3-Nitrooxypropanol supplementation had little effect on fiber degradation and microbial colonization of forage particles when evaluated using the in situ ruminal incubation technique

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ABSTRACT

3-Nitrooxypropanol (3-NOP) is an investigational compound that acts as an enzyme inhibitor to decrease ruminal methanogenesis. We hypothesized that when feeding 3-NOP to cattle fed a high-forage diet, H₂ would accumulate in the rumen, which could suppress microbial colonization of feed particles and fiber degradation. Therefore, the study investigated the effects of supplementing a high-forage diet with 3-NOP on ruminal fiber degradability and microbial colonization of feed particles using the in situ technique. Eight ruminally cannulated beef cattle were allocated to 2 groups (4 cattle/group) in a crossover design with 2 periods and 2 dietary treatments. The treatments were control (basal diet) and 3-NOP (basal diet supplemented with 3-NOP, 150 mg/kg of dry matter). The basal diet consisted of 45% barley silage, 45% chopped grass hay, and 10% concentrate (dry matter basis). Samples of dried, ground barley silage and grass hay were incubated in the rumen of each animal for 0, 4, 12, 24, 36, 48, 96, 120, 216, and 288 h to determine neutral detergent fiber (NDF) degradation kinetics. An additional 2 bags were incubated for 4 and 48 h to evaluate the bacterial community attached to the incubated forages. Dietary supplementation of 3-NOP decreased (−53%) the dissolved methane concentration and increased (+780%) the dissolved H₂ concentration in ruminal fluid, but did not substantially alter in situ NDF degradation. The addition of 3-NOP resulted in a decrease in the α-diversity of the microbial community with colonizing communities showing reduced numbers of amplicon sequence variants and phylogenetic diversity compared with control diets. Principal coordinate analysis plots

indicated that forages incubated in animals fed 3-NOP resulted in highly specific changes to targeted microbes compared with control diets based on unweighted analysis (considering only absence and presence of taxa), but did not alter the overall composition of the colonizing community based on weighted UniFrac distances; unchanged relative abundances of major taxa included phyla *Bacteroidetes*, *Firmicutes*, and *Fibrobacteres*. The effect of 3-NOP on colonizing methanogenic microbes differed depending upon the forage incubated, as abundance of genus *Methanobrevibacter* was decreased for barley silage but not for grass hay. In conclusion, 3-NOP supplementation of a high-forage diet decreased ruminal methanogenesis and increased dissolved H₂ concentration, but had no negative effects on ruminal fiber degradation and only minor effects on relative abundances of the major taxa of bacteria adhered to forage substrates incubated in the rumen.

Key words: enteric methane, fiber digestion, hydrogen, methane inhibitor, rumen microbiome

INTRODUCTION

Methane (CH₄) is a predominant greenhouse gas, and enteric CH₄ contributes about 17% of global total (natural and anthropogenic sources) CH₄ emissions (Knapp et al., 2014). Enteric CH₄ also represents 2 to 12% of the dietary energy consumed by livestock (Johnson and Johnson, 1995). Mitigation of enteric CH₄ emissions is an area of intensive research worldwide because of its potential contribution to global warming (Dangal et al., 2017). The investigational compound and CH₄ inhibitor 3-nitrooxypropanol (**3-NOP**) has been identified as an important potential mitigation strategy if it is approved by government authorities for use as a feed additive (Beauchemin et al., 2020). This compound is structurally similar to methyl-coenzyme M and can preferentially bind to the active site of methyl-coenzyme M reductase (**MCR**), oxidizing the Ni(I) cofactor

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to Ni(II), temporarily inactivating the enzyme (Duin et al., 2016). It is widely reported that 3-NOP supplementation decreases enteric CH₄ emissions between 10% and 80%, depending upon the dose and diet (Hristov et al., 2015; Romero-Pérez et al., 2015; Vyas et al., 2016, 2018).

Molecular hydrogen (H₂) is produced during carbohydrate fermentation to volatile fatty acids and is mainly consumed by methanogenic archaea for methanogenesis in the rumen. Ruminal H₂ disposal is important for fiber degradation, as increased ruminal H₂ partial pressure can inhibit bacterial hydrogenases, and therefore reduce carbohydrate fermentation rate and digestibility in the rumen (Leng, 2014). The use of 3-NOP in ruminant diets to inhibit methanogenesis has been shown to increase dissolved H₂ (dH₂) and gaseous H₂ in the rumen (Lopes et al., 2016). Different effects of 3-NOP on fiber digestibility in the rumen and total digestive tract have been observed. Some studies report that 3-NOP supplementation had negative (Reynolds et al., 2014), minor (Romero-Pérez et al., 2014; Jayanegara et al., 2018), or positive (Haisan et al., 2017) effects on NDF digestibility in cattle. Thus, it is unclear whether the consistent increase in H₂ accumulation in the rumen of animals fed 3-NOP leads to negative effects on ruminal fiber degradation.

Studies that have examined the effects of 3-NOP supplementation on ruminal microbial community have reported varying results. Haisan et al. (2014) reported significantly decreased 16S rRNA gene copy numbers for methanogens and a trend for decreased total bacteria in rumen contents from dairy cows fed 3-NOP. Martínez-Fernández et al. (2018) also reported decreased abundance of methanogen populations and lower diversity of ruminal microbiota (Shannon and Simpson indices) in cattle fed 3-NOP. Yet, other studies reported that 3-NOP supplementation did not affect the 16S rRNA gene copy numbers of methanogens or total bacteria in beef cattle or sheep (Martínez-Fernández et al., 2014; Romero-Pérez et al., 2014). It is evident that further research is needed to fully understand the effect of 3-NOP supplementation in cattle diets on ruminal microbial community composition.

