	NS	0.14
Fat (%)	4.46	4.28	4.24	4.47	0.22	NS	NS	0.13
Fat (g/d)	871	866	826	805	59	0.06	NS	NS
3.5% FCM (kg/d)	22.8	23.4	21.9	20.9	1.5	0.12	NS	NS
Protein (%)	3.74	3.74	3.59	3.58	0.07	<0.01	NS	NS
Protein (g/d)	741	776	700	652	51	<0.01	NS	0.13
Lactose (%)	4.61	4.65	4.60	4.57	0.05	0.02	NS	0.10
Lactose (g/d)	915	970	901	840	73	0.07	NS	0.13
BCS	2.89	2.92	2.92	2.93	0.126	NS	NS	NS
BW (kg)	445	448	446	445	19	NS	NS	NS
ADG (kg/d)	0.23	1.32	0.59	−0.30	0.41	0.06	NS	<0.01
SCC (× 1,000 cells/mL)	175	142	94	106	52	0.11	NS	NS
MUN (mg/dL)	9.61	8.94	9.67	9.64	1.15	NS	NS	NS
Milk NO ₃ −N (mg/L)	0.357	0.374	0.522	0.574	0.082	<0.01	NS	NS
Milk NE _L ² (Mcal/d)	15.9	16.2	15.2	14.5	1.1	0.05	NS	NS
Milk NE _L /DMI (Mcal/kg)	0.856	0.871	0.882	0.870	0.042	NS	NS	NS
Methane (mol/d)	22.6	22.7	18.9	18.8	1.8	<0.01	NS	NS
Methane/DMI (mol/kg)	1.186	1.113	1.042	1.085	0.056	0.14	NS	NS
Methane/milk NE _L (mol/Mcal)	1.385	1.366	1.292	1.457	0.190	NS	NS	NS
Blood methemoglobin (%)	0.50	0.59	1.60	1.45	0.36	0.01	NS	NS

¹Milk production and DMI data were collected from all 3 squares of cows; methane production and efficiencies (moles per DMI or milk NE_L) are only from 2 squares of intact cows (without rumen cannulas). The LYC was from Yea-Sacc TS (Alltech Inc., Nicholasville, KY).

²Milk $\text{NE}_L = [(0.0929 \times \% \text{ fat}) + (0.0563 \times \% \text{ protein}) + (0.0395 \times \% \text{ lactose})] \times \text{kg/d of milk (NRC, 2001)}.$

< 0.01) for cows fed $+\text{NO}_3^-$ than $-\text{NO}_3^-$, treatments averaged 1.5 and 0.5% methemoglobin, respectively, which are slightly lower than other reported values (van Zijderveld et al., 2011b; Veneman et al., 2015). Lack of LYC effect on methemoglobin does not support our hypothesis that LYC would help decrease NO_2^- accumulation.

Rumen Fermentation Characteristics

No treatment $\times$ time interactions ($P > 0.10$) existed for VFA (Table 5). Desnoyers et al. (2009) reported that yeast culture increased total VFA production in a meta-analysis, apparently because of increased OM digestibility with increasing yeast supplementation (especially with increasing dietary NDF percentage). In contrast, the main effect of adding LYC was numerically ($P = 0.11$) lower than without LYC for total VFA concentration; a similar trend was noted for decreased VFA production when LYC was administered to continuous cultures also when OM digestibility was not affected by treatment (Welty et al., 2019). Those authors discussed that LYC should lower redox potential, which might therefore increase flux toward more reduced end products, whereas NO_3^- should increase redox. Thus, LYC might have shifted carbon flux toward more reduced (and elongated) end products such as caproate

(not measured) or branched-chain VFA (Nerdahl and Weimer, 2015) or by increasing chain length of long-chain fatty acids in membranes (not measured) unless it enhanced digestibility to support more microbial growth (Ungerfeld and Kohn, 2006). The NO_3^- and LYC interacted ($P < 0.01$) in acetate, propionate, and acetate:propionate. The acetate:propionate ratio was increased ($P < 0.01$) for cows fed NO_3^- diets, especially when combined with LYC (interaction, $P = 0.01$). Contrary to most inverse relationships between methane and propionate (both are [2H] sinks), NO_3^- can inhibit CH_4 production while also increasing acetate concentration (Firkins and Yu, 2015). The acetate:propionate increased by 42 and 28% in the 2 studies reported by Veneman et al. (2015). The role of H_2 emission and formate accumulation in ruminal fluid to moderate shifts in VFA stoichiometry (including methanogenesis) needs further attention (Olijhoek et al., 2016), but unfortunately, these alternative H sinks were not recorded in our study.

