The effect of nitrate and monensin on in vitro ruminal fermentation¹

M. Capelari* and W. Powers†²

*Department of Animal Science, Michigan State University, East Lansing 48824; and

†University of California, Oakland 94607

ABSTRACT: Two experiments evaluated the effect of calcium ammonium nitrate decahydrate (calcium nitrate [NIT]) and monensin sodium (MON) on in vitro fermentation parameters of 2 contrasting diets (100:0 and 10:90 forage-to-concentrate ratios). Diet addition of NIT (0, 1.25, and 2.5 g/100 g DM) and MON (0, 3, and 6 mg/L) were tested alone and combined (9 treatments total; 5 bottles per treatment). Mixed ruminal microorganisms were incubated in anaerobic media containing 0.5 g of substrate diet, 1 of 9 treatments, and 40 mL buffer solution. Incubations were performed in batch cultures for 48 h at 39°C. Headspace gas volume was measured and sampled at 4, 8, 12, 24, and 48 h, and the VFA profile was assessed at the end of the experiment. Total gas production was reduced by NIT (87.9 vs. 94.6 mL; $P < 0.01$) and MON (78.6 vs. 94.6 mL; $P < 0.01$) and, in Exp. 2, further reduced by NIT+MON when the additives were combined (161.1 vs. 196.9 mL; $P < 0.01$). Methane production from control in Exp. 1 and Exp. 2 averaged 9.1 and 15.3 mL, respectively, and was decreased by NIT (3.4 and 8.3 mL in Exp. 1 and Exp. 2, respectively; $P < 0.01$), MON (4.1 and 7.7 mL; in Exp. 1 and Exp. 2, respectively; $P < 0.01$) and NIT+MON (1.1 and 1.5 mL; in Exp. 1 and Exp. 2, respectively;

$P < 0.01$). Both experiments demonstrated a significant increase in nitrous oxide (N_2O ; $P < 0.01$) when NIT was added. Compared to the control treatment, IVDMD was reduced when NIT+MON was added at the higher doses in EXP1 (31.7 vs. 37.4%; $P < 0.01$) and EXP2 (76.6 vs. 79.9 %; $P < 0.01$). Net VFA production was not affected by treatments ($P > 0.10$), but molar proportions of acetate and butyrate were reduced by MON ($P < 0.01$). Propionate molar proportion was increased in both experiments by MON ($P < 0.01$) and further increased in Exp. 2 when the additives were combined at lower doses ($P < 0.01$). Compared to the control treatment, the acetate:propionate (A:P) ratio was reduced by MON in Exp. 1 (1.2 vs. 2.8; $P < 0.01$) and Exp. 2 (1.0 vs. 2.3; $P < 0.01$). Fermentation efficiency (%) was increased by MON (81.7 vs. 73.7%; $P < 0.01$) and further increased in Exp. 2 when the additives were combined at lower doses (87.2 vs. 76.6%; $P < 0.01$). The combination of NIT and MON in 2 contrasting diets proved beneficial by altering fermentation products toward lower CH_4 and more propionate; however, the addition of NIT consistently increased N_2O production. Negative effects of the additives on IVDMD were found only when the additives were combined at higher doses.

Key words: in vitro, methane, monensin, nitrate, nitrous oxide

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INTRODUCTION

Methane (CH_4) production is an inherent consequence of microbial degradation of dietary carbohydrates in the foregut of ruminant animals that

allows continued microbial activity (Hungate, 1966). However, CH_4 is a potent greenhouse gas (Gerber et al., 2013) and a dietary energy loss for the host animal (Blaxter, 1966). As a greenhouse gas, the largest biogenic source of CH_4 is enteric fermentation from ruminants (USEPA, 2006), whereas 2 to 12% of dietary GE is lost in the form of CH_4 (Johnson and Johnson, 1995). Reducing CH_4 production from ruminants will contribute with global efforts to control global warming while offering potential improvements for animal energy metabolism.

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²Corresponding author: wendy.powers@ucop.edu

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Rumen CH_4 formation is dependent on carbon dioxide (CO_2) and hydrogen (H_2) availability (Miller, 1995), and the metabolic pathways involved in H_2 production and utilization are important factors that should be considered when developing strategies to reduce CH_4 emissions from ruminants (Martin et al., 2010). In the rumen, calcium nitrate (NIT) is reduced to ammonia (NH_3), and this reaction provides an alternative—and energetically more favorable—pathway for disposal of H_2 generated during fermentation (Ungerfeld and Kohn, 2006). Stoichiometrically, if all NIT is reduced to NH_3 , then CH_4 production should decrease by 25.8 g/100 g NIT (Leng and Preston, 2010). Monensin sodium (MON) reduces acetate-producing bacteria and protozoal populations, especially *Entodinium* spp., that utilize CO_2 for H_2 disposal (Chen and Wolin, 1979; Guan et al., 2006), consequently reducing CH_4 production.