The present study used the *in situ* ruminal fiber degradation technique to investigate the effects of feeding 3-NOP to beef cattle on ruminal fiber degradation and microbial community composition. Forage substrates (barley silage and grass hay) that composed the diet were incubated in the rumen for up to 288 h to determine the kinetics of NDF degradation. Ruminal dH₂ concentration and dynamic changes in the bacterial community directly attached to the incubated forages were also measured. We hypothesized that when feed-

ing 3-NOP to beef cattle fed a high-forage diet, H₂ would accumulate in the rumen and suppress microbial colonization of feed particles such that fiber degradation would decrease.

MATERIALS AND METHODS

The experiment was approved by the Lethbridge Research and Development Center Animal Care and Use Committee under the guidelines of the Canadian Council on Animal Care.

Animals

Eight ruminally cannulated beef cattle (515 ± 40.5 kg of BW) were used in a crossover design with 2 groups (blocks, 4 cattle/block), 2 periods, and 2 treatments [control, basal diet; 3-NOP, basal diet supplemented with 3-NOP (DSM Nutritional Products AG, Kaiseraugst, Switzerland)]. The basal diet was formulated to meet the nutrient requirements of growing beef cattle according to the National Academies of Sciences, Engineering, and Medicine (2016), and contained 45% barley silage, 45% chopped grass hay, and 10% concentrate on a DM basis (Table 1). Diets were offered for *ad libitum* intake with a 5% refusal level. The dose of 3-NOP (150 mg/kg of TMR, DM basis) was chosen based on a previous study that reported a 23% reduction in CH₄ from cattle fed a high-forage diet (Vyas et al., 2016). The 3-NOP (11.1% 3-nitrooxypropanol, 53.3% silica, 35.6% propylene glycol; as specified by the manufacturer) or carrier (60% silica, 40% propylene glycol) for the control was mixed into the TMR daily to reach the target concentration.

Ruminal Dissolved Gases

Dissolved gases were measured in ruminal fluid samples collected on d 17 and 28 via the fistula at 0 and 3 h relative to the commencement of feeding. The dissolved gas concentrations were measured using procedures described by Wang et al. (2014). Briefly, each ruminal fluid sample (35 mL) was quickly transferred into a 50-mL plastic syringe connected to a 20-mL syringe prefilled with 10 mL of N₂ gas. The N₂ gas was then injected into the 50-mL syringe, and the syringe was vigorously shaken for 5 min to extract the gases dissolved in the ruminal fluid into the N₂ gas phase. The gaseous H₂ and CH₄ concentrations were determined by gas chromatography (Agilent 7890A, Agilent Inc., Palo Alto, CA), and dH₂ and dissolved CH₄ (dCH₄) concentrations in the original ruminal fluid were calculated according to the equation given by Wang et al. (2016).

In Situ Ruminal Incubation and Sampling

Representative samples of barley silage and grass hay were collected, dried at 55°C, and ground to pass a 4-mm screen using a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA). Approximately 6.5 g of forage was weighed into individual polyester bags (10 cm × 20 cm; 50-μm porosity; R1020, ANKOM Technology, Macedon, NY) in duplicate. For each animal within a period, 22 bags were prepared for each forage with 20 bags used for kinetics of fiber degradation and 2 bags for changes in the microbial community. The bags were placed inside large mesh bags (40 cm × 40 cm) with 3 compartments to prevent them from aggregating together. The incubations were staggered in time, such that no more than 3 mesh bags were in the rumen of an animal at any one time.

The bags were incubated in each animal from d 31 to 43. Incubation times were: 0, 4, 12, 24, 36, 48, 96, 120, 216, and 288 h, with duplicate bags at each sampling time. Upon removal of the bags from the rumen, they were immediately submerged into ice water to arrest microbial activity. The bags were then washed, dried, weighed and stored until NDF content was measured. For the microbial analysis, additional duplicate bags were incubated for 4 and 48 h. Bags for microbial DNA extraction were gently washed with phosphate-buffered saline (pH = 7.4) to remove ruminal contents and loosely adhered microbes from the outer surface of the bag. Bags were then gently squeezed to extract the

liquid, which was then discarded. The rinsed solid residues were removed from the bags and transferred into sterile 50-mL tubes. These samples were flash frozen in liquid N₂ and stored at −80°C until DNA extraction.

Chemical Analysis

Dry matter and OM content were measured according to AOAC International (2005; methods 930.15 and 942.05, respectively). Neutral detergent fiber and ADF contents were sequentially measured (Van Soest et al., 1991) using an ANKOM200 fiber analyzer, with heat-stable α-amylase used in the NDF analysis. Values are expressed inclusive of residual ash. Starch content was determined by enzymatic hydrolysis of α-linked glucose polymers as described by Hall et al. (2015). Total nitrogen content was determined by flash combustion with gas chromatography and thermal conductivity detection (Carlo Erba Instruments, Milan, Italy; method 990.03; AOAC International, 2005) and the CP content was calculated as nitrogen × 6.25.

Microbial Analysis

The samples in 50-mL tubes were freeze-dried (Labconco FreeZone 1, Labconco Corporation, Kansas City) and blended with a coffee grinder. A 200-μg subsample from each tube was taken for DNA extraction using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA). The quality and quantity of DNA were measured based on absorbance of 260 and 280 nm using a NanoDrop One (NanoDrop Technologies Inc., Wilmington) and amplified using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') to check for the presence of PCR inhibitors. The amplification was conducted with the following conditions: 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, followed by a final elongation step at 72°C for 10 min.

