Rumen microbial responses to supplemental nitrate. II. Potential interactions with live yeast culture on the prokaryotic community and methanogenesis in continuous culture

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ABSTRACT

Nitrates have been fed to ruminants, including dairy cows, as an electron sink to mitigate CH₄ emissions. In the NO₃[−] reduction process, NO₂[−] can accumulate, which could directly inhibit methanogens and possibly other microbes in the rumen. *Saccharomyces cerevisiae* yeast was hypothesized to decrease NO₂[−] through direct reduction or indirectly by stimulating the bacterium *Selenomonas ruminantium*, which is among the ruminal bacteria most well characterized to reduce both NO₃[−] and NO₂[−]. Ruminal fluid was incubated in continuous cultures fed diets without or with NaNO₃ (1.5% of diet dry matter; i.e., 1.09% NO₃[−]) and without or with live yeast culture (LYC) fed at a recommended 0.010 g/d (scaled from cattle to fermentor intakes) in a 2 × 2 factorial arrangement of treatments. Treatments with LYC had increased NDF digestibility and acetate:propionate by increasing acetate molar proportion but tended to decrease total VFA production. The main effect of NO₃[−] increased acetate:propionate by increasing acetate molar proportion; NO₃[−] also decreased molar proportions of isobutyrate and butyrate. Both NO₃[−] and LYC shifted bacterial community composition (based on relative sequence abundance of 16S rRNA genes). An interaction occurred such that NO₃[−] decreased valerate molar proportion only when no LYC was added. Nitrate decreased daily CH₄ emissions by 29%. However, treatment × time interactions were present for both CH₄ and H₂ emission from the headspace; CH₄ was decreased by the main effect of NO₃[−] until 6 h postfeeding, but NO₃[−] and LYC decreased H₂ emission

up to 4 h postfeeding. As expected, NO₃[−] decreased methane emissions in continuous cultures; however, contrary to expectations, LYC did not attenuate NO₂[−] accumulation.

Key words: nitrate, yeast culture, methane emission, continuous culture

INTRODUCTION

Ruminal bacteria, fungi, and protozoa degrade and ferment carbohydrates to VFA, supplying nutrients to the cow in a multi-step process in which reducing equivalents ([2H]) are transferred to electron carriers such as NAD⁺ and ferredoxin. Electron carriers are re-oxidized when [2H] is recovered in reduced end products such as propionate or converted by hydrogenases to H₂ (Morgavi et al., 2010). After H₂ is released from the cell into the aqueous environment [hereafter referred to as H₂(aq)], it can be taken up by other microbes, particularly by hydrogenotrophic, methanogenic archaea. However, if production exceeds uptake, H₂ can evolve into the rumen headspace for eructation, particularly when dietary nitrate has been used as a methane abatement strategy (Olijhoek et al., 2016); consequently, inter-species H₂ transfers can complicate dietary efforts to inhibit methanogenesis without disrupting the rumen ecosystem (Knapp et al., 2014). Specific to our aims, further work is needed to explain the increased emission of H₂ when alternative electron sinks such as nitrate are available (Lund et al., 2014).

The potential for thermodynamics to influence VFA stoichiometry has been questioned (van Lingen et al., 2016). However, this model uses gaseous H₂ for model development and assumes that supersaturation (i.e., greater concentration than that predicted by Henry's law) of H₂(aq), which would influence the NAD:NADH in their calculations, is unlikely to occur in the rumen. In contrast, Wang et al. (2016a) argued that ruminal supersaturation could indeed influence thermodynam-

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ics calculations, which also was demonstrated in continuous culture (Wenner, 2016). Paradoxically, $H_2(aq)$ concentration was associated with greater numbers of methanogens and lower CH_4 emission (Wang et al., 2016b). Because many of the previously reported correlations ignored the fixed effects of diet, further research is needed to assess how $H_2(aq)$ can influence fermentation end products and community composition within and among diets. In continuous cultures from which production of CH_4 , H_2 , and VFA could be measured, low pH shifted $[2H]$ toward elongated VFA rather than suppressing methanogenesis (Wenner et al., 2017). Because $H_2(aq)$ spikes soon after feeding (Smolenski and Robinson, 1988; Wenner et al., 2017), thermodynamic control by elevated $H_2(aq)$ should transiently affect VFA, CH_4 , and H_2 production. Therefore, we assessed these end products over time after feeding diets without and with NO_3^- .

Live yeast culture (LYC), particularly with *Saccharomyces cerevisiae*, often improves dietary NDF digestibility (Desnoyers et al., 2009). Typically attributed to LYC's stimulation of cellulolytic bacteria and increased fiber digestibility, an increased DMI is expected to increase milk production (Williams et al., 1991; Wohlt et al., 1991). In addition to consuming O_2 , LYC could also stabilize pH by stimulating lactilytic strains of *Selenomonas ruminantium* (Pinloche et al., 2013), which also are important in NO_3^- and NO_2^- reduction (Yang et al., 2016). In our companion study (Roman-Garcia et al., 2019), we questioned if LYC would reduce NO_2^- in microaerophilic conditions and thereby help prevent its accumulation after NO_3^- is fed, but the hypotheses were not supported, perhaps because of a lack of microbial adaptation to NO_3^- . Our objectives were to evaluate the effect of dietary NO_3^- and its potential interaction with LYC on fermentation characteristics, microbial protein synthesis, and prokaryotic community composition in continuous cultures that are adapted to NO_3^- for 7 d. We hypothesized that NO_3^- would suppress methanogenesis and shift bacterial populations toward those reducing NO_3^- and NO_2^- , and LYC would suppress methanogenesis additively with feeding NO_3^- while decreasing NO_2^- accumulation to result in increased NDF digestibility and efficiency of bacterial growth.