Because NIT and MON offer different mechanisms to control methanogenesis, we hypothesized that combining the additives would further reduce CH_4 production compared with the additives alone with no impact to production parameters. The objective of this study was to assess the effect of a combination of NIT and MON, in vitro, on rumen fermentation parameters.

MATERIALS AND METHODS

Experiments were conducted at Michigan State University (East Lansing, MI) and consisted of 2 independent rumen batch culture trials. The 2 experiments were independently performed to assess the dose and combination effect of NIT and MON on rumen fermentation parameters (gas production, CH_4 , N_2O , VFA, pH, and IVDMD) using 2 contrasting diet substrates. All animal procedures were approved and followed internal guidelines recommended by the Animal Care and Use Committee of Michigan State University.

Experimental Design and Treatments

For each experiment, 3 different doses of calcium ammonium nitrate decahydrate (NIT; $5\text{Ca}(\text{NO}_3)_2 \cdot 2\text{NH}_4\text{NO}_3 \cdot 10\text{H}_2\text{O}$; Yara International ASA, Oslo, Norway; 0, 1.25, and 2.5 g/100 g DM) and MON (C36H61NaO1; Sigma-Aldrich Corp., St. Louis, MO; 0, 3, and 6 mg/L of buffered rumen fluid) were tested independently and in combination, resulting in 9 treatments. Serum bottles (165 mL volume) were used to simulate the rumen in vitro, and each bottle was considered an experimental unit. Each of the 9 treatments had 5 replicates, totaling 45 bottles. Six extra bottles served as blanks for VFA (3 bottles) and IVDMD (3 bottles). The VFA blanks contained the diet substrate (0.5 g) and

buffered rumen fluid (10 and 40 mL of processed rumen fluid and buffer solution, respectively) and bottles were immersed in ice-water bath for 15 min to immediately stop fermentation following addition of buffered rumen fluid. The average VFA concentration from blanks was then used to calculate net VFA production at the end of the 48-h incubation (net VFA production = final VFA accumulation at 48 h – blanks average).

Two stock solutions of MON were prepared 1 d before the experiments by dissolving either 15 or 30 mg of MON in 10 mL of 100% ethanol. Immediately before the serum bottles were sealed, 0.1 mL of the stock solution was transferred to the respective bottle already containing 50 mL of buffered rumen fluid, producing 3 and 6 mg of MON per liter of buffered rumen fluid. An equal amount of pure ethanol was added to all remaining bottles to equalize the amount of ethanol added to all treatments. The NIT source was directly mixed in the diets before incubation.

The basal diet used in the Exp. 1 consisted of 100% ground grass hay whereas in Exp. 2, the basal diet consisted of 90% ground corn and 10% ground grass hay (Table 1). Because the NIT source contained 17.6% N and 19.6% Ca, experimental diets were balanced with addition of urea and limestone to maintain the same levels of N and Ca across treatments. The basal diet was ground in a Wiley mill (Thomas Scientific, Swedesboro, NJ) to pass a 1-mm screen, and 0.5 g of the processed diet was added to all bottles.

Incubation Conditions and Rumen Inoculum Source

Inoculum donors were housed at the Michigan State University Dairy Cattle Teaching and Research Facility (East Lansing, MI). Rumen inoculum for Exp. 1 was collected from 2 ruminally fistulated dry cows consuming only mixed grass hay, whereas inoculum for Exp. 2 was collected from 3 ruminally fistulated lactating cows fed a 50:50 forage:concentrate ratio (DM basis; approximately 18% ground corn, 18% alfalfa haylage, 15% soybean meal, 15% high-moisture corn, and 18% corn silage) in the form of a total mixed ration. Diets of rumen fluid donors did not contain any ionophore or supplementary nitrate. Rumen fluid was collected 3 h following the morning feeding from 5 different rumen locations of each donor and then transferred to a preheated 10-L thermos. The rumen inoculum was immediately transported to the laboratory, where it was blended and filtered through 2 layers of cheesecloth.