Illumina sequencing was performed at McGill University's Genome Québec Innovation Centre (Montreal, Canada). The primers 515F (5'-GTGCCAGCMGC-CGCGGTAA-3') and 806R (5'-GGACTACHVGGGT-WTCTAAT-3') targeting the V4 region of the 16S rRNA gene were used to examine both bacterial and archaeal diversity (Caporaso et al., 2012). A 33-cycle PCR using 1 μL of a 1 in 10 dilution of genomic DNA samples and the FastStart High Fidelity PCR System (Roche, Montreal, QC, Canada) was conducted with the following conditions: 94°C for 2 min, followed by 33 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, followed by a final elongation step at 72°C for 7 min. Fluidigm Corporation (San Francisco, CA) barcodes

Table 1. The ingredients and nutrient composition of the basal diet (mean ± SD)

Item	Value
Ingredient, g/kg of DM	
Barley silage ¹	450
Grass hay ²	450
Barley grain, dry rolled	49.3
Canola meal	42.9
Urea	0.88
Salt	3.15
Molasses	2.16
Vitamin premix	0.50
Canola oil	1.09
Nutrient composition, g/kg of DM	
DM	931 ± 6.2 ³
OM	933 ± 1.9
CP	98.8 ± 3.94
NDF	529 ± 25.5
ADF	298 ± 17.4
Starch	128 ± 21.1

¹Barley silage: DM, 341 g/kg; OM, 930 g/kg; CP, 97.0 g/kg; NDF, 474 g/kg; ADF, 253 g/kg; and starch, 239 g/kg.

²Grass hay: DM, 851 g/kg; OM, 938 g/kg; CP, 62.0 g/kg; NDF, 649 g/kg; ADF, 380 g/kg; and starch, 26.6 g/kg.

³Multiple samples (n = 12, 2 periods × 3 sampling weeks × 2 replicates) were used for the nutrient composition analysis.

were incorporated in a second PCR reaction using the FastStart High Fidelity PCR System under the following conditions: 95°C for 10 min, followed by 15 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 1 min, followed by a final elongation step at 72°C for 3 min. After amplification, PCR products were checked in a 2% agarose gel to determine the success of amplification. All samples were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies Inc., Burlington, ON, Canada) and were pooled in equal proportions. Pooled samples were then purified using calibrated Ampure XP beads (Beckman Coulter, Mississauga, ON, Canada). The pooled samples (library) were quantified using the Quant-iT PicoGreen dsDNA Assay Kit and the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems Inc., Wilmington, MA). Average fragment size was determined using a LabChip GX (PerkinElmer, Waltham, MA) instrument. Sequencing was performed at McGill University's Génome Québec Innovation Centre on an Illumina MiSeq using the Illumina MiSeq Reagent Kit v2 (500 cycle) following the manufacturer's guidelines (Illumina Inc., San Diego, CA).

Quality analyses of the raw fastq files from the MiSeq were checked with the FastQC program (Andrews, 2010). Trimmomatic v0.33 (Bolger et al., 2014) was used to trim the raw reads, removing ambiguous and low-quality reads. Reads with average quality score <20 over a 4-bp sliding window and reads with lengths shorter than 36 bp were removed. Merging of the paired-end reads was done with PEAR version 0.9.8 using default options (Zhang et al., 2014). Unassembled reads were discarded. High-quality sequence reads from the various samples were then imported into Qiime2 for analysis of α - and β -diversity. The program DADA2 (Callahan et al., 2016) was used for quality control, filtering any phiX reads present in the sequencing data and removing chimeric sequences. Additionally, DADA2 models and corrects errors in Illumina sequence data and then generates a feature table containing count data (abundance) of amplicon sequences variants (**ASV**) at strain level resolution (>99.9% identity ASVs; Callahan et al., 2016). Following DADA2, the MAFFT program was used to perform multiple sequence alignment. Highly variable regions in the alignment were masked and a phylogenetic tree was generated with FastTree (Price et al., 2010). Taxonomy was assigned to sequences using a Naïve-Bayes classifier trained with the Silva 128 reference database and the feature-classifier plugin (Bokulich et al., 2018). Sequences from all samples were subsampled to 60,000 sequences to ensure that α - and β -diversity analysis used the same number of sequences per sample. The diversity plugin core-diversity

metrics was used to assess microbial diversity within (α -diversity) and between samples (β -diversity). Alpha-diversity measures for richness (Chao1 and Shannon's diversity index), phylogenetic diversity (Faith's phylogenetic diversity), number of observed ASV, evenness (Pielou's Evenness), and taxonomic abundance were evaluated. Beta-diversity analysis was carried out using weighted (accounting for relative abundances) and unweighted UniFrac (Lozupone et al., 2011). Sequences have been deposited to the Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) with accession number PRJNA557500.

Calculations and Statistical Analysis

The undegradable fraction of NDF (μNDF , g/kg) was calculated as

$$\mu\text{NDF} = 1,000 - \text{NDFD}_{288\text{h}},$$

where $\text{NDFD}_{288\text{h}}$ was the NDF disappearance (**NDFD**, g/kg) at 288 h of ruminal incubation.

The exponential model of Ørskov and McDonald (1979) was used to fit the kinetics of in situ ruminal **NDFD**:

$$p = a + b \times \{1 - e^{-c \times (t - \text{lag})}\},$$

where p is the **NDFD** at time t (g/kg); a is the rapidly soluble and washout fraction of NDF (g/kg); b is the potentially degradable fraction of NDF (g/kg); c is rate (%/h) of degradation of fraction b ; and lag is the lag time (h) before the start of degradation of fraction b . Data were fitted using the nonlinear model of R (version 3.6.1; <https://cran.r-project.org/web/packages/minpack.lm/index.html>). The effective ruminal degradation (ED, g/kg) was calculated according to the equation described by McDonald (1981) as follows:

$$\text{ED} = a + b \times c / (c + k),$$

where k is passage rate from the rumen (h^{-1}) and was set to 0.02 h^{-1} (**ED2**) and 0.06 h^{-1} (**ED6**) in the present study.