MATERIALS AND METHODS

Continuous Culture Operation

Four dual-flow continuous cultures designed to retain protozoa (Karnati et al., 2009) were used in a 5×4 Youden square design with 5 periods of 10 d; each

period included 7 d of adaptation followed by 3 d of collection. All 4 fermentors were fed 36.3 g of DM daily of 50:50 alfalfa and concentrate pellets (Table 1). Fermentors were administered isonitrogenous doses of either urea or $NaNO_3$ (2% as is; 2.25% of diet DM), which would be an average of 5.65 mM instantaneous dosage into fermentors of an average 1.71-L working volume. Fermentors were also administered a control without or with LYC (Yea-Sacc¹⁰²⁶, Alltech Inc., Nicholasville, KY) fed at the recommended 0.010 g/d (approximately 10×10^6 cfu per day, scaled from cow to fermentor). Treatments were arranged in a 2×2 factorial. Using an approved animal usage protocol, 4 L of rumen fluid was collected from 2 cows using an institutionally approved animal use protocol to inoculate all fermentors each period. The cows were fed a standard dairy diet of approximately 52% corn silage, 18% alfalfa hay, and mostly corn and soybean meal (about 17% CP); neither supplemental LYC nor NO_3^- was present in their diets. The rumen fluid was squeezed through 2 layers of cheesecloth, pooled, and added to an equal volume of prewarmed (39°C) buffer in the fermentor vessels (Karnati et al., 2009). The vessels were maintained anaerobically at pH ranging between 6.2 (never below 6.0) and 6.9 (never above 7.0) by a 7.0%/h influx of buffer (i.e., 7.0%/h liquid dilution rate) and 5.0%/h passage rate of solids through the overflow, temperature at 39°C, and agitation at 50 rpm. Buffer was modeled after Weller and Pilgrim (1974) but without urea. At d 5, fermentors with either NO_3^- or urea treatments were primed with 60 or 50 mg of $NaNO_3$ or $(NH_4)_2SO_4$ (each 10% atom % ^{15}N), respectively. After priming and through the rest of the period, fermentors were dosed with 200 mg/d of $Na^{15}NO_3$ or 167 mg/d of $(^{15}NH_4)_2SO_4$ (each 10 atom % ^{15}N), as dissolved and continuously infused via separate buffers.

Sample Collection, Processing, and Analyses

The continuous culture vessels were monitored constantly for CH_4 and H_2 headspace via a Micro-Oxymax Respirometer (Columbus Instruments Inc., Columbus, OH). At h 24 on d 4, 500 mL of the effluent was aspirated and combined with 50 mL of formalin for ^{15}N background analysis: (1) NAN, (2) NH_4^+ salt by diffusion (Hristov et al., 2001), (3) bacteria pellet, and (4) protozoa (Ye et al., 2018). On d 6 at h 0, 4, 8, and 12, a 10-mL sample was pulled from fermentors with a 60-mL pipette and combined with 10 mL of formalin for protozoa counts; a separate 30-mL sample (without formalin) was taken, frozen immediately, and stored at $-80^\circ C$ for subsequent DNA extraction. *Isotricha*, *Dasytricha*, *Epidinium*, *Ophryoscolex*, total

Table 1. Ingredient and nutrient composition of continuous culture treatment diets without (–) or with (+) nitrate or live yeast culture (LYC)^{1,2,3}

Item	–NO ₃ [–]		+ NO ₃ [–]	
	–LYC	+LYC	–LYC	+LYC
Ingredient, % of DM				
Alfalfa meal	49.6	49.6	49.0	49.0
Soybean hulls	18.3	18.3	18.1	18.1
Corn, ground	20.1	20.1	19.8	19.8
Distillers grains with solubles	4.95	4.95	4.89	4.89
Soybean meal, 48% CP	4.49	4.49	4.43	4.43
Corn oil	0.89	0.89	0.88	0.88
Trace mineral salt	0.45	0.45	0.44	0.44
Dicalcium phosphate	0.38	0.38	0.38	0.38
Magnesium oxide	0.10	0.10	0.10	0.10
Nitrate	0	0	2.06	2.06
Urea	0.73	0.73	0	0
Yea-Sacc ⁴	0	0.025	0	0.025
Nutrient				
DM, % as fed	90.5	90.5	90.6	90.6
OM, % of DM	91.5	91.5	89.7	89.7
NDF, % of DM	41.5	41.5	40.9	40.9
CP, % of DM	17.2	17.2	15.0	15.0

¹Diets were fed 40 g/d of 50:50 alfalfa to concentrate pellets.

²Nitrate was added to the diet at 1.5% of diet DM. It is not recovered in Kjeldahl analysis.

³Live yeast culture (LYC) was added to the diet at 0.010 g/d.

⁴Strain 1026; Alltech Inc. (Nicholasville, KY).

Entodinium, large *Entodinium* (>100 µm length), and the subfamily *Diplodiniinae* were characterized as per Dehority (1993). Following thawing of the nonformalin samples, DNA was extracted by repeated bead beating plus column purification (Yu and Morrison, 2004). The DNA was sequenced using the Illumina MiSeq platform (Illumina Inc., San Diego, CA) at the Molecular and Cellular Imaging Center (The Ohio State University, Ohio Agricultural and Research and Development Center, Wooster) with a single lane of Illumina MiSeq using 2 × 300-bp paired end reads following established HMP protocols (Caporaso et al., 2010). Data were analyzed as described by Faulkner et al. (2017). Briefly, processing was performed using QIIME 1.9.0 (Caporaso et al., 2010) and assigned taxonomy using the 97_SILVA_111 rep set (v123, <http://www.arb-silva.de/>; Edgar, 2013; Quast et al., 2013).