Measurements of ruminal pH tended to have a treatment $\times$ time interaction ($P = 0.10$) with a main effect of time ($P < 0.01$; Table 5). At 0 h, cows fed NO_3^- had decreased ($P = 0.05$) pH, but at 3 h ($P < 0.01$) and 6 h ($P = 0.07$), cows fed NO_3^- had higher pH than those not fed NO_3^- . As discussed previously, NO_3^- is unpalatable. Cows could also be sorting toward the

Table 5. Ruminal fermentation characteristics and protozoal counts from cows fed diets without (–) or with (+) NO_3^- and without (–) or with (+) live yeast culture (LYC)¹

Item	Diet				SEM	Contrast (<i>P</i> -value)		
	−NO ₃ [−]		+NO ₃ [−]			NO ₃ [−]	LYC	NO ₃ [−] × LYC
	−LYC	+LYC	−LYC	+LYC				
Total VFA (mM)	98.3	92.1	94.8	90.8	4.8	NS ²	0.11	NS
VFA (mol/100 mol)								
Acetate	63.6	61.5	61.9	63.7	1.3	NS	NS	<0.01
Propionate	20.1	22.4	20.1	18.5	1.3	<0.01	NS	<0.01
Butyrate	11.6	11.7	14.2	13.9	0.5	<0.01	NS	NS
Isobutyrate	0.98	1.07	0.84	0.98	0.07	0.11	0.14	NS
Isovalerate	1.44	1.68	1.43	1.58	0.20	NS	0.01	NS
Valerate	1.40	1.53	1.46	1.37	0.13	NS	NS	0.13
BCVFA ³	3.82	4.28	3.73	3.93	0.25	0.10	0.02	NS
Acetate:propionate	3.11	2.88	3.13	3.47	0.23	<0.01	NS	0.01
pH ⁴								
0 h	6.60	6.77	6.49	6.53	0.10	0.05	NS	NS
3 h	5.72	5.97	6.18	6.24	0.21	<0.01	0.09	NS
6 h	5.60	5.86	5.96	5.95	0.17	0.07	NS	NS
9 h	6.02	6.03	5.94	6.00	0.15	NS	NS	NS
NH ₃ -N ⁵ (mg/dL)	10.4	10.2	12.5	11.8	1.1	0.11	NS	NS
Protozoa (log ₁₀ of cells/mL)								
Total	5.94	5.98	6.17	6.15	0.10	0.02	NS	NS
Entodiniomorphids	5.93	5.97	6.16	6.14	0.10	0.02	NS	NS
Isotrichids	3.75	4.16	4.09	3.90	0.26	NS	NS	0.11
Other	2.68	2.79	4.13	3.91	0.64	0.03	NS	NS

¹Data are from one square of cannulated cows. The LYC was from Yea-Sacc TS (Alltech Inc., Nicholasville, KY).

²NS = $P > 0.15$.

³Branched-chain VFA = sum of isobutyrate, isovalerate, and valerate.

⁴Treatment $\times$ time was $P = 0.10$; main effect of time was $P < 0.01$.

⁵Treatment $\times$ time was $P > 0.15$; main effect of time was $P < 0.01$.

beginning of the day away from the concentrate mix containing NO_3^- . Because refusals were minimized by allowing only 3% orts, cows had to consume the grain mix before the next day. An increased feeding frequency could increase acetate:propionate (French and Kennelly, 1990), whereas restricted feeding of NO_3^- diets (van Zijderveld et al., 2011b) could maintain greater meal size and therefore help mediate the efficacy of CH_4 abatement. Feeding LYC increased ($P = 0.09$) ruminal pH at 3 h, which is consistent with the meta-analysis of Desnoyers et al. (2009).

