Inhibition of methanogenesis by nitrate, with or without defaunation, in continuous culture

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ABSTRACT

Within the rumen, nitrate can serve as an alternative sink for aqueous hydrogen [$H_2(aq)$] accumulating during fermentation, producing nitrite, which ideally is further reduced to ammonium but can accumulate under conditions not yet explained. Defaunation has also been associated with decreased methanogenesis in meta-analyses because protozoa contribute significantly to H_2 production. In the present study, we applied a 2×2 factorial treatment arrangement in a 4×4 Latin square design to dual-flow continuous culture fermentors ($n = 4$). Treatments were control without nitrate ($-NO_3^-$) versus with nitrate ($+NO_3^-$; 1.5% of diet dry matter), factorialized with normal protozoa (faunated, FAUN) versus defaunation (DEF) by decreasing the temperature moderately and changing filters over the first 4 d of incubation. We detected no main effects of DEF or interaction of faunation status with $+NO_3^-$. The main effect of $+NO_3^-$ increased $H_2(aq)$ by $11.0 \mu M$ (+117%) compared with $-NO_3^-$. The main effect of $+NO_3^-$ also decreased daily CH_4 production by $8.17 \text{ mmol } CH_4/d$ (31%) compared with $-NO_3^-$. Because there were no treatment effects on neutral detergent fiber digestibility, the main effect of $+NO_3^-$ also decreased CH_4 production by $1.43 \text{ mmol of } CH_4/g$ of neutral detergent fiber degraded compared with $-NO_3^-$. There were no effects of treatment on other nutrient digestibilities, N flow, or microbial N flow per gram of nutrient digested. The spike in $H_2(aq)$ after feeding NO_3^- provides evidence that methanogenesis is inhibited by substrate access rather than concentration, regardless of defaunation, or by direct inhibition of NO_2^- . Methanogens were not decreased by defaunation, suggesting a compensatory increase in non-protozoa-associated methanogens or

an insignificant contribution of protozoa-associated methanogens. Despite adaptive reduction of NO_3^- to NH_4^+ and methane inhibition in continuous culture, practical considerations such as potential to depress dry matter intake and on-farm ration variability should be addressed before considering NO_3^- as an avenue for greater sustainability of greenhouse gas emissions in US dairy production.

Key words: nitrate, protozoa, continuous culture, aqueous hydrogen

INTRODUCTION

The impact of dairy cattle on enteric CH_4 emissions is estimated to contribute approximate 3.9% of total anthropogenic CH_4 in the United States (US EPA, 2013). The primary route of methanogenesis consumes aqueous H_2 [$H_2(aq)$] via reduction of CO_2 or, alternatively, formate (Yang et al., 2016). Rumen protozoa produce both formate and H_2 , but ruminal formate metabolism is poorly understood compared with interspecies H_2 transfer (Tapio et al., 2017). The production of CH_4 pulls release of H_2 into the rumen via hydrogenases in bacteria, protozoa, and fungi. This interspecies H_2 transfer to methanogens is integrally linked in ruminal VFA metabolism; these 2 processes are readily studied in continuous culture because net (after recycling) VFA production can be measured without absorption or end-product feedback, and headspace CH_4 and $H_2(aq)$ can be directly measured rather than estimated (Wenner et al., 2017).

Some electron acceptors with a higher redox potential, such as NO_3^- or SO_4^{2-} (van Zijderveld et al., 2010; Leng, 2014), outcompete CO_2 . Thus, rumen microbial reducers of these electron acceptors can divert $H_2(aq)$ away from methanogenesis from CO_2 (Ungerfeld and Kohn, 2006; Morgavi et al., 2010). Nitrate has potential to contribute NH_3-N that is assimilated into microbial protein (Lee and Beauchemin, 2014; Wang et al., 2018); however, NO_3^- and especially its reduction product NO_2^- , can be toxic to some rumen bacteria and methanogens (Latham et al., 2016). When animals are adapted gradually, NO_3^- is a reliable CH_4 mitigation

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strategy (van Zijderveld et al., 2010; 2011a,b), albeit sometimes at the expense of decreased DMI (Lee and Beauchemin, 2014; Meller et al., 2019).

Nitrate represents a predictable treatment to test the theories of Janssen (2010) regarding concentration of $H_2(aq)$ and its role in shifting VFA production pathways away from acetate and CH_4 production. The relationship between $H_2(aq)$ and methanogenesis is clear (Janssen, 2010), but the feedback of increased $H_2(aq)$ concentration on VFA pathways is less clear (van Lingen et al., 2016; Wenner et al., 2017), particularly because of transcriptional regulation by methanogens and alternative pathways by bacteria (Greening et al., 2019). Both NO_3^- reducers and methanogens compete for $H_2(aq)$ (van Zijderveld et al., 2010; Leng, 2014; Zhao et al., 2015), so both processes increase total H_2 production. However, $H_2(aq)$ used in NO_3^- reduction should not be available to reduce fumarate and could decrease propionate production (Yang et al., 2016). Consequently, whether NO_3^- changes the acetate:propionate ratio probably depends on $H_2(aq)$.

Protozoa possess hydrogenosomes or mitosomes that release H_2 or formate (Hackstein and Tielens, 2010) that are the main substrates for ruminal methanogens and, as such, have a close relationship with some methanogens (Ushida, 2010; Belanche et al., 2014; Valle et al., 2015). The association of protozoa with bacteria and methanogens has led some researchers to suggest defaunation (complete removal of ruminal protozoa) as a method of improving efficiency of microbial protein synthesis (Eugène et al., 2004) and overall dietary N usage efficiency (Hristov and Jouany, 2005) while mitigating CH_4 production in ruminant agriculture, although the latter results are not always consistent (Tapio et al., 2017; Ungerfeld, 2018). Protozoa help stabilize ruminal pH, tend to increase butyrate concentration, and promote fiber degradation directly or indirectly by cellulolytic bacteria or fungi (Jouany and Ushida, 1999; Newbold et al., 2015) or by physically shredding fibrous feed particles (Dehority, 2010). Moreover, methanogens need not necessarily be close symbionts with protozoa but, instead, can be continuously recruited (Park and Yu, 2018). Thus, further research is needed to investigate potential interactions of protozoa with other CH_4 mitigation strategies such as feeding NO_3^- .

We hypothesized that dietary NO_3^- treatment would provide an alternate sink for $H_2(aq)$ to inhibit methanogens and shift their relative abundance toward those associating with protozoa. The combination of treatments was proposed to interact to suppress methanogenesis by removing protozoa as a supportive mechanism for associated methanogens. We also hypothesized that NO_3^- would shift fermentation toward acetate, especially after defaunation (a positive interaction),

unless $H_2(aq)$ increased enough to shift stoichiometry toward propionate. Our objective was to use continuous culture fermentors as a model to measure concentration of $H_2(aq)$, CH_4 production, VFA production, and fiber digestibility to test these hypotheses and compare microbial communities to help explain results.

MATERIALS AND METHODS

Experimental Design

The present study was conducted as a 2×2 factorial arrangement of treatments in a 4×4 Latin square experimental design using dual-flow continuous culture fermentors ($n = 4$). Once daily, all fermentors were fed 40 g (DM) of a pelleted 50:50 forage:concentrate diet (37.5% NDF, 19.5% NSC, 15.4% CP). The pelleted concentrate consisted of shelled corn (36.9%), soybean hulls (40.4%), dried distillers grains (10.0%), soybean meal (9.1%), corn oil (1.8%), and a vitamin/mineral mix (1.8%) containing 36.6% Na, 56.4% Cl, 3,500 mg/kg Zn, 2,800 mg/kg Mn, 1,750 mg/kg Fe, 350 mg/kg Cu, 70 mg/kg I, and 70 mg/kg Co. All treatments were fed the base diet. Isonitrogenous treatments were control without ($-NO_3^-$; supplemental urea at 0.7% and NaCl at 1.4% of diet DM, respectively) versus with ($+NO_3^-$; 1.5% of diet DM provided as NO_3^- in a sodium salt) nitrate, factorialized with faunated (**FAUN**) versus defaunated (**DEF**) fermentor contents. To prevent uneven adaptation exposure to NO_3^- between DEF and FAUN, $+NO_3^-$ was not dosed until defaunation was completed.