Hourly Samples

On d 7, 8, and 9, 40 mL of effluent was withdrawn from the liquid effluent line of each fermentor at h 0, 0.5, 1, 1.5, 2, 4, 8, and 24. From these samples, 10 mL was used for analyses of NO₃[–], NO₂[–], and NH₃ concentrations; the second 10-mL aliquot was added to a tube containing 1 mL of 6 N HCl to stop fermentation and then frozen at –20°C for subsequent VFA analysis. The last 20 mL of sample was injected into a culture bottle with 5 mL of 15 N H₃PO₄ and then

thoroughly flushed with N₂ before capping and later analysis of H₂(aq) within 3 d after sampling (Wenner et al., 2017). The samples were prepared and analyzed for NO₃[–] and NO₂[–] using ion-exchange chromatography using a Dionex ICS-2100 Ion Chromatography System equipped with a AS18 column (Thermo Fisher Scientific, Waltham, MA). An average peak concentration from the baseline buffer (control treatment) was subtracted from NO₂[–] peaks because of co-elution with HCO₃[–]. Ammonia was analyzed using a colorimetric assay (Chaney and Marbach, 1962). Aliquots were thawed, centrifuged at 15,000 × g for 15 min at 4°C, and filtered through a Whatman #1 (Sigma-Aldrich, St. Louis, MO) filter paper before VFA analysis by GLC according to Harvatine et al. (2002).

Daily Samples

Daily solid and liquid effluents were kept on ice baths and then pooled from each fermentor at h 24 on d 7, 8, and 9 after which sub-samples were aspirated. A 10-mL sample was combined with 10 mL of formalin for protozoal generic counts as described previously. Based on fermentor pool sizes and effluent flow, total protozoa generation time was calculated (Karnati et al., 2009). From the effluent, a 50-mL aliquot was stored and analyzed for VFA as described previously. Production of VFA was calculated by multiplying VFA concentration by each respective fermentor's average effluent

Table 2. Nutrient digestibilities of OM and NDF and fermentation characteristics in continuous cultures administered diets supplemented without (–) or with (+) nitrate or live yeast culture (LYC)^{1,2}

Item	–NO ₃ [–]		+NO ₃ [–]		Pooled SEM	P-value of effects ³		
	–LYC	+LYC	–LYC	+LYC		NO ₃ [–]	LYC	Interaction
OM, apparent	41.0	41.2	43.8	40.5	1.6	NS	NS	NS
NDF	45.8	56.6	50.6	55.0	3.9	NS	<0.01	NS
Total VFA, mmol/d	155	139	147	143	15	NS	0.08	NS
Individual VFA, mmol/100 mol								
Acetate	64.2	64.7	65.3	66.0	0.68	<0.01	0.05	NS
Propionate	19.6	19.4	19.4	18.9	0.30	0.17	0.13	NS
Isobutyrate	0.83	0.84	0.79	0.78	0.04	0.01	NS	NS
Butyrate	11.5	11.3	10.8	10.6	0.55	<0.01	0.16	NS
Isovalerate	2.27	2.26	2.27	2.21	0.17	NS	NS	NS
Valerate	1.55	1.50	1.45	1.50	0.06	<0.01	NS	<0.01
Acetate:propionate	3.27	3.34	3.37	3.51	0.07	0.03	0.07	NS
CH ₄ , mmol/d	30.3	31.7	24.3	20.0	6.8	0.02	NS	NS
H ₂ , mmol/d	0.191	0.170	0.165	0.097	0.053	NS	NS	NS
H ₂ (aq), ⁴ μM	14.1	9.0	25.0	6.0	9.2	NS	NS	NS

¹Nitrate added at 1.5% diet DM; urea was added to the diet isonitrogenously with the nitrate treatment.

²LYC added at 0.010 g/d (scaled from cow to fermentor).

³Main effects of nitrate or LYC treatments or their interaction. NS = $P > 0.15$.

⁴H₂(aq) = aqueous hydrogen.

flow. A 500-mL sample was combined with 50 mL of formalin for ¹⁵N analysis of NAN, as previously stated. Approximately 20% of effluent volume was sampled, stored frozen, and subsequently dried in a 55°C oven before grinding. Samples of feed and effluent were analyzed for DM and OM (AOAC, 1990), CP by Kjeldahl (AOAC, 1990), and NDF (Van Soest et al., 1991) using heat-stable amylase and sodium sulfite but not ash correction.

Statistical Analysis

The daily samples were analyzed using ANOVA by the MIXED procedure of SAS (version 8.2, SAS Institute Inc., Cary, NC) using the following model: $Y_{ijkl} = \mu + N_i + S_j + P_k + f_l + NS_{ij} + e_{ijkl}$, where Y_{ijkl} is the dependent variable, μ is the mean, N_i is the N source ($i = 1, 2$), S_j is *S. cerevisiae* addition ($j = 1, 2$), NS_{ij} is the interaction of N_i and S_j , P_k is the fixed effect of the k th period ($k = 1, 2, 3, 4, 5$), f_l is the random effect of the l th fermentor ($l = 1, 2, 3, 4$), and e_{ijkl} is the random error. Repeated measures from samples from the same fermentor within period were analyzed using the first-order autoregressive (for most variables) or heterogeneous first-order autoregressive (for gas emissions) covariate structures, depending on which had the lowest Bayesian information criterion. One fermentor's gas production was omitted for all periods resulting from a failed gas collection vessel that was not detected until period 3. Treatment main effects and interaction were analyzed by ANOVA. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Digestibility and VFA Production

Neither NO₃[–] nor LYC affected apparent OM digestibility ($P > 0.15$), but NDF digestibility increased ($P < 0.01$) for the main effect of LYC addition (Table 2). van Zijderveld et al. (2011) found no differences between urea or NO₃[–] diets for NDF digestibility in vivo. One mode of action by LYC is to increase NDF digestibility (Pinloche et al., 2013; Jeyanathan et al., 2014), perhaps by increasing abundance of fibrolytic bacteria (Pinloche et al., 2013; Zhao et al., 2015).