The statistical analyses were conducted to compare the 2 treatments using a linear mixed model in SAS (SAS Inst. Inc., Cary, NC). For the NDF degradation parameters, the statistical model included the fixed effect of treatment, random effects of group, period nested within group, and animal nested within group. A PERMANOVA based statistical test was used to examine the influence of treatment and sampling time

Table 2. The effects of 3-nitrooxypropanol (3-NOP; 150 mg/kg of DM) supplementation on kinetics of in situ NDF disappearance of barley silage and grass hay in the rumen of beef cattle fed a high-forage diet

Item ¹	Treatment		SEM	<i>P</i> -value
	Control	3-NOP		
Barley silage				
<i>a</i>	59.4	53.5	7.01	0.42
<i>b</i>	655	666	9.40	0.23
<i>c</i>	2.30	2.60	0.270	0.15
<i>a</i> + <i>b</i>	714	719	6.1	0.59
ED2	406	426	14.6	0.018
ED6	239	253	13.1	0.033
<i>lag</i>	5.46	5.26	0.537	0.72
μNDF	287	282	5.3	0.54
Grass hay				
<i>a</i>	142	121	9.20	0.023
<i>b</i>	512	530	10.3	0.11
<i>c</i>	2.70	3.10	0.170	0.018
<i>a</i> + <i>b</i>	654	651	3.3	0.60
ED2	435	444	6.60	0.17
ED6	300	303	8.40	0.78
<i>lag</i>	3.04	2.51	0.587	0.24
μNDF	330	327	4.0	0.63

¹*a* (g/kg of NDF) = the intercept of the degradation curve at time 0 h, which represents the very rapidly degradable and washout fraction of the NDF; *b* (g/kg of NDF) = the potential degradability of NDF and represents the more slowly degraded component; *c* (%/h) = the rate constant for the degradation of *b*; *lag* (h) = the lag time before the start of degradation of fraction *b*; μNDF (g/kg of DM) = undegradable fraction of NDF calculated as $1,000 - \text{NDFD}_{288\text{h}}$ (g/kg of DM) where $\text{NDFD}_{288\text{h}}$ is the NDF disappearance at 288 h of ruminal incubation. ED (g/kg of NDF) = effective ruminal degradation calculated as $a + b \times c/(c + k)$, where *k* is passage rate from the rumen (%/h) and was set to 2%/h (ED2) and 6%/h (ED6).

on the microbiome analysis. A principal coordinate analysis (PCoA) was also conducted using weighted and unweighted UniFrac matrices to visualize any sig-

nificant differences in the microbiome as a result of treatment and sampling time. For the rumen dissolved gas concentrations, α-diversity, and relative abundances of microbiota, the statistical model included the fixed effects of treatment, sampling and incubation time and interaction between treatment and time, random effects of group, period nested in group, and animal nested in group. All *P*-values of α-diversity and relative abundance of bacterial taxa were corrected for a false discovery rate of 0.05 using the Benjamini–Hochberg method (Benjamini and Hochberg, 2000). Significance was determined at $P \leq 0.05$.

RESULTS

3-Nitrooxypropanol supplementation greatly decreased dCH₄ (−53%, $P < 0.001$) and increased dH₂ concentrations (+780%, $P < 0.001$, Figure 1). The kinetics of in situ ruminal NDF disappearance indicated that 3-NOP supplementation did not alter the pattern of NDFD from either barley silage or grass hay (Table 2 and Supplemental Figure S1, <https://doi.org/10.3168/jds.2019-18077>). Analysis of the degradation kinetics of NDF for barley silage indicated that 3-NOP supplementation did not alter *a*, *b*, *c*, *a*+*b*, *lag*, or μNDF, although small increases were observed for ED2 ($P = 0.018$) and ED6 ($P = 0.033$). Kinetics of grass hay indicated that 3-NOP supplementation did not alter *b*, *a*+*b*, *lag*, ED2, ED6, or μNDF, although there was a small reduction in *a* ($P = 0.023$) and small increase in *c* ($P = 0.018$).

For incubated samples of barley silage, neither time of incubation nor treatment significantly altered the number of observed ASV or the species richness, as indicated by the Chao1 value or Shannon index (Table 3).

Figure 1. Effect of 3-nitrooxypropanol (3-NOP) supplementation on rumen dissolved CH₄ (A) and H₂ (B) concentrations in beef cattle fed a high-forage diet. Error bars are SEM. * indicates $P \leq 0.05$.

For grass hay, microbial species richness and α -diversity were not affected by incubation time (Table 4). However, 3-NOP supplementation reduced the microbial richness and diversity compared with control samples of grass hay at a 4-h incubation time (time $\times$ treatment; $P \leq 0.006$), as reflected by the lower observed ASV, Chao1 value, and Shannon index. Comparison of the overall bacterial and archaeal composition of the colonizing community was further analyzed by the PCoA. Unweighted UniFrac distances showed clear clustering of samples based on incubation time ($P < 0.01$) and 3-NOP supplementation ($P < 0.01$; Figure 2A and 2B). However, weighted UniFrac distances showed a clear clustering of samples based on incubation time ($P < 0.01$), but not for 3-NOP supplementation (Figure 2C and 2D).