Continuous Culture Operation

Dual-flow continuous culture fermentors ($n = 4$; Hoover et al., 1976) were given 8 d of adaptation with sampling periods lasting 3 d, for a total of 11 d per experimental period. Each experimental period was initiated by sampling the rumen contents of 2 ruminally cannulated lactating Jersey cows. These cows were fed a standard lactating diet similar to that used in a previous continuous culture study (Welty et al., 2019) consisting of 52% corn silage, 18% alfalfa hay, and 30% corn/soybean meal premix; no cows used in this study had been previously fed NO_3^- . Rumen contents were squeezed through 2 layers of cheesecloth into warmed, insulated containers and transported to the laboratory at 39°C within 30 min. Inocula sources were pooled from 2 cows per period and added to fermentors at 50% of total working volume. Clarified rumen fluid (centrifuged at $15,000 \times g$, 4°C, 15 min, and then autoclaved) was diluted 1:20 with anaerobic buffer for the first 2 d of each period to better adapt microbial contents to fermentors.

Average fermentor volume was 1.71 L (± 0.09 L), stirring was set to 50 rpm, temperature was set at 39°C, total buffer dilution rate was fixed at 7.0%/h for all treatments, and solids dilution rate was fixed at 5.0%/h for all treatments. Buffer pH was maintained at 6.75 under continuous bubbling of CO₂ to maintain anaerobic conditions; constant buffer pH allowed a diurnal post-feeding shift in pH to better reflect normal rumen function (Wenner et al., 2017). Fermentor pH ranged from 6.8 pre-feeding to 6.2 at its nadir, approximately 8 h post-feeding, before returning to 6.8 gradually for the last 16 h of the daily cycle. Fermentor buffer was made according to Weller and Pilgrim (1974); 10 mg/dL urea was added to buffer to maintain approximately 5 to 10 mg/dL NH₃-N at 4 h post-feeding after 5 d of adaptation.

Fermentor CH₄ and H₂ emissions were measured using the Micro-Oxymax detection system (Columbus Instruments Inc., Columbus, OH) as previously described (Wenner et al., 2017; Wagner et al., 2018). Gas production data were taken in tandem with fermentor effluent samples on the last 3 d of each experimental period. Ammonium sulfate enriched with 10% ¹⁵N was dosed in a priming dose of 50 mg on d 5 and added to the buffer at 25 mg/L from d 5 to 10 for a desired enrichment of 0.2% atom excess.

Defaunation of Fermentors

Fermentors with FAUN treatments were equipped with filters designed to retain protozoa with impeller speeds of about 50 rpm and a prefilter wrapped around a 50- μ m mesh filter (Karnati et al., 2009a). Increased stirring to prevent settling of isotrichids combined with 300- μ m wire mesh filters to wash out particle-associating entodiniomorphids defaunated fermentors in one previous study (Karnati et al., 2009a) but not another (Fowler et al., 2015), nor in our preliminary studies. Hence, for DEF treatments, fermentors were fitted with 300- μ m wire mesh filters (Karnati et al., 2009a), mixed at 100 rpm, and had temperature decreased to 34°C. Decreased temperature increased protozoal generation time and eliminated protozoa in batch cultures after 5 transfers (Dehority, 2010). Given our turnover rate of 1.68/d, we expected 3 to 4 d would be sufficient to fully defaunate fermentors (5.0 to 6.7 turnovers). At the end of the d 4, DEF fermentors were returned to 50 rpm and 39°C (same as FAUN).

Sample Collection and Analysis

From d 9 to 11, daily fermentor effluent was collected cumulatively for 24 h post-feeding in containers on ice. A 30% subsample by weight of the mixed total

effluent was aspirated with a 6.35-mm-diameter tube to prevent sampling bias against larger particles and pooled by fermentor within period. Sampled fermentor effluent was dried at 55°C and stored for various analyses at -20°C. A subsample of dried effluent was used to determine DM at 105°C and subsequently ashed at 550°C (AOAC, 1990). Effluent N was determined by the Kjeldahl method (AOAC, 1990), and NH₃-N remaining in oven-dried samples was determined by dissolving approximately 0.2 g of sample in 1 mL of distilled H₂O followed by colorimetric analysis (Chaney and Marbach, 1962) to determine NAN flow by subtraction. Kjeldahl analysis only recovers a portion of escaped NO₃⁻ or NO₂⁻, which Meller et al. (2019) estimated to be 29.4%. However, NAN should be removing NO₃⁻ or NO₂⁻ along with NH₄⁺ (Welty et al., 2019). Neutral detergent fiber was determined with use of heat-stable amylase and sodium sulfite (Van Soest et al., 1991). Starch and water-soluble carbohydrates were commercially analyzed by Dairy One Forage Lab (Ithaca, NY) and summed as NSC. A 50-mL aliquot of daily effluent was acidified with 3 mL of 6 N HCl and composited by fermentor within period and stored at -20°C. Samples were later thawed, centrifuged (15,000 $\times g$, 4°C, 15 min), and filtered through Whatman No. 1 filter paper (Whatman International, Maidstone, UK) for analysis of NH₃-N (Chaney and Marbach, 1962) and VFA by gas-liquid chromatography (Harvatine et al., 2002). Analysis of ¹⁵N enrichment was performed by ion-ratio mass spectrometry after combustion and ionization (Delta V Advantage Isotope-Ratio Mass Spectrometer; ThermoFinnigan MAT GmbH, Bremen, Germany) by C. Lee (The Ohio State University, Ohio Agricultural Research and Development Center, Wooster).

Using the protozoal N:cell ratio previously reported by Sylvester (2005), we estimated protozoal N based on daily outflow of protozoal cells in effluent. Sylvester's (2005) samples were primarily small entodiniomorphids and were approximated at 0.5 ng/cell, but N:cell can range to more than 40 times that value (Weller and Pilgrim, 1974; Karnati et al., 2009a; Ye et al., 2018). Given that larger cells should contain more N but N concentration could not be directly determined in the present study, large entodiniomorphids and all *Isotrichidae* were assigned a conservative value of 1 ng of N/cell, whereas *Charonina* and small entodiniomorphids were assigned 0.5 ng of N/cell. This approximated protozoal N was subtracted from daily NAN flow before using ¹⁵N enrichment of bacterial to estimate bacterial N flow.

Microbial Sample Processing

On d 7 of each experimental period, fermentors were mixed at 150 rpm for 15 s to sample mixed fermentor

contents by transfer pipette with a 6.25-mm-diameter orifice. Samples were taken at 0, 1.5, 3, 6, and 12 h post-feeding. One 10-mL aliquot was sampled and stored at -80°C for DNA extraction. An additional 8-mL aliquot of effluent and 10-mL aliquot of agitated fermentor contents were taken and fixed in 2 mL and 5 mL, respectively, of 50% formalin for protozoal counts according to Dehority (1993). Because of the unexpected enrichment of *Charonina ventriculi* (confirmed by B. Dehority, The Ohio State University, Columbus; personal communication), protozoal counts were differentiated morphologically into the following categories: *Charonina*, small entodiniomorphids ($<100\text{ }\mu\text{m}$ in length), large entodiniomorphids ($>100\text{ }\mu\text{m}$ in length), and family *Isotrichidae*.

Microbial DNA was extracted from fermentor samples pooled by fermentor within period and extracted in a manner similar to that of Faulkner et al. (2017). Briefly, frozen samples of fermentor contents were thawed overnight at 4°C , mixed by inversion, pooled, and a 1-mL subsample centrifuged ($16,000 \times g$, 4°C , 10 min). Supernatant was discarded and DNA extraction proceeded with the repeated bead beating and column purification approach of Yu and Morrison (2004). Quantification of DNA was performed using a NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington, DE) for sequencing and using the Quant-iT ds DNA assay (Thermo Fisher Scientific Inc., Waltham, MA) for quantitative real-time PCR (qPCR).

A pooled reference of all samples was used for qPCR analyses of total bacteria and total archaea by targeting the 16S rRNA gene using an Mx3000 real-time polymerase chain reaction system (Stratagene California, La Jolla, CA). Domain-specific primers used in qPCR are listed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2020-18325>) and qPCR conditions matched that of Stiverson et al. (2011).