Total daily VFA production tended to decrease ($P = 0.08$) with LYC but was not affected by NO₃[–] (Table 2). Desnoyers et al. (2009) reported an increase in total VFA concentration in a meta-analysis of *S. cerevisiae* studies. Differences might be because DMI was increased, on average, in those studies. However, the increased NDF digestibility with lower total VFA production in our study might suggest a slight difference in carbon diverted into microbial cell matter rather than VFA. Similar to Yang et al. (2016), NO₃[–] increased ($P < 0.01$) acetate and decreased ($P < 0.01$) butyrate molar proportion, likely because NO₃[–] is an alternate electron acceptor compared with intracellular fumarate, which is used in propionate production. Live yeast tended to increase ($P = 0.07$) acetate:propionate by increasing ($P = 0.05$) acetate and numerically ($P = 0.13$) decreasing propionate. Isobutyrate and valerate were also decreased ($P < 0.01$) by NO₃[–], potentially resulting from a disruption of branched-chain AA deamination, although responses are mixed and probably

depend on various factors (Martinez-Fernandez et al., 2016). Desnoyers et al. (2009) reported no effect on acetate:propionate ratio in vivo in a meta-analysis of *S. cerevisiae* studies.

Treatment $\times$ time interactions ($P \leq 0.05$) were detected for acetate, propionate, and butyrate, so data were analyzed at each time point and presented in Figure 1. Nitrate generally increased ($P \leq 0.05$) molar

percentage of acetate (panel A) and decreased ($P \leq 0.05$) propionate (panel B) at all times measured, as discussed previously, so the interaction with time was mostly explained by increased acetate ($P \leq 0.05$) and trends for decreased ($P \leq 0.09$) propionate at 2 and 4 h for LYC. Molar percentage of butyrate decreased ($P \leq 0.01$) when NO_3^- was fed only for the time points ranging from 0.5 to 4 h postfeeding (panel C).

Figure 1. Molar percentages of acetate (top), propionate (middle), and butyrate (bottom) in continuous cultures over time after being administered diets supplemented without or with NO_3^- or live yeast culture (LYC). Treatment by time interaction was $P < 0.01$. Main effect of NO_3^- , LYC, or their interaction (Inter) at each time point (** $P \leq 0.01$, * $P \leq 0.05$, NS = $P > 0.15$). The SEM is pooled across treatment per time and shown only for one series per plot.

Figure 2. Incremental (i.e., rate between 0.5-h measurements) CH₄ (top) or H₂ (bottom) production in continuous cultures over time after administration of diets supplemented without or with NO₃[−] or live yeast culture (LYC). Treatment by time interaction was *P* < 0.01 for both panels. Main effect of NO₃[−], LYC, or their interaction (Inter) at each time point (***P* ≤ 0.01, **P* ≤ 0.05, NS = *P* > 0.15). Dashed line indicates NS for the entire span of time points. The SEM is pooled across treatment per time and shown only for one series per plot.

Methane and Hydrogen Production

As expected, CH₄ emission was decreased (*P* = 0.02) by 28.5% by the main effect of adding NO₃[−], but our hypothesis for an interaction with LYC could not be supported (Table 2). Nitrate thermodynamically outcompetes for electrons compared with methanogenesis, and its reduction pathway intermediates (NO₂[−] and NO) are known inhibitors of methanogenesis (Latham et al., 2016; Yang et al., 2016). According to a review from Knapp et al. (2014), electron acceptors NO₃[−] and SO₄^{2−} decreased emission of CH₄ by an average of 16% in cited reports. A treatment by time interaction (*P* < 0.01) was observed for incremental rate of CH₄ (Figure 2, panel A). Because there were no differences for LYC or the NO₃[−] × LYC interaction, the interaction of treatment × time is explained by the differences (*P* ≤ 0.07) between NO₃[−] treatments from 1.5 to 7 h. Simi-

lar to results of Nolan et al. (2010), CH₄ production decreased in the first hour after feeding and increased in the second; however, our study had a more obvious decline for the NO₃[−] treatment compared with the control. The overall shape of our CH₄ production curve follows that of van Zijderveld et al. (2010), with peak CH₄ production occurring 6 h postfeeding and significant differences between the control and NO₃[−] from 2 to 6 h. Daily emission of gaseous H₂ and concentration of H₂(aq) were not different among treatments (Table 2). An interaction was observed between treatment and time (*P* < 0.01) for incremental rate of H₂ emission, so incremental H₂ emission data were analyzed statistically at each time point (Figure 2, panel B). The interaction between NO₃[−] and LYC was not significant. The main effect of NO₃[−] tended (*P* = 0.09) to decrease H₂ emission at h 0.5 and decreased (*P* ≤ 0.05) until 4 h. Zhou et al. (2012) reported an inhibition of H₂ produc-

tion when NO_3^- was fed to a batch culture of ruminal microbes, and abundance of archaea decreased when fed NO_3^- salts in sheep (van Zijderveld et al., 2010) and continuous culture (Wenner, 2016); consequently, these results coincide with decreased methanogenesis in our study but probably were diluted out when evaluating H_2 emission over the full day (Table 2). In contrast, NO_3^- suppressed methane emission but increased H_2 emission in dairy cows (van Zijderveld et al., 2011). The main effect of LYC decreased ($P \leq 0.05$) at h 2 and 2.5 and tended ($P = 0.07$) to decrease H_2 emission rate at h 3. The amount of H_2 emitted when methanogenesis is depressed appears to depend on the varying degree of $\text{H}_2(\text{aq})$ supersaturation and greater release into the gas phase (Wang et al., 2016b). Yeast are thought to decrease the redox potential (a more negative E_h), either directly by scavenging O_2 or indirectly by stimulation of the general population of bacteria (Pinloche et al., 2013).