Incubation time increased relative abundances of phyla *Firmicutes*, *Fibrobacteres*, and *Euryarchaeota* for both forages ($P \leq 0.05$, Table 3 and Table 4). Incubation time increased relative abundances of genera *Ruminococcaceae* UCG-011, *Fibrobacter*, and *Methanobrevibacter* ($P \leq 0.05$) and decreased relative abundances of genera *Bacteroidetes* and *Prevotella* 1 for both forages ($P \leq 0.05$). Consistent with the β -diversity analysis, 3-NOP supplementation did not alter ($P > 0.10$) most of the major phyla including *Bacteroidetes*, *Firmicutes*, and *Fibrobacteres*, and the major genus *Prevotella* 1. Importantly, there were no effects of 3-NOP supplementation on the abundance of major fiber-degrading microbes, such as genera *Fibrobacter*, *Butyrivibrio*, and *Treponema* sp. ($P > 0.10$). However, 3-NOP supplementation altered the methanogen community, with decreased relative abundance of the phylum *Euryarchaeota* ($P = 0.004$) and genus *Methanobrevibacter* ($P = 0.012$) in barley silage. Furthermore, there was an interaction ($P \leq 0.05$) between treatment and time for some low-abundant methanogens, with decreased genera *Methanosphaera* and uncultured *Thermoplasmatales incertae sedis* ($P \leq 0.05$) for both forages at the incubation time of 4 h. The differences in relative abundances of other genera ($0.5\% < \text{relative abundance} < 1.0\%$) between control and 3-NOP attached to barley silage and grass hay are shown in Supplemental Tables S1 and S2 (<https://doi.org/10.3168/jds.2019-18077>), respectively.

DISCUSSION

3-Nitrooxypropanol supplementation can be highly effective in reducing CH_4 emissions from ruminants (Jayanegara et al., 2018). In the present study, 3-NOP supplementation decreased ruminal dCH_4 concentration by 53%, which is comparable with our previous study

in which 2 g/d of 3-NOP supplementation to beef cattle reduced enteric CH_4 emissions by 59% (Romero-Pérez et al., 2015). Methanogenesis in the rumen is catalyzed by MCR in the rumen, which is a nickel enzyme in which the nickel is bound in the tetrapyrrole derivative cofactor F_{430} (Shi et al., 2014). The 3-NOP binds to MCR because it has a chemical structure similar to that of methyl-coenzyme M, and once bound, 3-NOP inactivates the enzyme and inhibits methanogenesis (Duin et al., 2016).

In addition to a decrease in methanogenic activity, an inhibition of methanogenesis can also be caused by a decrease in the methanogen population. Haisan et al. (2014) reported that 3-NOP supplementation decreased the methanogen population in dairy cows, yet other studies reported that 3-NOP supplementation did not affect the methanogen population in sheep (Martínez-Fernández et al., 2014) or beef cattle (Romero-Pérez et al., 2014). In our study, 3-NOP supplementation decreased the relative abundances of *Methanosphaera* and uncultured *Thermoplasmatales incertae sedis* in both forages at 4 h, and reduced the abundance of *Methanobrevibacter* in only barley silage. As there was a substantial decrease in ruminal dCH_4 concentration with 3-NOP, it appeared that the methanogen population adhered to forages might not play a major role in the inhibition of methanogenesis due to 3-NOP supplementation. The differing effect of 3-NOP on methanogen groups for barley silage compared with grass hay might have been caused by their different carbohydrate composition because barley silage is composed of greater starch and less fiber compared with grass hay. Starch promotes the formation of propionate, which decreases H_2 production, the substrate and energy source for methanogens. Thus, greater starch content of barley silage may explain the decrease in hydrogenotrophic methanogens when supplemented with 3-NOP.

Molecular hydrogen is a major substrate for ruminal methanogenesis. Ruminal CH_4 inhibition causes a reduction in H_2 consumption, leading to an increase in H_2 accumulation. In our study, 3-NOP supplementation greatly increased dH_2 concentration in the rumen. Ruminal methanogenesis occurs in 2 important steps: conversion of reducing equivalents to H_2 by hydrogenases (i.e., [FeFe]-hydrogenases, [NiFe]-hydrogenases and [Fe]-hydrogenases; Greening et al., 2019) and the ultimate reduction of CO_2 by H_2 in an enzymatic cascade when MCR catalyzes the last step before releasing CH_4 . The 3-NOP inactivates MCR activity (Duin et al., 2016), which is only involved in the last step of methanogenesis and may have no direct effect on hydrogenases, leading to increased H_2 accumulation. Previous studies also reported that 3-NOP supplementation increased

Table 3. The α -diversity of microbial community and differentially abundant phyla and genera (average relative abundance $\geq 1\%$ for bacteria) attached to barley silage at different incubation times for beef cattle fed control or 3-nitrooxypropanol (3-NOP)