The diversity and composition of the prokaryotes in the fermentor samples were analyzed using metatranscriptomics similar to that described by Faulkner et al. (2017). Briefly, amplicon libraries were prepared by amplifying the V4–V5 hypervariable region of the 16S rRNA gene using a universal primer, with each library having a unique barcode for multiplexing. The amplicon libraries were pooled and sequenced (2×300 bp paired-end sequencing) on a MiSeq platform (Illumina Inc., San Diego, CA). The sequences were processed and then analyzed using QIIME version 1.5 (Caporaso et al., 2010). Only sequences ($\sim 247,000$) with a Phred score ≥ 20 and free from chimera were analyzed. Operational taxonomic units (OTU) were clustered at 97% similarity with Uclust (Edgar, 2013; Quast et al., 2013) and the Silva database (v123, <http://www.arb-silva.de/>), which was also used to taxonomically classify

the OTU. Only major phyla, family, and genera with a relative sequence abundance $\geq 0.5\%$ were included in statistical analysis.

Hourly Filtrate Sampling

Using a y-valve septum on the filtrate line (Wenner et al., 2017), 10 mL each of filtrate was sampled from the fermentors at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, and 12 h post-feeding on d 9, 10, and 11. Samples were acidified with 1 mL of 6 N HCl and analyzed for $\text{NH}_3\text{-N}$ (Chaney and Marbach, 1962), VFA (Harvatine et al., 2002), and both NO_3^- and NO_2^- (O'Dell, 1993). At each of the same time points, 20 mL of filtrate was sampled from the septum and injected into 60-mL glass culture bottles (Wheaton Glass, Millville, NJ) that were prefilled with 5 mL of 5 M H_3PO_4 , flushed with N_2 , and sealed with a butyl rubber stopper for $\text{H}_2(\text{aq})$ measurement according to Wenner et al. (2017). Partial pressure of H_2 in the headspace of the fermentors was also used to predict $\text{H}_2(\text{aq})$ using Henry's Law for comparison of predicted versus measured $\text{H}_2(\text{aq})$. Hydrogen recovery was estimated using VFA production, including valerate but not caproate (Guyader et al., 2017) and including the $\text{H}_2(\text{aq})$ incorporation from NO_3^- reduction.

Statistical Analysis

Daily effluent nutrient measurements and ^{15}N enrichment data were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC) according to the following model:

$$Y_{ijklm} = \mu + N_i + D_j + (N \times D)_{ij} + p_k + f_l + \varepsilon_{ijklm},$$

where Y_{ijklm} is the dependent variable, μ is the overall population mean, N_i is the fixed effect of i th NO_3^- treatment ($i = -\text{NO}_3^-, +\text{NO}_3^-$), D_j is the fixed effect of j th faunation treatment ($j = \text{faunated, defaunated}$), $(N \times D)_{ij}$ is the interaction of NO_3^- and defaunation, p_k is the random effect of k th period ($k = 1, 2, 3, 4$), f_l is the random effect of l th fermentor ($l = 1, 2, 3, 4$), and ε_{ijklm} is the residual error, assumed independent and $\sim N(0, \sigma_e^2)$.

For evaluations of protozoa, the effect of DEF (and its interaction with N) was removed. A Kenward-Roger degrees of freedom correction was applied to all dependent variables. For daily CH_4 production, the random effect of m th day ($m = 1, 2, 3$) was added to the model due to large daily variation in CH_4 production between days. All hourly data were analyzed using repeated measures and the covariance structure with the lowest

Bayesian information criterion. Gas production data over time post-feeding were fitted to an autoregressive heterogeneous (first-order) covariance structure using the PARM statement in SAS to specify starting variance parameters obtained using the MIVQUE0 statement; all other dependent variables were fitted to a first-order autoregressive covariance structure. Contrast statements tested the main effect of NO_3^- , faunation, or the $\text{NO}_3^- \times$ faunation interaction when there were no repeated measures or for main effect treatment means when there was no interaction ($P > 0.10$); when treatment interacted with time, contrasts were performed at each time point simultaneously.

The overall prokaryotic microbiota among the treatments were compared using nonmetric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity using ANOSIM in R project (<https://www.R-project.org/>). Relative abundance of individual taxa based on sequencing data and quantification based on qPCR were analyzed using the same statistical model as above but through the R project (package vegan, <http://cran.r-project.org/web/packages/vegan/>). All qPCR data were $\log_{10}$ -transformed before analysis, but relative abundance data were left untransformed because of limitations in several popular transformation techniques described previously by Faulkner et al. (2017).

RESULTS AND DISCUSSION

Protozoal Counts and Generation Time

Our experiment required an initial drop of temperature to fully defaunate the continuous cultures. The increased stirring and filter porosity might have been less effective in fresh culture compared with fermentor contents 14 d into the experiment (Karnati et al., 2009a) due to the abundance of protozoa in fresh inocula (average = 7.56×10^5 cells/mL) that were given the opportunity to adapt to DEF treatment conditions. Reducing the temperature to 34°C (Dehority, 2010) was effective in eliminating protozoa from fermentors and should have minimal long-lasting effects on prokaryotes (B. Dehority, The Ohio State University; personal communication). However, we would caution that there was no way to verify that our defaunation protocol could not bias bacterial populations compared with control populations; there was no baseline bacterial population for comparison because DEF was imposed from inoculation. Following inoculation, protozoal abundance declined in both FAUN and DEF fermentors but stabilized at 1.35×10^4 cells/mL for FAUN, whereas protozoa were microscopically undetectable for DEF after 4 d. Methanogenesis, a key indicator of continuous culture

viability, returned to pre-defaunation ranges <2 d after returning temperature to 39°C (data not shown).

We observed no effects ($P > 0.10$) of $+\text{NO}_3^-$ on protozoa counts, effluent flow of protozoa, or generation time (Table 1). The large, fibrolytic entodiniomorphids were almost washed out, leaving small *Entodinium* spp. as the primary group, which is typical in our conditions (Karnati et al., 2009a; Ye et al., 2018). Although NO_2^- interfered with protozoal motility (particularly that of isotrichids) and chemotaxis (Roman-Garcia et al., 2019), addition of NO_3^- to continuous cultures at the same dose as used in the present study (Welty et al., 2019) tended to increase *Entodinium* spp., which was attributed to nitrate reduction. Protozoal concentrations were not affected by feeding NO_3^- in sheep (van Zijderveld et al., 2010) but increased in dairy cows (Meller et al., 2019). Responses of protozoa to NO_3^- addition are poorly understood but might depend on better characterization of nitrate reductase activity (Yang et al., 2016; Roman-Garcia et al., 2019).

Nutrient Digestibility and Nitrogen Flow

Despite extensive bag washing, minute particles inherent in fermentors were not separable from protozoal cells per the method of Sylvester et al. (2005). Although feed N would dilute the ^{15}N label, the high enrichment in washed samples from both FAUN and DEF treatments was presumably a result of extensive bacterial adherence to contaminating particulates because there were no protozoa present in DEF. We attempted to subtract FAUN from DEF washed samples to estimate protozoal N flow, but bacterial contamination of particles was either too severe or protozoal N contribution was relatively too small to be reliably detected in this manner. This prompted the use of estimated N composition of protozoal cells as referenced in the methods previously.