Nitrate and Nitrite Concentrations

Both NO_3^- and NH_3 concentrations interacted with time ($P < 0.01$), but NO_2^- concentration did not ($P > 0.15$); however, because NO_2^- is the intermediate, it was also analyzed at each time point (Figure 3). Nitrate treatment increased ($P < 0.01$) NO_3^- concentration at h 0.5 and 1 postfeeding of NO_3^- and tended ($P = 0.06$) to increase NO_3^- concentration at 2 h after feeding (panel A). Similarly, the NO_3^- treatment increased ($P < 0.01$) NO_2^- concentration at 0.5 and 1.5 h, whereas it had a tendency ($P \leq 0.10$) to increase at 2 and 4 h (panel B). Nitrate feeding had a minimal effect on NH_3 concentration, which only decreased ($P < 0.05$) for the NO_3^- treatments at 1 h (panel C). As in our study, nitrate rapidly increased in the first 0.5 h after dosing nitrate and then declined until 2 h. In our study, NO_2^- peaked at 1 to 2 h, which is generally consistent with studies with sheep dosed nitrate (Leng, 2008). Although no treatment $\times$ time interaction was detected for NO_2^- (panel B), that it persisted past the peak of NO_3^- (panel A) without much change in NH_3 (panel C) supports previous studies that NO_3^- is more rapidly reduced to NO_2^- , which can accumulate until it is reduced to NH_3 . Active *S. cerevisiae* might have the ability to efflux NO_3^- or NO_2^- from stores produced in NO signaling pathways (Cabrera et al., 2014); however, the lack of significance for the main effect of LYC or its interaction with NO_3^- in Figure 3 and its inhibition of yeast growth (Roman-Garcia et al., 2019) suggests internal stores in yeast were unlikely to prolong NO_3^- or NO_2^- reduction.

Nitrogen Outflow to Effluent

Total N flows tended ($P = 0.06$) to decrease with the addition of NO_3^- and the addition of LYC (Table 3). The main effect of LYC tended ($P = 0.08$) to increase $\text{NH}_3\text{-N}$ flow. The flow of NAN was less ($P = 0.05$) when NO_3^- was fed. The N flows from NO_3^- treatments are, in part, a result of Kjeldahl analyses not recovering all residual NO_3^- or NO_2^- escaping into effluent (Bremner and Mulvaney, 1982); however, NAN and nonammonia, nonbacteria N (NANBN) should not be affected by this artifact because any lost recovery of inorganic NAN would be factored out. Treatment did not affect ($P > 0.15$) bacterial N flow or assimilation of protozoal N from bacterial N (Table 3). The percentage of protozoal N derived from bacterial N is low in the current study, probably because of feed N contamination of protozoal samples; thus, protozoal ^{15}N enrichment was diluted and could not be fixed (data not shown) by the boiling approach described by Ye et al. (2018). Although the potential for denitrification of nitrates to N_2 or N_2O has also been speculated to become a more prevalent route of NO_2^- reduction while CH_4 emission is suppressed (Liu et al., 2017), Latham et al. (2016) summarized denitrification to be minor in the rumen but suggested that such alternative routes deserve future research. The lack ($P > 0.15$) of treatment effect on bacterial N flow or bacterial N derived from $\text{NH}_3\text{-N}$ does not support decreased $\text{NH}_3\text{-N}$ assimilation. There was an interaction ($P < 0.01$) between NO_3^- and LYC on NANBN flow such that LYC numerically decreased NANBN without NO_3^- but increased it when combined with NO_3^- .

The NO_3^- main effect tended ($P = 0.10$) to increase abundance of total protozoa and genus *Entodinium*, the dominant genus recovered (Table 3), perhaps indicating that they have nitrate reductase linked to electron transport phosphorylation. Preliminary searches of the genome of *Entodinium caudatum* returned annotated nitrate reductase (personal communication, Tansol Park, The Ohio State University, Columbus), which should be expected based on previous studies comparing protozoal enriched samples versus bacterial samples (Yang et al., 2016). Increasing concentration of NO_2^- profoundly inhibited motility of entodiniomorphids (of which *E. caudatum* is a predominant member) and entodiniomorphid chemotaxis in our companion study (Roman-Garcia et al., 2019), so NO_2^- reduction by protozoa awaits further inquiry. There was no difference in generation time between NO_3^- , LYC, or their interaction. Protozoal counts were low in this study compared with our other studies (Karnati et al., 2009; Wenner et al., 2017). In this study, NO_3^- or NO_2^- concentrations

Figure 3. Concentrations of nitrate (top), nitrite (middle), and ammonia (bottom) in continuous cultures administered diets supplemented without or with NO₃[−] or live yeast culture (LYC). Treatment by time interaction was $P < 0.01$ for all. Main effect of NO₃[−], LYC, or their interaction (Inter) at each time point (** $P \leq 0.01$, * $P \leq 0.05$, NS = $P > 0.15$). The SEM is pooled across treatment per time and shown only for one series per plot.