Item	4 h				48 h				FDR ¹ corrected P-value			
	Control	3-NOP	Control	3-NOP	Control	3-NOP	SEM	Time	Treatment	Time	Treatment	Time
Observed ASV ²	485	452	475	473	475	473	9.8	0.71	0.17	0.71	0.17	0.19
Chao 1 value	488	454	477	473	477	473	9.7	0.61	0.28	0.61	0.28	0.16
Shannon index	7.67	7.58	7.59	7.57	7.59	7.57	0.030	0.29	0.28	0.29	0.28	0.30
Evenness	0.860	0.860	0.854	0.853	0.854	0.853	0.0013	0.037	0.77	0.037	0.77	0.70
Relative abundances, %												
p <i>Euryarchaeota</i>	2.64	1.68	3.22	2.97	3.22	2.97	0.220	0.099	0.004	0.099	0.004	0.18
g <i>Methanobrevibacter</i>	2.18	1.41	2.79	2.60	2.79	2.60	0.233	<0.001	0.012	<0.001	0.012	0.12
g <i>Methanospira</i>	0.088 ^a	0.057 ^b	0.127	0.160	0.127	0.160	0.0099	<0.001	0.94	<0.001	0.94	0.022
g Uncultured <i>Thermoplasmatales</i> g <i>incertae sedis</i>	0.355 ^a	0.070 ^b	0.219	0.153	0.219	0.153	0.0249	0.50	<0.001	0.50	<0.001	0.002
p <i>Actinobacteria</i>	3.46	3.16	2.04	1.91	2.04	1.91	0.255	<0.001	0.21	<0.001	0.21	0.62
g <i>Senegalimassilia</i>	1.17	1.16	0.87	0.99	0.87	0.99	0.051	<0.001	0.19	<0.001	0.19	0.15
p <i>Bacteroidetes</i>	40.5	40.3	30.3	31.4	30.3	31.4	0.95	<0.001	0.98	<0.001	0.98	0.73
g Uncultured rumen bacterium f <i>Bacteroidales</i> BS11 gut group	1.89	1.84	2.03	2.53	2.03	2.53	0.372	0.19	0.47	0.19	0.47	0.38
g Unknown f <i>Bacteroidales</i> BS11 gut group	1.58	1.03	2.03	1.57	2.03	1.57	0.383	0.21	0.21	0.21	0.21	0.90
g Uncultured rumen bacterium f <i>Bacteroidales</i> S24-7 group	1.75	2.54	2.48	3.65	2.48	3.65	0.308	0.002	0.001	0.002	0.001	0.40
g <i>Prevotella</i> 1	21.9	20.2	9.99	10.0	9.99	10.0	0.877	<0.001	0.22	<0.001	0.22	0.21
g <i>Prevotellaceae</i> NK3B31 group	1.56	2.09	1.00	1.25	1.00	1.25	0.216	<0.001	0.005	<0.001	0.005	0.26
g <i>Prevotellaceae</i> UCG-001	3.71	3.28	1.93	1.64	1.93	1.64	0.222	0.049	<0.001	0.049	<0.001	0.69
g <i>Rikenellaceae</i> RC9 gut group	2.30	2.51	4.48	4.46	4.48	4.46	0.407	<0.001	0.61	<0.001	0.61	0.52
p <i>Fibrobacteres</i>	2.20	2.96	7.12	7.75	7.12	7.75	0.626	<0.001	0.71	<0.001	0.71	0.98
g <i>Fibrobacter</i>	2.34	2.85	7.07	7.69	7.07	7.69	0.647	<0.001	0.27	<0.001	0.27	0.91
p <i>Firmicutes</i>	42.8	43.6	46.9	45.1	46.9	45.1	1.22	0.024	0.71	0.024	0.71	0.68
g <i>Acetivomaculum</i>	1.29	1.11	1.61	1.34	1.61	1.34	0.178	0.017	0.048	0.017	0.048	0.72
g <i>Christensenellaceae</i> R-7 group	1.88	2.32	2.42	2.60	2.42	2.60	0.246	0.13	0.056	0.13	0.056	0.52
g <i>Butyrivibrio</i> 2	1.30	1.59	1.64	1.73	1.64	1.73	0.112	0.043	0.17	0.043	0.17	0.39
g <i>Lachnospiraceae</i> NK3A20 group	2.97	3.63	3.04	3.42	3.04	3.42	0.356	0.58	<0.001	0.58	<0.001	0.27
g <i>Ruminococcaceae</i> UCG-011	2.90	3.02	3.86	4.35	3.86	4.35	0.451	<0.001	0.18	<0.001	0.18	0.41
g <i>Ruminococcus</i> 1	1.65	1.19	1.02	0.90	1.02	0.90	0.092	<0.001	0.003	<0.001	0.003	0.16
g <i>Ruminococcus</i> 2	5.43	6.35	2.93	2.92	2.93	2.92	0.296	<0.001	0.091	<0.001	0.091	0.29
g <i>Ruminococcaceae</i> NK4A214 group	2.08	1.60	3.22	2.47	3.22	2.47	0.175	<0.001	<0.001	<0.001	<0.001	0.19
g Uncultured f <i>Ruminococcaceae</i>	2.59	1.59	1.37	0.92	1.37	0.92	0.259	<0.001	0.001	<0.001	0.001	0.15
g Unknown f <i>Ruminococcaceae</i>	1.28	0.41	1.08	0.46	1.08	0.46	0.188	0.60	<0.001	0.60	<0.001	0.38
g <i>Succinidlasticum</i>	3.67	4.44	3.26	3.20	3.26	3.20	0.300	0.006	0.19	0.006	0.19	0.13
p <i>Proteobacteria</i>	1.56	2.24	0.81	0.69	0.81	0.69	0.232	<0.001	0.26	<0.001	0.26	0.11
p <i>Spirochaetae</i>	2.91	2.60	5.12	6.20	5.12	6.20	0.680	0.006	0.87	0.006	0.87	0.73
g <i>Treponema</i> 2	2.07	1.89	4.41	5.66	4.41	5.66	0.605	<0.001	0.26	<0.001	0.26	0.14
p <i>Verrucomicrobia</i>	1.26	1.14	1.06	1.04	1.06	1.04	0.096	<0.001	0.17	<0.001	0.17	0.28

^{a,b}Within the same row, mean values with a different superscripted letter differ significantly between treatments within an incubation time ($P \leq 0.05$).¹FDR = false discovery rate.²ASV = amplicon sequence variants.³p = phylum, g = genus, f = family.