There were neither interactions nor main effects ($P > 0.10$) of treatment on apparent digestibilities of OM or NSC; NDF digestibility; $\text{NH}_3\text{-N}$ concentration; or effluent outflows of total N, microbial N, NAN, or non-ammonia, non-microbial N (Table 2). The lack of effects for $+\text{NO}_3^-$ was consistent with results using a similar NO_3^- dosage in continuous culture (Welty et al., 2019) at low urea inclusion. Similarly, nutrient digestibility was previously unaffected by the addition of NO_3^- in vivo (Nolan et al., 2010; van Zijderveld et al., 2011b). Estimated protozoal N was a relatively small fraction of total microbial N flow under our conditions, possibly contributing to the lack of effects for DEF treatment on NDF digestibility in the present study compared with in vivo conditions. The effect of defaunation on

Table 1. Counts of protozoa populations within fermentors, calculated effluent flow, and generation time for continuous culture fermentors¹

Item	−NO ₃ [−]		+NO ₃ [−]		SEM	P-value ²
	FAUN	DEF ³	FAUN	DEF		
Fermentor counts [cells (×10 ³)/mL]						
Total	7.85	—	10.0	—	3.11	NS
<i>Isotrichidae</i>	0.819	—	0.764	—	0.147	NS
Large entodiniomorphids	0.326	—	0.573	—	0.160	NS
Small entodiniomorphids	4.82	—	5.75	—	1.50	NS
<i>Charonina</i>	1.88	—	2.93	—	2.16	NS
Effluent flow [cells (×10 ⁶)/d]						
Total	9.95	—	9.14	—	3.44	NS
<i>Isotrichidae</i>	0.120	—	0.0693	—	0.0495	NS
Large entodiniomorphids	0.550	—	0.536	—	0.159	NS
Small entodiniomorphids	7.76	—	6.69	—	2.24	NS
<i>Charonina</i>	1.78	—	1.79	—	1.57	NS
Generation time ⁴ (h)	40.6	—	45.1	—	9.3	NS

¹Treatments: −NO₃[−] = no nitrate, +NO₃[−] = nitrate added at 1.5% of diet DM, FAUN = no defaunation, DEF = defaunated.

²P-values reported for the main effects of +NO₃[−], DEF, and their interaction. NS = *P* > 0.15.

³DEF were confirmed to lack protozoa; as such, their numbers were zero and excluded from analysis.

⁴Generation time = total pool size of cells (i.e., fermentor counts × fermentor volume)/effluent flow of cells/d × 24 h.

methanogenesis is variable (Morgavi et al., 2012) and typically accompanied by depressed NDF digestibility (Guyader et al., 2014; Newbold et al., 2015). Depressed NDF digestibility should decrease production of VFA

and, consequently, also H₂ and formate (Newbold et al., 2015). The association of methanogens with differing protozoal taxa is inconsistent (Tapio et al., 2017). Thus, further research is needed to sort out these interactions.

Table 2. Nutrient digestibilities and N flows to effluent in continuous culture fermentors¹

Item	−NO ₃ [−]		+NO ₃ [−]		SEM	P-value ²		
	FAUN	DEF	FAUN	DEF		NO ₃ [−]	DEF	NO ₃ [−] × DEF
Apparent OM digestibility (%)	43.7	43.1	39.5	42.9	2.4	NS	NS	NS
Apparent NSC digestibility (%)	91.1	91.7	93.6	92.4	1.3	NS	NS	NS
NDF digestibility (%)	54.1	52.3	54.9	56.3	3.3	NS	NS	NS
NH ₃ -N (mg/dL)	6.40	6.17	6.05	5.51	0.51	NS	NS	NS
Effluent N flows ³								
Total N (g/d)	1.10	1.08	1.09	1.09	0.06	NS	NS	NS
Microbial N ⁴ (g/d)	0.539	0.543	0.525	0.554	0.037	NS	NS	NS
Protozoal N (g/d)	0.00525	—	0.00475	—	0.00177	NS	—	—
Bacterial N (g/d)	0.534	0.543	0.520	0.554	0.037	NS	NS	NS
Bacterial N (g/kg of OM apparently degraded)	33.2	34.3	35.8	35.1	3.0	NS	NS	NS
Bacterial N (g/kg of NSC apparently degraded)	61.6	63.4	61.7	65.5	4.5	NS	NS	NS
Bacterial N (g/kg of NDF degraded)	73.6	78.1	69.5	72.6	7.4	NS	NS	NS
NH ₃ -N (g/d)	0.182	0.175	0.170	0.157	0.015	0.12	NS	NS
NAN (g/d)	0.919	0.904	0.922	0.93	0.06	NS	NS	NS
NAN (% of N intake) ⁵	83.7	82.3	83.9	84.7	5.3	NS	NS	NS
NANMN ⁶ (g/d)	0.381	0.361	0.397	0.377	0.042	NS	NS	NS
NANMN (% of N intake)	34.7	32.9	36.1	34.3	3.8	NS	NS	NS

¹Treatments: −NO₃[−] = no nitrate, +NO₃[−] = nitrate added at 1.5% of diet DM, FAUN = no defaunation, DEF = defaunated. Effects of DEF are precluded, by design, from protozoal N flow.

²P-values reported for the main effects of −NO₃[−], DEF, and their interaction. NS = *P* > 0.15.

³Total N does not contain N from NO₃[−] or NO₂[−] that was not recovered by Kjeldahl analysis, but these should be subtracted in derivation of NAN anyway (Welty et al., 2019).

⁴Microbial N includes protozoal N for FAUN but bacterial N only for DEF.

⁵Percent of dietary N intake does not include N from buffer.

⁶Non-ammonia, non-microbial N.

Hourly Ammonia, Nitrate, and Nitrate Flows

Analysis of filtrate effluent indicated that the main effect of $+NO_3^-$ decreased ($P < 0.05$) the concentration of NH_3-N for the first 1.5 h post-feeding (Figure 1A), but this effect was negated thereafter. The main effect of $+NO_3^-$ also increased NO_3^- ($P < 0.01$) and NO_2^- ($P \leq 0.07$) concentration post-feeding (0.25 to 2 h and 0.25 to 4 h post-feeding for Figure 1B and C, respectively), documenting not only delivery of dose but also the reduction of NO_3^- to NO_2^- and then to NH_3 . Nitrate reduction in adapted rumen fluid or cultures is rapid (Lee and Beauchemin, 2014; Welty et al., 2019). Protozoa might mediate a rapid response in unadapted cultures (Lin et al., 2011) and perhaps benefit from electron-transport phosphorylation linked to nitrate reductase (Welty et al., 2019). The latter authors reported that ^{15}N -labeled NO_3^- was incorporated into microbial protein in continuous cultures, presumably via NH_3 assimilation. A similar numerical decrease in ruminal NH_3-N concentration was observed in sheep with the addition of NO_3^- to the diet (van Zijderveld et al., 2010).

Daily Production of VFA and Methane

There was neither an interaction ($P > 0.10$) of $NO_3^- \times DEF$ for any VFA nor any main effect of treatment ($P > 0.10$) on total VFA concentration (Table 3). However, numerical differences contributed to trends for increased daily VFA production for both the main effect of $+NO_3^-$ (9.6% increase by adding NO_3^- ; $P = 0.07$) and of faunation status (8.9% increase by DEF vs. FAUN; $P = 0.09$). The increase in VFA production in the DEF treatment contrasts with a recent meta-analysis in which defaunation typically decreased total VFA concentration (Newbold et al., 2015) as explained by reduced NDF digestibility, which did not occur in our study. In addition, the bacteria displacing the protozoa probably rely more on de novo synthesis of long-chain fatty acids and diverting carbon away from VFA production (Karnati et al., 2009a). However, that study had supplementary fat and nearly twice the counts of protozoa; thus, the loss of protozoa (16.7% of total microbial N) likely represented a greater impact on VFA production than in the present study. Production of total VFA on a molar basis also ignores the chain length of VFA. The main effect of DEF increased ($P < 0.01$) the molar proportion of propionate and valerate and decreased ($P \leq 0.05$) molar proportion of isovalerate and acetate:propionate. Defaunation previously increased the molar proportion of propionate (Demeyer and Van Nevel, 1979); because protozoa consume lactate more quickly than bacteria (Newbold et al., 1986),

Figure 1. Concentrations of NH_3-N (A), NO_3^- (B), and NO_2^- (C) over time post-feeding in continuous culture fermentors treated or not with NO_3^- and with or without defaunation. Treatments: $-NO_3^-$ = no nitrate added, $+NO_3^-$ = nitrate added at 1.5% of diet DM, FAUN = no defaunation, DEF = defaunated. There was no interaction of $+NO_3^- \times DEF$ ($P > 0.10$). The main effect of $+NO_3^-$ decreased ($P < 0.05$) NH_3-N from 0.5 to 1.5 h post-feeding. The addition of NO_3^- increased ($P < 0.01$) concentration of NO_3^- from 0.25 to 2 h post-feeding, and increased ($P < 0.01$) NO_2^- concentration in solution for 0.25 h through 2 h post-feeding. There were trends for the main effect of DEF that decreased ($P < 0.10$) NH_3-N at 2 and 12 h post-feeding. Further, DEF tended to increase ($P = 0.09$) NO_2^- concentration in solution at 4 h post-feeding only. Pooled SEM: 0.947 mg/dL for NH_3-N , and 0.154 and 0.059 μM for NO_3^- and NO_2^- , respectively.