Table 3. Nitrogen flows, protozoal counts, and total generation time in continuous cultures administered diets supplemented without (–) or with (+) nitrate or live yeast culture (LYC)^{1,2}

Item ³	–NO ₃ [–]		+NO ₃ [–]		Pooled SEM	P-value of effects ⁴		
	–LYC	+LYC	–LYC	+LYC		NO ₃ [–]	LYC	Interaction
N flow, g/d	0.873	0.924	0.800	0.874	0.045	0.06	0.06	NS
NH ₃ -N flow, g/d	0.160	0.187	0.154	0.173	0.015	NS	0.08	NS
NAN flow, g/d	0.711	0.738	0.641	0.699	0.036	0.05	0.12	NS
Bacteria N flow, g/d	0.364	0.432	0.360	0.356	0.031	NS	NS	NS
NANBN, g/d	0.347	0.307	0.281	0.342	0.017	NS	NS	<0.01
g of bacteria N/kg of OMD	27	33.2	25.5	28.2	3.3	NS	NS	NS
g of bacteria N/kg of NDFD	55	53.3	51.1	46.8	7.9	NS	NS	NS
Bacteria N from NH ₃ -N, %	68.9	67.1	65.0	62.6	3.9	NS	NS	NS
Protozoa N from bacteria N, %	33.5	35.4	34.5	33.2	2.0	NS	NS	NS
Total <i>Entodinium</i> , cells/mL	530	525	653	718	108	0.10	NS	NS
Total protozoa	560	541	656	758	104	0.12	NS	NS
Generation time, h	32.7	36.6	35.1	29.8	4.8	NS	NS	NS

¹Nitrate added at 1.5% diet DM; urea was added to the diet isonitrogenously with the nitrate treatment. Kjeldahl analysis does not recover NO₃[–] or NO₂[–], so total N flow is underestimated for the +NO₃[–] treatments.

²LYC added at 0.010 g/d (scaled from cow to fermentor).

³NANBN = nonammonia, nonbacteria N; OMD = OM digestibility; NDFD = NDF digestibility.

⁴Main effects of nitrate or LYC treatments or their interaction. NS = $P > 0.15$.

(Figure 3) peaked below half the initial low doses that blunted entodiniomorphid chemotaxis in the companion paper (Roman-Garcia et al., 2019); consequently, higher concentration of NO₂[–] that was not typically measured in other studies might explain some of the negative results reported by Yang et al. (2016). The LYC treatments did not affect protozoa populations. Mathieu et al. (1996) noted a trend for increased protozoal counts in sheep, and Arakaki et al. (2000) reported that yeast culture increased protozoa concentration in steers.

Bacterial and Archaeal Relative Abundance

Consistent with in vivo results summarized by Firkins and Yu (2015), *Prevotellaceae*, a member of the *Bacteroidetes* phylum, was the most abundant family (Table 4), whereas *Ruminococcaceae* and *Lachnospiraceae* (Table 5) were the second and third most abundant of the other dominant phylum, *Firmicutes*. The representation by minor phyla (Table 6), as well as uncharacterized taxa are also consistent with other sequencing results from the literature (Firkins and Yu, 2015). The greater passage of fiber compared with starch in the particulate fraction probably decreased the relative abundance of some strictly fibrolytic taxa, such as the dominant members of the *Fibrobacteraceae* (Ransom-Jones et al., 2012) when the particulate fraction is combined as in our approach. Relative abundance provides useful treatment comparisons, but key findings should be confirmed by quantitative PCR (Firkins and Yu, 2015).

A trend ($P = 0.07$) was observed for an interaction between NO₃[–] and LYC on relative abundance of

Prevotella spp. such that LYC numerically increased ($P = 0.14$) and NO₃[–] tended to increase ($P = 0.07$) relative abundance of *Prevotella* spp., but the combination of NO₃[–] and LYC was not additive (Table 4). Nitrate increased ($P < 0.05$) the relative abundance of the genus *Xylanibacter* and decreased ($P = 0.05$) the abundance of uncultured *Prevotellaceae*. The *Bacteroidales* S24–7 family tended to increase ($P = 0.06$) with LYC. As described by Hackmann and Firkins (2015), we searched the Hungate 1000 genomes (Creevey et al., 2014) using the IMG database (Markowitz et al., 2014) for genus *Prevotella* or family *Prevotellaceae* (*Xylanibacter* or *Bacteroidales* S24–7 were not specifically represented when we searched in June 2018). We did not detect an annotated nitrate reductase. However, we did note that 14 of the 31 genomes had annotated genes for periplasmic nitrite reductase complex NrfHA (Kyoto Encyclopedia of Genes and Genomes identification: K15876 flanked by K03385; data not shown) and YgbA (promotor activated by nitrous oxide; conserved protein pfam11756; data not shown). Kern and Simon (2009) described this gene complex for *Wolinella succinogenes* as a mechanism to link reduction of NO₂[–] to NH₄⁺ to the menaquinone pool used in electron transport phosphorylation. Genus *Prevotella* also has a full complement of genes needed for electron transport phosphorylation (Hackmann et al., 2017), as does *Bacteroidales* S24–7 (Ormerod et al., 2016). Thus, induction of NrfHA would be beneficial by either generating more ATP or by more effective electron disposal (Latham et al., 2016). The NO₃[–] main effect tended to increase ($P = 0.09$) and LYC numerically increased ($P = 0.11$) the relative abundance of family *Rikenellaceae*.