Table 4. The α -diversity of microbial community and differentially abundant phyla and genera (average relative abundance $\geq 1\%$ for bacteria) attached to grass hay at different incubation times for beef cattle fed control or 3-nitrooxypropanol (3-NOP)

Item	4 h			48 h			FDR ¹ corrected <i>P</i> -value		
	Control	3-NOP	Control	3-NOP	SEM	Time	Treatment	Time × treatment	
Observed ASV ²	506 ^a	425 ^b	472	485	6.4	0.33	0.033	0.005	
Chao 1 value	509 ^a	427 ^b	474	489	5.6	0.38	0.044	0.006	
Shannon index	7.78 ^a	7.52 ^b	7.61	7.66	0.021	0.75	0.025	0.003	
Evenness	0.867	0.862	0.859	0.859	0.0015	0.007	0.12	0.21	
Relative abundances, %									
p <i>Euryarchaeota</i>	2.18	1.57	3.54	3.62	0.288	0.002	0.20	0.46	
g <i>Methanobrevibacter</i>	1.65	1.41	2.96	3.13	0.289	<0.001	0.89	0.43	
g <i>Methanospira</i>	0.089 ^a	0.044 ^b	0.183	0.198	0.0139	<0.001	0.26	0.031	
g Uncultured <i>Thermoplasma</i> g <i>incertae sedis</i>	0.419 ^a	0.085 ^b	0.291	0.228	0.0327	0.87	<0.001	0.007	
p <i>Actinobacteria</i>	3.34	2.76	2.20	2.05	0.219	<0.001	0.012	0.12	
g <i>Senegalimassilia</i>	1.38	1.29	1.29	1.07	0.055	<0.001	0.57	0.25	
g <i>Olsenella</i>	1.29	0.95	0.95	0.32	0.157	<0.001	0.030	0.52	
p <i>Bacteroidetes</i>	45.0	45.3	30.0	31.4	0.946	<0.001	0.93	0.72	
g Uncultured rumen bacterium f <i>Bacteroidales</i> BS11 gut group	2.21	1.98	1.98	2.10	0.493	0.906	0.89	0.70	
g Unknown f <i>Bacteroidales</i> BS11 gut group	1.76	1.36	1.36	2.35	0.436	0.012	0.25	0.90	
g Uncultured rumen bacterium f <i>Bacteroidales</i> S24-7 group	1.92	2.70	2.70	3.99	0.330	0.001	0.001	0.32	
g <i>Prevotella</i> 1	24.9	24.2	8.33	8.29	0.899	<0.001	0.67	0.72	
g <i>Prevotellaceae</i> NK3B31 group	1.19	1.50	1.50	1.46	0.185	0.289	0.008	0.44	
g <i>Prevotellaceae</i> UCG-001	3.87	3.37	3.37	1.65	0.240	<0.001	0.023	0.64	
g Unknown f <i>Prevotellaceae</i>	1.18	1.55	1.55	0.85	0.306	0.036	0.47	0.36	
g Unknown p <i>Bacteroidetes</i>	1.11	0.93	0.93	0.60	0.131	0.005	0.26	0.81	
g <i>Rikenellaceae</i> RC9 gut group	2.41	2.60	4.91	4.96	0.358	<0.001	0.53	0.72	
p <i>Fibrobacteres</i>	3.01	3.91	5.62	5.88	0.591	0.003	0.36	0.72	
g <i>Fibrobacter</i>	2.98	3.87	5.58	5.84	0.582	<0.001	0.22	0.55	
p <i>Firmicutes</i>	37.4	37.5	49.0	47.7	1.25	<0.001	0.92	0.72	
g <i>Acetivomaculum</i>	1.20	0.97	0.97	1.55	0.192	<0.001	0.031	0.71	
g <i>Christensenellaceae</i> R-7 group	2.25	2.84	2.84	2.87	0.178	0.089	0.096	0.12	
g <i>Butyrivibrio</i> 2	1.93	2.33	2.33	2.53	0.157	0.461	0.11	0.19	
g <i>Lachnospiraceae</i> NK3A20 group	2.32	3.02	3.02	3.59	0.321	<0.001	0.004	0.22	
g <i>Ruminococcaceae</i> UCG-011	2.80	3.68	3.42	4.54	0.414	0.011	0.001	0.67	
g <i>Ruminococcaceae</i> NK4A214 group	2.16	1.61	1.61	2.91	0.158	<0.001	<0.001	0.96	
g <i>Ruminococcus</i> 1	2.06	1.57	1.57	0.79	0.108	<0.001	<0.001	0.18	
g <i>Ruminococcus</i> 2	1.62	1.54	1.54	3.06	0.203	<0.001	0.437	0.23	
g Unknown f <i>Lachnospiraceae</i>	1.55	1.87	1.87	1.73	0.174	0.906	0.297	0.30	
g <i>Succinidlasticum</i>	3.95	4.61	2.77	3.56	0.310	0.001	0.022	0.83	
p <i>Spirochaetes</i>	4.19	4.26	4.02	4.40	0.407	0.82	0.94	0.74	
g <i>Treponema</i> 2	3.07	3.95	3.40	3.93	0.378	0.044	0.64	0.61	
p <i>Verrucomicrobia</i>	1.22	1.39	1.00	1.10	0.099	<0.001	0.033	0.65	

^{a,b}Within the same row, mean values with a different superscripted letter differ significantly between treatments within an incubation time ($P \leq 0.05$).¹FDR = false discovery rate.²ASV = amplicon sequence variants.³p = phylum, g = genus, f = family.

Figure 2. Comparison of surface colonizing microbial community based on sequencing V4 region of the 16s rRNA gene. Principal coordinate analysis (PCoA) of samples incubated in the rumen was calculated using unweighted and weighted UniFrac distances. Top panels are based on unweighted UniFrac distances. Bottom panels are based on weighted UniFrac distances. A and C: barley silage; B and D: grass hay. Treatment is shown by color (red = control; blue = 3-NOP), time is shown by shape (circle = 4 h, stars = 48 h). Percentages of data variation explained by the analysis are shown on the axes. 3-NOP = 3-nitrooxypropanol. PERMANOVA *P*-values are listed as follows. Barley silage: unweighted UniFrac distances, treatment effect, *P* = 0.002; time effect, *P* = 0.001; weighted UniFrac distances, treatment effect, *P* = 0.18; time effect, *P* = 0.001. Grass hay: unweighted UniFrac distances, treatment effect, *P* = 0.001; time effect, *P* = 0.001; weighted UniFrac distances, treatment effect, *P* = 0.26; time effect, *P* = 0.001. Number of permutations = 999.

ruminal dH₂ concentration and gaseous H₂ emissions (Hristov et al., 2015; Vyas et al., 2018).