lactate concentration has often increased after defaunation (Williams and Coleman, 1992). However, Newbold et al. (2015) noted a lack of significance for lactate concentration (although numerically increased by 11%) and for propionate molar percentage by defaunation. In contrast, those authors noted that defaunation typically decreases butyrate concentration, which did not occur in our study. The main effect of $+NO_3^-$ decreased ($P \leq 0.02$) the molar proportion of isobutyrate and valerate. The decrease in branched-chain VFA by feeding NO_3^- has been previously reported (van Zijderveld et al., 2010; Guyader et al., 2015) but not explained. Although acetate was expected to increase because added NO_3^- provides an alternate electron acceptor to fumarate, a precursor in propionate formation (Yang et al., 2016), neither molar proportion of acetate nor acetate:propionate was affected by NO_3^- in the present study.

Daily CH_4 production was decreased ($P < 0.01$) by 30.8% by $+NO_3^-$ compared with $-NO_3^-$, with no effect ($P > 0.10$) of DEF or interaction (Table 3). Thus, CH_4 produced per unit of NDF degraded was decreased by 35.6% ($P = 0.01$) because there was no treatment effect on NDF digestibility. The decrease in CH_4 production in the present study is similar to that in previous publications in sheep (Nolan et al., 2010; van Zijderveld et al., 2010, 2011a), dairy cattle (van Zijderveld et al., 2011b), and in vitro (Patra and Yu, 2013; Welty et al., 2019), with greater inhibition of methanogenesis in unadapted batch culture (Zhou et al., 2012).

Production Rates of Methane and Hydrogen

Methanogenesis was inhibited ($P < 0.01$) by $+NO_3^-$ from 2 to 9 h post-feeding, with no effect of DEF or

interaction ($P > 0.10$, Figure 2A). The temporal effect of $+NO_3^-$ is similar to results in sheep (Alaboudi and Jones, 1985) and limit-fed dairy cows (van Zijderveld et al., 2011a). The temporal and declining efficacy of $+NO_3^-$ in the present study is likely a 3-fold response of dosed NO_3^- being reduced by microbes, its passage in effluent, and more rapid fermentation post-feeding generating greater metabolic hydrogen (termed [2H], with brackets indicating various cofactors) that declined with remaining degradable carbohydrate.

The lack of effect of DEF compared with FAUN or interaction of DEF with $+NO_3^-$ for CH_4 production is contrary to our hypothesis and some previous studies that DEF would decrease methanogenesis (Newbold et al., 2015). Smaller cells should have lower activity relative to larger cells (Whitelaw et al., 1984); our low concentration of protozoa, especially large ones, might have contributed to the lack of effect of defaunation in the present study. However, defaunation has previously failed to decrease methanogenesis in continuous culture with more protozoa (Karnati et al., 2009a) and in vivo (Machmüller et al., 2003; Hegarty et al., 2008; Morgavi et al., 2012). Tapio et al. (2017) also described inconsistent responses to protozoal community structure among multiple studies.

Another possible explanation for the lack of effect in DEF or its interaction with NO_3^- is the compensatory contribution of fungi or cellulolytic bacterial consortia within biofilms toward total H_2 production. Biofilms harbor a micro-environment with complex H_2 transfer capacity where fibrolytic microbes and their symbiotic constituents benefit from methanogens decreasing the concentration of $H_2(aq)$ within the close proximity of their local environment (Hegarty and Gerdes, 1999; Leahy et al., 2010; Leng, 2014). Fungi were not

Table 3. Effluent flow, VFA concentrations and production, and methane production for continuous culture fermentors¹

Item	$-NO_3^-$		$+NO_3^-$		SEM	<i>P</i> -value ²		
	FAUN	DEF	FAUN	DEF		$+NO_3^-$	DEF	$+NO_3^- \times DEF$
Effluent (L/d)	2.88	2.88	2.87	2.91	0.09	NS	NS	NS
VFA (mM)	48.7	52.9	53.5	57.3	2.9	0.12	NS	NS
Individual VFA (mol/100 mol)								
Acetate (A)	64.4	63.7	64.8	64.7	0.7	0.14	NS	NS
Propionate (P)	21.2	22.5	21.1	22.3	1.3	NS	<0.01	NS
Isobutyrate ³	0.623	0.588	0.524	0.496	0.037	0.02	NS	NS
Butyrate	10.5	10.4	10.7	9.77	0.77	NS	NS	NS
Isovalerate	1.75	1.01	1.47	1.02	0.13	NS	<0.01	NS
Valerate	1.60	1.83	1.45	1.71	0.10	0.01	<0.01	NS
A:P	3.06	2.87	3.11	2.96	0.20	NS	0.02	NS
VFA (mmol/d)	140	152	153	167	8	0.07	0.09	NS
Methane (mmol/d)	30.3	30.4	21.2	20.8	3.6	<0.01	NS	NS
Methane per NDF (mmol/kg degraded)	4.14	4.36	2.78	2.69	0.57	0.01	NS	NS

¹Treatments: $-NO_3^-$ = no nitrate, $+NO_3^-$ = nitrate added at 1.5% of diet DM, FAUN = no defaunation, DEF = defaunated.

²*P*-values reported for the main effects of $-NO_3^-$, DEF, and their interaction. NS = $P > 0.15$.

³Under current column conditions, 2-methylbutyrate was not separated from isobutyrate.

quantified in the present study, but they might have contributed more $H_2(aq)$ in the absence of protozoa, given their hydrogenase activity similar to protozoa (Hackstein and Tielens, 2010) and antagonism with protozoa but symbiosis with methanogens (Edwards et al., 2017).

Figure 2. Rates of CH_4 production (A) and emission of H_2 (B) measured in headspace gas post-feeding for continuous culture fermentors treated or not with NO_3^- and with or without defaunation. Treatments: $-NO_3^-$ = no nitrate added, $+NO_3^-$ = nitrate added at 1.5% of diet DM, FAUN = no defaunation, DEF = defaunated. For H_2 escape, there was an interaction ($P = 0.01$) of $+NO_3^- \times DEF$ at 1.5 h post-feeding. Escape of H_2 was increased ($P = 0.04$) by the main effect of $+NO_3^-$ at 5 h post-feeding. Escape of H_2 was increased ($P = 0.03$) by DEF at 3 h post-feeding. There was no effect of DEF or interaction of $+NO_3^- \times DEF$ ($P > 0.10$) on CH_4 production rate. The main effect of $+NO_3^-$ decreased ($P < 0.05$) CH_4 production rate from 2 to 9 h post-feeding. Pooled SEM: 13.1 and 210 μmol/h for H_2 and CH_4 , respectively.

H_2 Escape and $H_2(aq)$ Concentration

The rate of H_2 escape was increased by DEF at 3 h post-feeding ($P < 0.05$) and by $+NO_3^-$ at 5 h post-feeding ($P < 0.05$, Figure 2B). Treatments interacted ($P < 0.05$) at 1.5 h, with H_2 rate being greater with the $-NO_3^-/FAUN$ and $+NO_3^-/DEF$ treatments than with $+NO_3^-/FAUN$ or $-NO_3^-/DEF$. Escape of H_2 from culture is typically low but can result when $H_2(aq)$ is incompletely captured by treatments that suppress methanogenesis (Ungerfeld, 2015a). Defaunation decreased ($P = 0.02$) $H_2(aq)$ concentration at 1 h post-feeding, but there was an interaction ($P = 0.03$) among treatments, with $H_2(aq)$ of $+NO_3^-/FAUN$ being greatest, $+NO_3^-/DEF$ intermediate, and $-NO_3^-/FAUN$ and $-NO_3^-/DEF$ being not different from each other but less than both NO_3^- treatments (Figure 3). Shortly after feeding (0.5 to 2.5 h), the concentration of $H_2(aq)$ was increased ($P < 0.05$) by the main effect of $+NO_3^-$. Concentrations of $H_2(aq)$ were within a similar range of previously published measurements of $H_2(aq)$ for rumen fluid (Wenner et al., 2017; Welty et al., 2019).