Ammonia numerically increased ($P = 0.11$) for the main effect of NO_3^- , which could be a result of increased ($P = 0.02$) protozoal abundance when cows were fed the NO_3^- diets. Protozoa actively deaminate AA and probably do not assimilate $\text{NH}_3\text{-N}$ as do the bacteria with which they compete (Firkins et al., 2007). Isotrichids were not affected by treatment, but entodiniomorphid ($P = 0.02$) and other protozoa ($P = 0.03$) counts increased when the diets contained NO_3^- . Roman-Garcia et al. (2019) noted that increasing NO_2^- dosage blunted chemotaxis to glucose or peptides but only inhibited entodiniomorphid chemotaxis to peptides (not glucose), thus suggesting a role for NO_2^- in NO-mediated chemotaxis. Those protozoa had acute exposure to NO_3^- or

NO_2^- , whereas adaptation to prevent accumulation of NO_2^- might help entodiniomorphids and others (primarily *Charonina*, which clusters closer to entodiniomorphids than to isotrichids; Kittelmann et al., 2015) make ATP via electron transport phosphorylation. Unfortunately, rumen protozoal NO_3^- or NO_2^- reductases have not yet been characterized. Results for the role of NO_3^- on protozoal abundance are mixed, but protozoa appear to have nitrate reductase activity (Latham et al., 2016; Yang et al., 2016), although a distinction for isotrichids versus entodiniomorphids has not been made to our knowledge. Protozoa are positively associated with methanogenesis, but direct association with symbiotic methanogens is still being questioned (Tapio et al., 2017). When considering other covariates such as a tendency for depressed NDF digestibility and fermentation pathways directed away from acetate, CH_4 mitigation strategies relying on protozoal inhibition have not been consistently successful (Hristov et al., 2013).

16S rRNA Gene Sequence Analysis

We had over 1.2 million high quality reads (~77,000 per each of 16 samples), resulting in 3,005 unique operational taxonomic units ($1,176 \pm 185$ per sample)

from all samples (data not shown). Analyses for microbial sequence richness and Shannon diversity were not different ($P > 0.15$) between treatments (Table 6). Bray-Curtis dissimilarity was expressed as a nonmetric multidimensional scaling plot, and treatment clustering was $P > 0.15$ (data not shown). Diet primarily drives observed diversity differences (Henderson et al., 2015), and several yeast products did not shift diversity (Pinloche et al., 2013; AlZahal et al., 2014). Relative sequence abundance for phylum *Actinobacteria* was increased by the main effect ($P = 0.01$) of LYC, and a trend ($P = 0.08$) existed for an interaction for *Actinobacteria* to increase most when LYC was added to $+NO_3^-$ diets (Table 6). Within this phylum, results for the LYC main effect (but not interaction) were confirmed for the genus *Bifidobacterium* (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-16008>). Relative sequence abundance of *Bifidobacterium* increased considerably after a subacute ruminal acidosis challenge (Mao et al., 2013). Increasing live yeast also significantly increased *Bifidobacterium* relative abundance (Pinloche et al., 2013). Those authors also noted that relative sequence abundance of lactilytic bacteria increased when cattle were fed live yeast, explaining their increased relative abundance of fibrolytic bacteria as a result of more stable ruminal pH. Bifidobacteria are acid-tolerant bacteria that do not flux glucose-6-phosphate to pyruvate via the standard Embden-Meyerhof-Parnas

(glycolysis) pathway (O'Callaghan and van Sinderen, 2016), probably because they can make more ATP via the *Bifidobacterium* pathway (Hackmann et al., 2017). Yeast increase availability of dicarboxylic acids that fill key central metabolic intermediates (Callaway and Martin, 1997) and serve as electron carriers (Martin, 1998); therefore, perhaps carbon flux could be shifted toward the ATP-generating acetate pathway and away from lactate, thus improving the competitiveness of bifidobacteria. After searching the Hungate 1000 genome as done by Hackmann and Firkins (2015), the bifidobacterial genomes suggested limited malate dehydrogenase or malic enzyme activities, so organic acids such as fumarate or aspartate might be increased by LYC and provide central intermediates and electron sinks that stimulate growth by bifidobacteria. Feeding LYC should decrease redox (Pinloche et al., 2013). However, feeding NO_3^- should increase redox (Latham et al., 2016), so redox does not appear to explain the trend for the positive interaction for *Actinobacteria* (Table 6). We could find no evidence that ruminal bifidobacteria have activities of NO_3^- or NO_2^- reductases. Relative sequence abundance of bifidobacteria was not affected in continuous cultures dosed with NO_3^- and LYC (Welty et al., 2019).