Glycolysis of carbohydrates in the rumen needs the participation of oxidized cofactors (e.g., NAD⁺) and produces reduced cofactors (e.g., NADH), with H₂ liberated by the reoxidation of NADH to NAD⁺ (Hegarty and Gerdes, 1999). Increased H₂ partial pressure has a negative effect on the reoxidation of NADH, leading to inhibition of the glycolysis cycle and carbohydrate degradation (Leng, 2014). This increased dH₂ concentration in rumen fluid is thought to have a negative effect on fiber degradation. Ma et al. (2019) reported that increased ruminal dH₂ concentration caused by elemental magnesium supplementation inhibited NDF digestibility in dairy cows. Similarly, Zhang et al. (2019) reported that decreased ruminal dH₂ concentration was associated with improved NDF digestibility. Yet, in the present study, increased dH₂ concentration caused by 3-NOP supplementation did not substantially alter in situ ruminal NDF degradation. Previous studies also reported that 3-NOP supplementation had little effect on total-tract NDF digestion in ruminants (Reynolds

et al., 2014; Romero-Pérez et al., 2014; Jayanegara et al., 2018). Reasons for the apparent contradiction between the observed lack of effect of 3-NOP on NDF degradation kinetics and an expected decrease in fiber degradation due to elevated ruminal dH₂ concentration with 3-NOP supplementation are not clear. It seems that increased H₂ partial pressure caused by 3-NOP supplementation may not inhibit the reoxidation of NADH and fiber degradation. It is possible that other alternative H₂ sinks, such as butyrate, lactate, succinate, and ethanol from pyruvate might have facilitated the oxidation of NADH (van Lingen et al., 2016).

Carbohydrate degradation in the rumen occurs through a consortium of microbes that function together in a synergistic manner (Wang and McAllister, 2002). Many studies demonstrate that ruminal microbes colonize ingested feed particles rapidly, and the colonized microbial community is often dominated by phyla *Bacteroidetes*, *Firmicutes*, and *Fibrobacteres* (Huws et al., 2016; Liu et al., 2016). The composition of the microbial community attached to forage substrates in the rumen changes over time, reflecting the change in substrate

composition as degradation proceeds (Liu et al., 2016; Cheng et al., 2017). Consequently, we examined the microbial community composition associated with the forages at 4 and 48 h of in situ incubation. Consistent with previous studies, samples had greater abundance of phylum *Bacteroidetes* and lower abundances of phyla *Firmicutes* and *Fibrobacteres* at 4 h compared with samples incubated for 48 h. *Bacteroidetes* have a role in mobilizing easily accessible nutrients (e.g., soluble sugars and protein), thus the initial stage of fermentation favors the colonization of phylum *Bacteroidetes*. Taxa within the phyla *Firmicutes* and *Fibrobacteres* are known to degrade the more recalcitrant polysaccharides of the plant cell wall, consistent with a later stage of fermentation. Cheng et al. (2017) also reported that forage samples had lower abundance of phylum *Bacteroidetes* and greater abundance of phylum *Firmicutes* after 6 h of in situ ruminal incubation in dairy cows. Liu et al. (2016) reported increased phylum *Fibrobacteres* was associated with rice straw after 6 h when incubated in the rumen of cows receiving a diet contain 75% grass hay. The results from our study confirm that in situ incubation time alters the microbial community attached to forage fibers, reflecting the varied functions of the community members and the change in substrate carbohydrate composition over time.

The microbial community attached to the forages was affected by 3-NOP supplementation even though NDFD of the forages was not affected. Unweighted UniFrac PCoA indicated that the microbial community of forages incubated in the rumen of animals fed 3-NOP was distinctly separated from that of the control. In contrast, a PCoA plot based on weighted UniFrac distances did not show clear separation of the control and 3-NOP samples. These data suggest that 3-NOP targets specific taxa within the rumen microbial community, and that the effect on these specific microbes does not result in substantial changes to the overall community composition. This is consistent with the mechanism of action of this inhibitor, and consistent with the observed effect of 3-NOP on genus *Methanobrevibacter*. Romero-Pérez et al. (2015) reported that 3-NOP supplementation did not alter the total bacterial population in beef cattle, and Lopes et al. (2016) reported little change in relative abundances of major bacterial taxa in ruminal contents of dairy cows fed 3-NOP. It appears that 3-NOP supplementation does not greatly alter the composition of the bacterial community adhered to forage incubated in the rumen. Importantly, 3-NOP did not significantly change the relative abundance of major taxa involved in fiber degradation, indicating that this inhibitor can be used to reduce CH₄ emissions without reducing the efficiency of ruminal fiber degradation.

CONCLUSIONS

Supplementation of a high-forage diet with 3-NOP inhibited ruminal methanogenesis, leading to a decrease in ruminal dCH₄ concentration and an augmentation of ruminal dH₂ concentration, but did not affect fiber degradation of barley silage or grass hay during long-term in situ ruminal incubations. Although 3-NOP supplementation reduced the relative abundance of some microbes including genera *Methanobrevibacter* and *Methanospaera*, it did not affect the most abundant taxa and did not significantly change the overall composition of the microbial community colonizing the surface of feed in the rumen. In conclusion, 3-NOP supplementation inhibited (−53%) ruminal methanogenesis and increased ruminal dH₂ concentration (+780%) without negative effects on in situ fiber degradation or substantial changes in the bacterial community adhered to the forages in the rumen of cattle.

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