The lag in measured versus predicted $H_2(aq)$ appears extended in FAUN versus DEF, especially with $+NO_3^-$ (Figure 4, panel B vs. A and especially panel D vs. C). Nitrate reductase inside protozoa might temporarily sequester $H_2(aq)$, but this potential effect has not been tested. Instead, this lag likely supports temporary

Figure 3. Concentration of aqueous hydrogen [$H_2(aq)$] over time post-feeding for continuous culture fermentors treated or not with NO_3^- and with or without defaunation. Treatments: $-NO_3^-$ = no nitrate added, $+NO_3^-$ = nitrate added at 1.5% of diet DM, FAUN = no defaunation, DEF = defaunated. There was an interaction of $NO_3^- \times DEF$ ($P = 0.03$) at 1 h post-feeding and a tendency ($P = 0.07$) for an interaction at 0 h post-feeding. The main effect of $+NO_3^-$ increased ($P < 0.05$) $H_2(aq)$ from 0.5 to 2.5 h post-feeding, and the main effect of DEF decreased ($P = 0.02$) $H_2(aq)$ at 1 h post-feeding. Pooled SEM: 5.33 μM.

Figure 4. Pattern of predicted and observed aqueous hydrogen [$H_2(aq)$] during early fermentation post-feeding in continuous culture fermentors treated or not with NO_3^- and with or without defaunation. Treatments: $-NO_3^-$ = no nitrate added, $+NO_3^-$ = nitrate added at 1.5% of diet DM, FAUN = no defaunation, DEF = defaunated. Predicted (Pred.) $H_2(aq)$ calculated from H_2 detection in the headspace is represented by the dotted lines and measured $H_2(aq)$ is represented by solid lines for each treatment.

supersaturation of $H_2(aq)$ (Wang et al., 2016a,c) and highlights the importance for a direct measurement of $H_2(aq)$ rather than estimating from partial pressure in the headspace (Wang et al., 2016b; Guyader et al., 2017; Wenner et al., 2017). Direct measurement of $H_2(aq)$ is perhaps more important in rumen-cannulated animals from which gases can leak (Wang et al., 2019).

The increase in concentration of $H_2(aq)$ in the present study probably resulted from NO_3^- reduction diverting H_2 away from methanogenesis (Ungerfeld and Kohn, 2006). However, NO_2^- reduction is not nearly as broadly shared within the microbial community, and proximity of NO_3^- or NO_2^- reducers to H_2 producers might be important (Leng, 2014). Also, removal of the remaining H_2 may not be completed as quickly as it would be through the Wolfe cycle (Thauer, 2012), leading to accumulation of nitrite in the interim (Yang et

al., 2016), as in Figure 1. Toxicity toward methanogens could also partially explain the increase in $H_2(aq)$ with $+NO_3^-$ treatments (Latham et al., 2016). If so, depressed disposal of reducing equivalents through CH_4 should ultimately either directly decrease production of H_2 or thermodynamically shift fermentation away from H_2 -producing acetate pathways.

An increase in $H_2(aq)$ of even 10 μM (less than the main effect of $+NO_3^-$ in the present study) could be enough to increase the Gibbs free energy by approximately 30 kJ/mol of glucose in the rumen and shift the stoichiometry of fermentation pathways (Ungerfeld and Kohn, 2006; Janssen, 2010). That the shift in $H_2(aq)$ is substantial but not permanent throughout the day likely explains the minimal shift in daily average acetate: propionate (van Lingen et al., 2016). Increasing availability of H_2 or electron donors did little to ameliorate

the inhibition of soil methanogens by NO_3^- (Roy and Conrad, 1999), and NO_3^- or NO_2^- directly inhibits rumen prokaryotes (Latham et al., 2016). Thus, the spike of $\text{H}_2(\text{aq})$ post-feeding might not just influence VFA production but instead affect VFA interconversions (Ungerfeld, 2015a) and direct [2H] into cellular matter (Ungerfeld, 2015b), supporting the subsequent analysis of H recovery.

Estimated H Recovery

There was no interaction of $+\text{NO}_3^- \times \text{DEF}$ nor an effect of DEF on H recovery ($P > 0.10$). The main effect of $+\text{NO}_3^-$ decreased ($P < 0.01$) H recovery by 16.6 percentage units if the reduction of NO_3^- was not taken into consideration (77.2% vs 60.5%, SEM: 4.3). However, there was no effect ($P > 0.10$) if NO_3^- reduction to either NH_3 or NO_2^- was factored into the estimates (77.2% vs 78.6%, SEM: 4.4). Nitrate inhibition of methanogenesis typically decreases H recovery (Lee and Beauchemin, 2014), perhaps resulting from an increased $\text{H}_2(\text{aq})$ concentration that increases escape into the headspace, increased production of formate (Olijhoek et al., 2016), or into major cellular products (Ungerfeld, 2015b); only H_2 emission was measured in our study. Generally, CH_4 mitigation decreases H recovery resulting from H_2 emission (Ungerfeld, 2015b), which represents lost metabolizable energy (Morgavi et al., 2010; Lee and Beauchemin, 2014).

Microbial Responses

Quantification of total bacterial 16S rRNA gene copies revealed no main effect or interaction ($P > 0.10$) among treatments (Figure 5). The total archaeal 16S rRNA gene copies were not affected ($P > 0.10$) by the main effect of DEF or its interaction with NO_3^- . Methanogen abundance is inconsistently associated with methanogenesis (Tapio et al., 2017), partially due to a flexibility in efficiency of ATP generation coupled with H_2 production (Thauer et al., 2008; Wallace et al., 2017). However, the main effect mean of $+\text{NO}_3^-$ was 47% lower ($P < 0.01$) for total archaeal 16S rRNA gene copies. Because methane production was 31% lower, $+\text{NO}_3^-$ probably not only decreased methanogenesis by thermodynamic competition for H_2 but also through inhibition of methanogens by NO_3^- or (more likely) NO_2^- (Latham et al., 2016). Similar decreases in copies of methyl coenzyme M reductase A (a conserved gene involved in methanogenesis) were reported in dairy cows fed NO_3^- (Veneman et al., 2015).

Between 180 and 190 OTU were found in the fermentors (Supplemental Table S2; <https://doi.org/10.3168/jds.2020-18325>), which is much lower than the richness reported for rumen samples (Meller et al., 2019) and for untreated continuous cultures (Salfer et al., 2018). Prokaryote population richness was not affected by treatment ($P > 0.10$), but measures of α -diversity had an interaction with treatment ($P = 0.05$), whereby $-\text{NO}_3^-/\text{DEF}$ and $+\text{NO}_3^-/\text{FAUN}$ had greater index values for both Shannon-Wiener and inverse Simpson diversities than $+\text{NO}_3^-/\text{DEF}$ and $-\text{NO}_3^-/\text{FAUN}$ (Supplemental Table S2). This decreased diversity may have led to greater sensitivity to the current treatment conditions due to decreased functional microbial redundancy. Analysis using Bray-Curtis dissimilarity (Supplemental Figure S1; <https://doi.org/10.3168/jds.2020-18325>) indicated no difference ($P > 0.20$) in overall microbiota for treatment, interaction of treatment, or fermentor; however, experimental periods 1 and 3 had uniquely different microbiota ($P < 0.01$) from periods 2 and 4. The separation of microbial populations within continu-

Figure 5. Quantification of total bacterial (A) and total archaeal (B) 16S rRNA gene copies per mL by qPCR for continuous culture fermentors treated or not with NO_3^- and with or without defaunation. Treatments: $-\text{NO}_3^-$ = no nitrate added, $+\text{NO}_3^-$ = nitrate added at 1.5% of diet DM, FAUN = no defaunation, DEF = defaunated. Concentrations of 16S rRNA gene copies are $\log_{10}$ transformed. There was no effect of treatment or interaction ($P > 0.10$) on total bacteria copies (pooled SEM: 0.11 $\log_{10}$ copies/mL). There was no effect of DEF or interaction of $\text{NO}_3^- \times \text{DEF}$ ($P > 0.10$) on total archaea copies, but the main effect of NO_3^- decreased ($P < 0.01$) archaeal 16S rRNA gene copies (pooled SEM: 0.09 $\log_{10}$ copies/mL).