Although no other significant differences besides in *Actinobacteria* were seen at the phylum taxonomic rank (Table 6), numerous effects or trends existed for differ-

Table 6. Prokaryotic community indices and phyla relative abundance (% of total) from cows fed diets without (–) or with (+) NO_3^- and without (–) or with (+) live yeast culture (LYC)¹

Item	Diet				SEM	Contrast (<i>P</i> -value)		
	−NO ₃ [−]		+NO ₃ [−]			NO ₃ [−]	LYC	NO ₃ [−] × LYC
	−LYC	+LYC	−LYC	+LYC				
OTU indices ²								
Richness	1,116	1,209	1,176	1,202	134	NS ³	NS	NS
Shannon-Wiener	5.57	5.59	5.42	5.70	0.24	NS	NS	NS
Archaea								
<i>Euryarchaeota</i>	1.26	0.95	0.76	1.48	0.46	NS	NS	NS
Bacteria								
<i>Bacteroidetes</i>	48.7	46.4	49.4	44.3	4.3	NS	NS	NS
<i>Firmicutes</i>	40.3	43.7	37.1	45.7	6.7	NS	NS	NS
<i>Proteobacteria</i>	3.29	2.29	5.44	0.38	2.47	NS	NS	NS
<i>Actinobacteria</i>	1.10	2.28	0.38	5.38	0.91	NS	0.01	0.08
<i>Spirochaetes</i>	1.61	1.06	2.38	0.74	0.59	NS	0.12	NS
<i>Fibrobacteres</i>	0.79	0.69	1.33	0.10	0.72	NS	NS	NS
<i>Tenericutes</i>	0.69	0.73	0.75	0.65	0.16	NS	NS	NS
<i>Cyanobacteria</i>	0.32	0.20	0.38	0.03	0.20	NS	NS	NS
Candidate division TM7	0.17	0.17	0.13	0.14	0.04	NS	NS	NS
<i>Verrucomicrobia</i>	0.03	0.12	0.05	0.22	0.08	NS	NS	NS
<i>Lentisphaerae</i>	0.13	0.07	0.14	0.04	0.05	NS	0.15	NS
<i>Planctomycetes</i>	0.09	0.05	0.09	0.04	0.03	NS	NS	NS
<i>Chloroflexi</i>	0.07	0.03	0.11	0.01	0.05	NS	NS	NS

¹Data are from one square of cannulated cows.

²Operational taxonomic unit (OTU at 97% 16S rRNA gene sequence similarity) richness (number of OTU) and diversity (Faulkner et al., 2017).

³NS = $P > 0.15$.

ences among main effect means in families or genera within phyla (Supplemental Tables S1–S3; <https://doi.org/10.3168/jds.2018-16008>); therefore, main effect means of LYC (averaged over NO_3^- supplementation) will be presented in text. Relative sequence abundance of family *BS11* gut group decreased ($P = 0.08$) from 2.15 to 0.70% for $-\text{LYC}$ versus $+\text{LYC}$ (Supplemental Table S2; <https://doi.org/10.3168/jds.2018-16008>; phylum *Bacteroidetes*). Family *Lachnospiraceae* decreased ($P = 0.06$) from 12.8 to 10.3% primarily because one of its members, genus *Butyrivibrio*, decreased ($P = 0.04$) from 3.45 to 1.07% when LYC was fed (Supplemental Table S3; <https://doi.org/10.3168/jds.2018-16008>; phylum *Firmicutes*). In contrast, the *Firmicutes* families *Peptococcaceae* and *Peptostreptococcaceae* tended ($P = 0.09$) to increase (0.104 vs. 0.367 and 0.064 vs. 0.209%, respectively, for $-\text{LYC}$ vs. $+\text{LYC}$). Family *Spirochaeta* (phylum *Spirochetes*) tended ($P = 0.07$) to decrease when LYC was fed (0.308 vs. 0.047%), but genus *Akkermansia* (phylum *Verrucomicrobia*) tended ($P = 0.10$) to increase from 0.030 to 0.176% when LYC was fed (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-16008>).