Table 4. Within the phylum *Bacteroidetes*, family and genus relative abundance (% of total sequences) in continuous cultures fed without (–) or with (+) NO₃[–] and without (–) or with (+) live yeast culture (LYC)¹

Item ²	−NO ₃ [−]			+NO ₃ [−]			Contrast (<i>P</i> -value) ³					
	−LYC			+LYC			−LYC			+LYC		
	Family	Genus		Family	Genus		Family	Genus		Family	Genus	
<i>Prevotellaceae</i>	28.9			28.1			27.8			27.4		
<i>Prevotella</i>		16.9			20.1			20.6			20.2	
<i>Paraprevotella</i>		0.275			0.341			0.325			0.365	
<i>Xylanibacter</i>		0.267			0.253			0.475			0.565	
Unc. <i>Prevotellaceae</i>		8.27			7.41			6.38			6.27	
<i>Bacteroidales</i> S24–7	6.77			7.55			6.23			9.93		
Unc. rumen bacterium		5.87			6.70			5.18			8.47	
Unc. bacterium		0.831			0.991			0.848			1.310	
Other		0.072			0.066			0.055			0.083	
<i>Rikenellaceae</i>	7.58			8.76			8.83			9.79		
Sp3-e08		0.499			0.847			0.821			0.732	
RC9 gut group		6.99			7.91			7.99			8.96	
BS11 gut group	4.51			6.07			5.25			6.76		
Unc. bacterium		2.32			3.19			3.16			3.88	
Unc. rumen bacterium		2.01			2.77			2.06			2.78	
<i>Bacteroidales</i> RF16	0.258			0.291			0.252			0.289		
Other <i>Bacteroidales</i> ⁴	0.955			0.702			1.052			0.901		

¹All data presented had at least one treatment with ≥0.50% representation (see Materials and Methods), so the sum of genera will be ≤ the respective family. Total sequences included bacteria and archaea.

²Unc. = uncultured.

³Main effects of nitrate or LYC treatments or their interaction. NS = *P* > 0.15.

⁴Represented by the single genus other *Bacteroidales*.

Table 5. Within the phylum *Firmicutes*, family and genus relative abundance (% of total sequences) in continuous cultures fed without (–) or with (+) NO_3^- and without (–) or with (+) live yeast culture (LYC)¹

Item ²	– NO_3^-			+ NO_3^-			Contrast (<i>P</i> -value) ³		
	–LYC			+LYC			–LYC		
	Family	Genus		Family	Genus		Family	Genus	
<i>Ruminococcaceae</i>	9.57			8.38			8.16		
Unc. bacterium		3.80			3.96			4.14	
<i>Ruminococcus</i>		4.43			3.23			3.84	
<i>Saccharofermentans</i>		0.537			0.485			0.619	
<i>Incertae sedis</i>		0.563			0.486			0.513	
Other <i>Ruminococcaceae</i>		0.181			0.122			0.131	
<i>Lachnospiraceae</i>	24.1			19.2			17.0		
<i>Butyrivibrio</i>		10.72			6.96			6.93	
<i>Pseudobutyribrio</i>		4.65			4.31			4.96	
Uncultured		4.93			3.99			4.30	
<i>Incertae sedis</i>		1.586			1.544			1.540	
Other <i>Lachnospiraceae</i>		1.178			0.997			1.301	
<i>Roseburia</i>		0.393			0.456			0.514	
<i>Anaerospiribacter</i>		0.160			0.207			0.200	
<i>Christensenellaceae</i>	2.85			2.38			2.60		
Unc. <i>Christensenellaceae</i>		2.76			2.30			2.40	
<i>Clostridiaceae</i>	2.23			1.36			1.60		
<i>Clostridium</i>		2.22			1.35			1.56	
<i>Veillonellaceae</i>	2.45			2.71			2.50		
<i>Succinilasticum</i>		1.58			1.93			1.74	
<i>Selenomonas</i>		0.276			0.290			0.391	
Other <i>Selenomonas</i> ⁴		0.147			0.162			0.207	
Unc. <i>Selenomonas</i> ⁴		0.0680			0.0816			0.1063	
Unc. rumen <i>Selenomonas</i> ⁴		0.0516			0.0347			0.0552	
<i>Selenomonas ruminantium</i> ⁴		0.0104			0.0137			0.0207	
Family XIII <i>Incertae sedis</i> ⁵	0.884			0.947			0.925		
Other <i>Clostridiales</i>	0.349			0.325			0.347		

¹All data presented had at least one treatment with $\geq 0.50\%$ representation (see Materials and Methods), so the sum of genera will be $\leq$ the respective family. Total sequences included bacteria and archaea.

²Unc. = uncultured.

³NS = $P > 0.15$.

⁴Species-level operational taxonomic units reported because nitrate and nitrite reduction expected a priori.

⁵Within order *Clostridiales* (family “other *Clostridiales*” represented only by the single genus “other *Clostridiales*”).

Item ²	-NO ₃ ⁻			+NO ₃ ⁻			Contrast (<i>P</i> -value) ³					
	-LYC			+LYC			-LYC			+LYC		
	Family	Genus		Family	Genus		Family	Genus		Family	Genus	
<i>Succinivibrionaceae</i> ⁴	0.819			1.122			1.157			0.913		
<i>Succinivibrio</i>		0.271			0.412			0.445			0.408	
Unc. <i>Succinivibrionaceae</i>		0.371			0.492			0.468			0.360	
<i>Campylobacteraceae</i> ⁴	0.044			0.220			0.139			0.192		
<i>Moraxellaceae</i> ⁴	0.199			0.152			0.406			0.109		
<i>Desulfuronomonadales</i> GR-WP33-58 ⁴	0.161			0.175			0.270			0.264		
<i>Bifidobacteriaceae</i> ⁵	0.0011			0.0009			0.0004			0.1079		
<i>Spirochaetaceae</i> ⁶	2.56			2.65			2.50			1.66		
<i>Treponema</i>		1.943			1.925			1.810			1.035	
<i>Spirochaeta</i>		0.588			0.755			0.696			0.613	
<i>Fibrobacteraceae</i> ⁷	0.071			0.090			0.066			0.045		
<i>Anaeroplasmataceae</i> ⁸	0.475			0.552			0.439			0.513		
<i>Mollicutes</i> Unc. RF9 ⁸	0.215			0.228			0.333			0.222		
Unc. RFP12 ⁹	0.199			0.271			0.160			0.174		
<i>Methanobacteriaceae</i> ¹⁰	0.200			0.112			0.223			0.148		
<i>Thermoplasmatales</i> Vadin CA11 gut group ¹⁰	0.011			0.014			0.015			0.008		

¹⁰Methanogenic *Euryarchaeota* (families *Methanobacteriaceae* and *CAL1* gut group represented only by single genera *Methanobrevibacter* and uncultured archaeon, respectively; not shown).