Figure 5. Quantification of total bacterial (A) and total archaeal (B) 16S rRNA gene copies per mL by qPCR for continuous culture fermentors treated or not with NO_3^- and with or without defaunation. Treatments: $-\text{NO}_3^-$ = no nitrate added, $+\text{NO}_3^-$ = nitrate added at 1.5% of diet DM, FAUN = no defaunation, DEF = defaunated. Concentrations of 16S rRNA gene copies are $\log_{10}$ transformed. There was no effect of treatment or interaction ($P > 0.10$) on total bacteria copies (pooled SEM: 0.11 $\log_{10}$ copies/mL). There was no effect of DEF or interaction of $\text{NO}_3^- \times \text{DEF}$ ($P > 0.10$) on total archaea copies, but the main effect of NO_3^- decreased ($P < 0.01$) archaeal 16S rRNA gene copies (pooled SEM: 0.09 $\log_{10}$ copies/mL).

ous culture fermentor periods has been documented in previous publications (Karnati et al., 2009b), probably due to continuous ecological successions, and accentuates the importance of sampling multiple cows.

Prokaryote phyla (Supplemental Table S2; <https://doi.org/10.3168/jds.2020-18325>) represent the primary expected phyla within the rumen (Henderson et al., 2015). Among the phyla of archaea and bacteria detected, only *Proteobacteria* was decreased ($P = 0.02$) by DEF and tended to be increased ($P = 0.09$) by $+NO_3^-$ (Supplemental Table S2). Some members of *Proteobacteria* are enriched in likely endosymbionts of protozoa (Park and Yu, 2018). The decreased proteobacterial relative abundance in DEF might be related to the decrease of protozoa-associated bacteria.

Part of the downward shift in *Proteobacteria* was mediated through genus *Succinivibrio* (Table 4) in which $+NO_3^-$ /FAUN was highest and $+NO_3^-$ /DEF was lowest (interaction $P = 0.09$). This genus increased dramatically within *Epidinium caudatum* 4 to 8 h post-feeding (Park and Yu, 2018). *Succinivibrio* numerically increased with the addition of NO_3^- at a dose similar to that (1.5%) in the present study (Welty et al., 2019). Zhao et al. (2015) reported a quadratic increase in *Succinivibrio* in response to 1 or 2% added NO_3^- . *Succinivibrio* also increased in 1 of 2 studies with dairy cattle fed NO_3^- (Veneman et al., 2015) and in grazing steers supplemented with slow-release NO_3^- (Granja-Salcedo et al., 2019). The latter authors discussed succinate-producing bacteria like *Succinivibrio* harnessing H_2 -reducing power in fumarate reduction. However, one *Succinivibrionaceae* strain was documented to produce ATP via carboxylation of phosphoenolpyruvate and subsequent succinate formation using $[2H]$ derived from fermenting hexoses rather than from $H_2(aq)$ (Pope et al., 2011). Therefore, the inverse relationship between methane production and *Succinivibrionaceae* abundance (Tapio et al., 2017) awaits further mechanistic explanation.

Within phylum *Bacteroidetes*, $+NO_3^-$ interacted ($P < 0.05$) with DEF to affect relative sequence abundance of uncultured genera *RC9 Gut Group* within family *Rikenellaceae* and uncultured genus within family *S24-7* along with a trend ($P = 0.06$) for interaction in the uncultured rumen *BS11 Gut Group* (Table 4); all were highest for $+NO_3^-$ /DEF. Welty et al. (2019) discussed the relative abundance of these or closely related genera to increase in abundance with dosing NO_3^- or live yeast, suggesting potential for nitrite reductase linked to electron transfer phosphorylation for ATP synthesis.

Within phylum *Bacteroidetes*, the *Other RF16* genus-level OTU tended ($P = 0.06$) to decrease in relative sequence abundance by the main effect of feeding

NO_3^- (Table 4). We know very little about this group currently; however, *RF16* was associated with higher (77:23) than lower (49:51) forage:concentrate ratio (AlZahal et al., 2017). Within phylum *Firmicutes*, the *insertae sedis* in order *Clostridiales* ($P = 0.08$) and genus *Butyrivibrio* ($P = 0.07$) tended to be decreased by the main effect of $+NO_3^-$. Some *Butyrivibrio* were projected to reduce NO_3^- and NO_2^- (Latham et al., 2016), but others; namely, the characterized type strain of *Butyrivibrio hungatei*, are incapable of NO_3^- reduction (Kopečný et al., 2003). Other studies have shown no effect of supplemental NO_3^- on *Butyrivibrio* spp. (Zhao et al., 2015; Popova et al., 2017); however, these studies had far lower relative sequence abundance of *Butyrivibrio* than that in the present study. Dosing of NO_3^- depressed *Butyrivibrio* in continuous culture in a similar fashion (Welty et al., 2019). Potentially, the energy-conserving hydrogenase complex in many *Butyrivibrio* spp. (Hackmann and Firkins, 2015) might be interrupted by accumulating $H_2(aq)$, but they also have a Na^+ -driven circuit to compensate (Schoelmerich et al., 2020). There were no significant increases in known nitrate-reducing bacteria such as *Selenomonas*, which agrees with results from dairy cattle (Veneman et al., 2015) and suggests NO_3^- or NO_2^- reductases distributed in other community members. Welty et al. (2019) discussed potential NO_2^- reductase in the highly prevalent genus *Prevotella* and family *Prevotellaceae*, which increased in relative abundance along with other *Bacteroidales* genera. However, our study had no difference in these taxa, which decreased in vivo (Veneman et al., 2015; Granja-Salcedo et al., 2019).

Defaunation increased ($P = 0.05$) both uncultured *Christensenellaceae* and other *Clostridiales* and tended ($P = 0.09$) to increase uncultured *Ruminococcaceae* with phylum *Firmicutes* (Table 4). Using qPCR, Mosoni et al. (2011) reported that *Ruminococcus albus* and *Ruminococcus flavefaciens* increased in response to defaunation of sheep, hypothesizing a response to decreased predation; however, a more recent meta-analysis (Newbold et al., 2015) had the opposite relationship. Genus *Ruminococcus* (known cellulolytics) was unaffected, so the *Ruminococcaceae* affected herein probably occupy a different niche (e.g., amylolytic). Little is known about either *Christensenellaceae* or the *RC9 Gut Group*, but they were both reported to be among the most abundant unclassified bacterial OTU within the rumen (Seshadri et al., 2018). Perhaps, either or both of these groups are closely related to the roles filled by protozoa (e.g., amylolytic). Several members of the *RC9 Gut Group* decreased in relative abundance in cows switched from corn silage-based TMR to fresh pasture (Schären et al., 2017).