One of the purported modes of action of LYC is to scavenge O_2 (Jeyanathan et al., 2014), which is important after ingestion of new feed, but Mann et al. (2018) explained that epimural bacteria in particular need to cope with increased O_2 tension and oxygen stress resulting from O_2 diffusion from blood. Numerous bacterial taxa associated with the rumen epithelium included *Bifidobacterium*, *Butyrivibrio*, and *Treponema* (Mann et al., 2018). The increased bifidobacterial abundance discussed previously would explain our decrease in the relative abundance of other competing members such as *BS11*, *Butyrivibrio*, and *Spirochaeta* (closely related to *Treponema*).

Family *BS11* probably is not a major cellulolytic but still fills different fibrolytic niches based on preference for differing hemicellulosic monomers (Solden et al., 2017). They also have enzymes capable of degrading glycoproteins such as those found in mucins. Family *Prevotellaceae* (in *Bacteroidales* order, as is *BS11*) and *Lachnospiraceae* (which includes genus *Butyrivibrio*, which exceeded 10% of the relative sequence abundance in the rumen epithelium) were associated with degrading specific compounds in the rumen mucosa of goats (Shen et al., 2017). *Peptostreptococcus* was greater in the rumen and especially the feces than the rumen epithelium but still was included as a dominant member of the epithelium (Liu et al., 2016). Popova et al. (2017) detected relative sequence abundance of family *Verrucomicrobia* comparable to that in our study but more abundance in the cecum of growing cattle. Its main characterized genus, *Akkermansia*, is an important mu-

cinolytic bacterium in the colon and has been widely associated with healthfulness of animals and humans (Derrien et al., 2017).

Transcripts for membrane-bound, but not secreted, mucins were detected in rumen tissue (Hoorens et al., 2011), which could indicate that luminal bacteria potentially interact with epimural bacteria or those that are adherent to sloughed epithelial cells. The epimural bacteria might help explain the resilience of the luminal bacteria in the rumen even after near complete ruminal exchange (Bickhart and Weimer, 2018). *Coproccoccus* (numerically increased by LYC, $P = 0.11$, in Supplemental Table S3; <https://doi.org/10.3168/jds.2018-16008>) was among the bacteria most responsive to ruminal exchange and was proposed as a candidate to improve feed efficiency (Zhou et al., 2018), although Weimer et al. (2017) suggested that this taxon's role in feed efficiency is not yet clear. Although speculative at this time, the potential role of LYC influencing the interaction of epimural and luminal bacteria deserves further research attention.

Relative sequence abundance of *Ruminococcus* decreased from 2.23 to 1.16% and *Anaerotruncus* increased from 0.202 to 0.325% of total sequences when NO_3^- was fed (Supplemental Table S3; <https://doi.org/10.3168/jds.2018-16008>). Effects of NO_3^- on relative bacterial abundance are mixed (Latham et al., 2016; Yang et al., 2016). We could find little information on *Anaerotruncus* other than it increased when corn stover was increased as a forage source (Zhang et al., 2014), suggesting a role in fiber degradation. *Ruminococcus* was not affected in one of the reported studies but decreased in the solids when NO_3^- was fed in a second study (Veneman et al., 2015). Popova et al. (2017) suggested that *Ruminococcus* adapts to NO_3^- , but *Fibrobacter* does not. In contrast, feeding NO_3^- increased relative abundance of both *Ruminococcus* and *Fibrobacter* in steers (Zhao et al., 2015). Relative abundance of *Fibrobacter* was relatively low (averaging 0.77%) and highly variable (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-16008>) even though we blended ruminal samples to recover particle-associated bacteria.

Genus *Selenomonas* and other NO_3^- -reducing members of family *Veillonellaceae* had low relative sequence abundance in our study (Supplemental Table S3; <https://doi.org/10.3168/jds.2018-16008>). Relative abundance of *Selenomonas* was increased when NO_3^- was fed (Wang et al., 2018). However, NO_3^- had few differences in relative sequence abundance of expected NO_3^- reducers, including only one group of *Selenomonas* (Popova et al., 2017). Strain differences of the lactilytic selenomonads are linked to their activity of NO_3^- reduction (Yang et al., 2016; Welty et al., 2019). *Selenomonas* was not even detected in all animals (Popova et al., 2017), so those

authors suggested other bacteria were filling this niche, which is consistent with a review of the literature (Yang et al., 2016). Clearly, more functional research such as metatranscriptomics is needed to characterize taxa that reduce NO_3^- or NO_2^- and their potential interactions with other community members that do not reduce NO_3^- or NO_2^- .