Nitrate treatment numerically increased ($P = 0.15$) the relative sequence abundance of the BS11 gut group and increased ($P < 0.05$) the genus of BS11 characterized as an uncultured rumen bacterium. The LYC increased ($P = 0.02$) the BS11 gut group family, including both uncultured (nonrumen) and uncultured rumen genera. Solden et al. (2017) distinguished the 2 genera functionally based on substrate preference but also noted the potential for electron-transport phosphorylation (e.g., encoding cytochrome b1 oxidase). The addition of LYC could increase the uptake of lactate (conversion to pyruvate generates [2H]) or decrease the redox potential by consuming traces of O_2 (Chaucheyras-Durand et al., 2008), whereas NO_2^- buildup could increase the redox potential (Latham et al., 2016); thus, the increased abundance of bacteria able to dispose of electrons and accompanying [2H] under these conditions probably helps explain the LYC effects and interactions with NO_3^- .

The main effect of LYC decreased ($P = 0.04$) relative abundance of the *Ruminococcaceae* family (Table 5). The genus *Butyrivibrio* tended ($P = 0.07$) to be decreased by both NO_3^- and LYC, which could explain the decrease in butyrate production observed at some time points. Compared with killed yeast culture, LYC increased genus *Ruminococcus* but did not affect genus *Butyrivibrio* relative sequence abundance (Jiang et al., 2017). *Clostridiaceae* and *Veillonellaceae* families had trends ($P \leq 0.10$) for an interaction; when NO_3^- was present, LYC did not have an effect. *Succiniclasticum ruminis* (family *Veillonellaceae*) grows on succinate but does not reduce NO_3^- (van Gylswyk, 1995), so succinate accumulation with LYC could be counteracted by inhibition by NO_3^- or NO_2^- . In the ruminal solids fraction in dairy cattle, *Lachnospiraceae* tended to be decreased by a lower dose of LYC or killed *S. cerevisiae*, and the active LYC had lower abundance of *Clostridiaceae* than the killed culture (Jiang et al., 2017). Without a likely mechanism for inhibition, the decreased relative abundance in our study is likely explained by increased concentration of stimulated taxa of Bacteroidetes because a mode of action of LYC is increasing bacterial abundance (Chaucheyras-Durand et al., 2008; Pinloche et al., 2013).

The genus *Selenomonas* is among the most widely recognized ruminal bacteria that reduce both NO_3^- and NO_2^- (Latham et al., 2016; Yang et al., 2016). However, in the current study, their relative sequence abundance might be lower than would be expected because relative sequence abundance is not fully quantitative (Firkins and Yu, 2015). Unlike most other taxa (which were comparable), Illumina sequencing resulted in a much lower relative abundance compared with quantitative PCR for *Selenomonas* (Jiang et al., 2017). Uncultured

rumen *Selenomonas* was increased by NO_3^- ($P < 0.05$), not affected by LYC, but increased with the combination of both ($P = 0.06$ trend for an interaction), perhaps displacing taxa such as genus *Succiniclasticum*. The lactilytic selenomonads respond to LYC (Chaucheyras-Durand et al., 2008) and are likely more active in NO_3^- reduction compared with *S. ruminantium* strains that rely more on glucose, perhaps because lactate is an electron donor that can be used in NO_2^- reduction (Yang et al., 2016).

Desulfuronomonadales GR-WP33-58 was increased ($P = 0.05$) by the main effect of NO_3^- (Table 6). To our knowledge, little is known of this taxon's role in the gut. However, it is found in other anoxic environments such as aquaculture (Lin et al., 2017), where it reduces sulfate and most likely NO_3^- or NO_2^- , or both. A trend was observed for an interaction between NO_3^- and LYC on *Spirochaetacea* relative abundance, which appeared comparable to *Fibrobacteraceae* (i.e., $P \leq 0.09$ for NO_3^- main effect and its interaction with LYC). Although a reason for the treatment response is not clear, an apparent symbiosis between *Fibrobacter* and *Treponema* strains has previously been discussed (Shinkai et al., 2010; Leng, 2014).

Contrary to the expectation of NO_2^- inhibition of methanogens (Latham et al., 2016), *Methanobacteriaceae* was not affected by the NO_3^- treatment (Table 6). We noted that LYC decreased ($P = 0.03$) their relative abundance, supporting a potential role for LYC to decrease methanogenesis (Jeyanathan et al., 2014). However, methanogen abundance is not always consistently positively associated with methanogenesis (Firkins et al., 2015).

CONCLUSIONS

As expected, dietary nitrate suppressed methanogenesis and shifted populations of bacteria that likely have either NO_3^- or NO_2^- reductase activities. Contrary to our hypothesis, the combination of LYC with dietary nitrate did not interact to limit NO_2^- accumulation, which is an important consideration if dietary nitrates were to be used to suppress methanogenesis in vivo. The H_2 emission and bacterial sequence abundance were influenced positively by feeding LYC, supporting previously described modes of action. That LYC shifted relative sequence abundances of functionally important bacteria inconsistently with other reports suggests further research is needed to explain variation associated with supplementing LYC under differing dietary conditions. No evidence indicated NO_3^- toxicity in continuous cultures, although further research is needed in studies with a higher abundance of protozoa.

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