Table 4. Relative sequence abundance of prokaryote genera sorted by phyla¹

Genera-level OTU ²	-NO ₃ ⁻		+NO ₃ ⁻		SEM	P-value ³		
	FAUN	DEF	FAUN	DEF		+NO ₃ ⁻	DEF	+NO ₃ × DEF
<i>Archaea</i>								
<i>Methanobrevibacter</i>	0.449	1.041	0.379	0.751	0.375	NS	NS	NS
Uncultured <i>Vadin CA11 Gut Group</i>	0.060	0.006	0.137	0.121	0.088	NS	NS	NS
Other <i>Vadin CA11 Gut Group</i>	0.007	0.000	0.054	0.008	0.027	NS	NS	NS
<i>Bacteria</i>								
Phylum <i>Actinobacteria</i>								
<i>Bifidobacterium</i>	0.719	1.217	2.181	1.088	1.006	NS	NS	NS
<i>Denitrobacterium</i>	0.012	0.295	0.123	0.000	0.156	NS	NS	NS
Phylum <i>Bacteroidetes</i>								
Order <i>Bacteroidales</i>								
<i>Bacteroides</i>	2.57	2.46	2.81	2.72	1.43	NS	NS	NS
Uncultured <i>BS11 Gut Group</i>	0.535	0.629	0.666	1.328	0.603	NS	NS	NS
Uncultured Rumen <i>BS11 Gut Group</i>	0.637	0.458	0.215	0.784	0.351	NS	NS	0.06
<i>Phocaeicola</i>	0.232	0.262	0.199	0.167	0.126	NS	NS	NS
<i>Prevotella</i>	10.4	10.1	9.0	8.2	6.0	NS	NS	NS
Uncultured <i>Prevotellaceae</i>	7.43	5.57	4.76	5.68	1.53	NS	NS	NS
<i>Xylanibacter</i>	0.652	0.542	1.014	0.533	0.222	NS	NS	NS
Uncultured <i>RF16</i>	0.787	1.017	0.546	0.491	0.401	0.12	NS	NS
Other <i>RF16</i>	1.12	1.28	0.82	0.76	0.59	0.06	NS	NS
<i>Alistipes</i>	1.25	1.72	0.89	1.50	0.80	NS	0.11	NS
<i>RC9 Gut Group</i>	7.39	8.01	6.00	8.57	1.59	NS	0.01	0.04
Uncultured <i>S24-7</i>	1.21	0.83	0.83	1.56	0.27	NS	NS	0.01
Uncultured Rumen <i>S24-7</i>	2.79	2.01	2.96	4.36	2.16	0.13	NS	NS
Other <i>S24-7</i>	0.281	0.173	0.362	0.374	0.135	NS	NS	NS
Other <i>Bacteroidales</i>	0.919	1.193	1.038	0.893	0.341	NS	NS	NS
Phylum <i>Cyanobacteria</i>								
Other <i>Chloroplast</i>	0.064	0.102	0.285	0.064	0.134	NS	NS	NS
Phylum <i>Fibrobacteres</i>								
<i>Fibrobacter</i>	0.100	0.211	0.763	0.320	0.357	NS	NS	NS
Phylum <i>Firmicutes</i>								
Order <i>Lactobacillales</i>								
<i>Streptococcus</i>	0.275	2.023	0.079	0.005	1.019	NS	NS	NS
Order <i>Clostridiales</i>								
Uncultured <i>Christensenellaceae</i>	2.28	4.13	2.15	2.92	0.81	NS	0.05	NS
<i>Clostridium</i>	3.14	0.72	3.05	4.85	2.98	NS	NS	NS
<i>Incertae sedis</i>	0.309	0.371	0.131	0.250	0.116	0.08	NS	NS
Uncultured <i>incertae sedis</i>	0.440	0.450	0.403	0.545	0.101	NS	NS	NS
Uncultured <i>Clostridiales</i>	0.358	0.361	0.262	0.381	0.193	NS	NS	NS
Other <i>Clostridiales</i>	0.242	0.452	0.253	0.313	0.095	NS	0.02	0.15
Family <i>Lachnospiraceae</i>								
<i>Acetitomaculum</i>	0.084	0.242	0.179	0.187	0.094	NS	NS	NS
<i>Blautia</i>	0.233	0.292	0.204	0.218	0.107	NS	NS	NS
<i>Butyrivibrio</i>	7.02	3.63	3.28	2.18	2.68	0.07	0.11	NS
<i>Coprococcus</i>	0.327	0.184	0.244	0.275	0.145	NS	NS	NS
<i>Incertae sedis</i>	1.93	2.27	2.12	2.26	0.46	NS	NS	NS
<i>Moryella</i>	0.024	0.142	0.015	0.074	0.072	NS	NS	NS
<i>Oribacterium</i>	0.192	0.196	0.182	0.056	0.112	NS	NS	NS
<i>Pseudobutyrvibrio</i>	1.56	1.53	4.60	1.05	1.51	NS	NS	NS
Uncultured <i>Lachnospiraceae</i>	3.52	3.55	3.82	3.24	0.79	NS	NS	NS
Other <i>Lachnospiraceae</i>	1.09	1.08	1.45	1.16	0.43	NS	NS	NS
Family <i>Ruminococcaceae</i>								
<i>Incertae sedis</i>	0.432	0.374	0.467	0.704	0.189	NS	NS	NS
<i>Ruminococcus</i>	1.46	2.04	1.14	1.43	0.51	NS	NS	NS
<i>Saccharofermentans</i>	0.202	0.503	0.206	0.258	0.197	NS	NS	NS
Uncultured <i>Ruminococcaceae</i>	14.6	16.4	12.5	15.7	6.4	NS	0.09	NS
Other <i>Ruminococcaceae</i>	0.513	0.485	0.376	0.456	0.240	0.08	NS	NS
Family <i>Veillonellaceae</i>								
<i>Anaerovibrio</i>	0.334	0.347	0.842	0.111	0.267	NS	NS	0.15
<i>Selenomonas</i>	0.391	0.305	0.458	0.067	0.241	NS	NS	NS
<i>Succiniclasticum</i>	0.726	0.749	0.416	0.432	0.397	NS	NS	NS
Order <i>Erysipelotrichales</i>								
Uncultured <i>Erysipelotrichaceae</i>	0.216	0.195	0.307	0.341	0.072	0.13	NS	NS

Continued

Table 4 (Continued). Relative sequence abundance of prokaryote genera sorted by phyla¹

Genera-level OTU ²	−NO ₃ [−]		+NO ₃ [−]		SEM	P-value ³		
	FAUN	DEF	FAUN	DEF		+NO ₃ [−]	DEF	+NO ₃ × DEF
Phylum <i>Proteobacteria</i>								
<i>Ruminobacter</i>	0.041	0.013	0.908	0.193	0.398	NS	NS	NS
<i>Succinivibrio</i>	0.422	0.269	1.765	0.287	0.387	0.08	0.05	0.09
Uncultured <i>Succinivibrionaceae</i>	1.05	0.27	0.85	0.39	0.58	NS	0.15	NS
Phylum <i>Spirochaetes</i>								
<i>Spirochaeta</i>	0.541	0.358	0.416	0.322	0.300	NS	NS	NS
<i>Treponema</i>	0.842	1.037	1.272	0.574	0.386	NS	NS	NS
Phylum <i>Tenericutes</i>								
<i>Anaeroplasma</i>	0.447	0.218	0.422	0.250	0.194	NS	0.12	NS
Phylum <i>Verrucomicrobia</i>								
<i>Akkermansia</i>	0.080	0.286	0.119	0.065	0.128	NS	NS	NS
Unassigned	11.8	10.8	15.2	14.3	3.1	NS	NS	NS

¹Treatments: −NO₃[−] = no nitrate added, +NO₃[−] = nitrate added at 1.5% diet DM, FAUN = no defaunation, DEF = defaunated.

²Operational taxonomic units are included in this table only if one measurement had ≥0.50% relative abundance.

³P-values reported for the main effects of +NO₃[−], DEF, and their interaction. NS = *P* > 0.15.

CONCLUSIONS

Expectations for NO₃[−] to suppress methanogenesis are corroborated in the present study, but increased concentration of H₂(aq) and escape of gaseous H₂ explained previous observations for the poor response in ME by ruminants fed NO₃[−] for CH₄ mitigation. Meanwhile, the lack of effect of DEF adds to the known complexity of protozoal contribution to methanogenesis via symbiotic methanogens. Shifts in propionate, valerate, and branched-chain VFA with DEF are contrary to previous studies in continuous culture and in vivo, but this result could point to the importance of other dietary factors unique to continuous culture fermentors rather than a clear responsibility of protozoa. Under our culture conditions, the removal of protozoa had little effect on the nitrate-reducing capacity or methanogenic symbiosis with H₂ producers. More work is needed to better explain the complex relationship of protozoa with other microbial community members in the rumen under diverse fermentative conditions. The use of continuous cultures and NO₃[−] as an alternate electron sink provide a valuable model for future research to investigate the complexities of microbial interactions affecting fermentation stoichiometry in the rumen.

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