Archaea were not well represented by our sequencing approach and did not differ among treatments (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-16008>). Archaea decreased in the solid phase in the one study in which NO_3^- was fed (Veneman et al., 2015) but only modestly. In that study, *Methanobrevibacter* dominated recovered archaeal sequences and was numerically decreased. Methanogens in the rumen are not known to possess nitrate reductase (Yang et al., 2016). Methanogenesis per abundance of methanogens varies among studies using a variety of agents to mitigate methane (Firkins and Yu, 2015) and could be even more variable because of the potential direct inhibition by accumulated NO_2^- (Yang et al., 2016).

Several factors could influence lack of responsiveness of prokaryote community to dietary NO_3^- . Cow-period is the replicating unit in Latin square designs such as ours, and the random effect of cow is critical for statistical power. However, microbial resilience within cow might lessen treatment effects on community composition (Weimer et al., 2017). Welty et al. (2019) noted shifts in known NO_3^- reducers in continuous cultures that have common inoculum among treatments within period (i.e., diminish the random effect of cow) and obviously lack ruminal mucosa, which could help explain the cow's role in bacterial resilience. Those authors also discussed potential for protozoa and prominent bacteria such as genera in the family *Prevotellaceae* to reduce NO_3^- or NO_2^- . While searching the Hungate 1000 genomes (as described previously) for potential NO_3^- and NO_2^- reductases, we noted that 104 of 415 genomes have annotated genes for the nitrogenase (N_2 reduced to NH_3) complex NifDHK (K02586, K02588, and K02591 annotations by KEGG; <https://www.genome.jp/kegg/>). Almost all were *Firmicutes*, including many *Lachnospiraceae* (particularly the butyrivibrios), *Selemonomonas* (11 of 16 strains), and 8 of 20 strains of *Ruminococcus*, the latter of which was decreased by dietary NO_3^- in our study but was variable in other studies (see previous discussion). One soil *Lachnospiraceae* isolate was characterized for nitrogenase activity (Haas and Blanchard, 2017), and this isolate's bacterial fatty acid profile most closely associated with a characterized strain of *Ruminococcus*. Those authors noted flanking by NitT/TauT (sulfonate/nitrate/taurine transport system), which is widely represented in almost all *Ruminococcus* and *Ruminococcaceae* genomes we surveyed.

Although we could find little information supporting nitrogenase activity in the rumen, transcripts of *nifD-HK* were expressed at low levels and associated with *Fibrobacter* (we noted only in the genome of *Fibrobacter succinogenes elongatus* in our search) and *Clostridium* in epimural bacteria (Mann et al., 2018). Finally, N_2O might be emitted when animals are fed NO_3^- (Yang et al., 2016). Therefore, metabolic routes that are considered negligible in most feeding conditions should be reconsidered in future studies feeding NO_3^- .

CONCLUSIONS

Feeding NO_3^- decreased CH_4 by 16.6% but was not efficacious because CH_4 :DMI decreased by 7.5% and CH_4 :milk NE_L was unaffected. Cows sustained milk production and fat concentration on either diet even with decreased DMI for the NO_3^- diet. Percentage milk protein was decreased for cows fed NO_3^- , which tended to decrease milk NE_L . However, depressed DMI beyond our 4-wk periods would eventually increase the need for more cows and their replacements. These negative effects reduce the overall benefit to NO_3^- supplementation, although it never increased methemoglobin to a concerning level in our study. The hypothesis was supported for the capability of NO_3^- to decrease CH_4 . Although shifts existed in the bacterial community associated with feeding LYC, there were few interactions. Thus, LYC did not provide an additive benefit to feeding NO_3^- to further decrease CH_4 or prevent NO_2^- accumulation. Further research is needed to reduce variability in efficacy of methane abatement and determine an acceptable additive to NO_3^- to facilitate its use as an alternative NPN source that mitigates enteric CH_4 production.

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