The processed fluid (10 mL) was transferred under continuous CO_2 flow to prewarmed serum bottles containing 40 mL of buffered artificial saliva (pH 8.1; buffered rumen fluid pH not measured at 0 h; 35 g/L NaHCO_3 , 4 g/L $(\text{NH}_4)\text{HCO}_3$, 5.7 g/L

Table 1. Ingredients and chemical composition of experimental diets for in vitro Exp. 1 (100:0 roughage:concentrate ratio) and Exp. 2 (10:90 roughage:concentrate ratio)¹

Item, DM, % (unless otherwise stated)	0 g NIT/100 g DM			1.25 g NIT/100 g DM			2.5 g NIT/100 g DM		
	0 mg MON/L	3 mg MON/L	6 mg MON/L	0 mg MON/L	3 mg MON/L	6 mg MON/L	0 mg MON/L	3 mg MON/L	6 mg MON/L
Experiment 1									
Grass hay	97.5	97.5	97.5	97.0	97.0	97.0	97.0	97.0	97.0
Experiment 2									
Grass hay	9.8	9.8	9.8	9.7	9.7	9.7	9.7	9.7	9.7
Ground corn	87.7	87.7	87.7	87.5	87.5	87.5	87.5	87.5	87.5
Experiments 1 and 2									
Nitrate source ²				1.5	1.5	1.5	2.9	2.9	2.9
Monensin, ³ mg/L		3	6		3	6		3	6
Urea	1.3	1.3	1.3	0.7	0.7	0.7			
Limestone	1.2	1.2	1.2	0.6	0.6	0.6			
Calculated chemical composition, % of DM									
Experiment 1									
DM	90.7	90.7	90.7	90.7	90.7	90.7	90.7	90.7	90.7
CP	12.4	12.4	12.4	12.5	12.5	12.5	12.3	12.3	12.3
NDF	65.1	65.1	65.1	65.1	65.1	65.1	65.1	65.1	65.1
NFC ⁴	15.3	15.3	15.3	15.3	15.3	15.3	15.3	15.3	15.3
Ash	8.1	8.1	8.1	8.1	8.1	8.1	8.1	8.1	8.1
Ca	1.1	1.1	1.1	1.2	1.2	1.2	1.2	1.2	1.2
Experiment 2									
DM	91.1	91.1	91.1	91.1	91.1	91.1	91.1	91.1	91.1
CP	11.8	11.8	11.8	11.9	11.9	11.9	11.7	11.7	11.7
NDF	14.3	14.3	14.3	14.3	14.3	14.3	14.3	14.3	14.3
NFC	68.9	68.9	68.9	68.9	68.9	68.9	68.9	68.9	68.9
Ash	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2
Ca	0.5	0.5	0.5	0.6	0.6	0.6	0.6	0.6	0.6

¹NIT = calcium nitrate (0, 1.25, and 2.5% of diet DM); MON = monensin sodium (0, 3, and 6 mg/L).

²Nitrate source was manufactured by Yara International ASA, Oslo, Norway. The source of nitrate was the double salt of calcium ammonium nitrate decahydrate [5Ca(NO₃)₂·NH₄NO₃·10H₂O]; 85.6% DM; 17.6% N, 19.6% Ca, and 71.4% nitrate on a DM basis.

³Monensin sodium (C₃₆H₆₁NaO₇; Sigma-Aldrich Corp., St. Louis, MO).

⁴NFC = Non-fiber carbohydrates.

Na₂HPO₄, 6.2 g/L KH₂PO₄, 0.6 g/L MgSO₄·7H₂O, 13.2 g/L CaCl₂·2H₂O, 10 g/L MnCl₂·4H₂O, 1 g/L CoCl₂·6H₂O, and 8.0 g/L FeCl₃·6H₂O; Goering and Van Soest, 1970) fitted with a rubber stopper and crimp-sealed with aluminum caps. Before incubation (0 h), any additional pressure was released from bottles by inserting a 16-gauge needle through the rubber septa. Bottles were incubated at 39°C (±0.3) in an incubator (Ambi-Hi-Low chamber; Lab-Line Instruments Inc., Melrose Park, IL) and hand-shaken every 2 h.

Sampling and Measurements Procedures

Gas pressure in the headspace was measured with a relative pressure gauge (MediaGauge; SSI Technologies, Janesville, WI) at 4, 8, 12, 24, and 48 h of incubation and used for calculation of total gas produced during each interval (Eq. [1]). At the same time points, a 5-mL sample of gas was collected from each bottle using a precise syringe (Hamilton Co., Reno,

NV) and immediately transferred to 20-mL vials containing 15 mL of ultra-high-purity N₂ gas. Following headspace sampling, additional accumulated gas was released from the system by inserting a 16-gauge needle through rubber septa of all serum bottles. Methane concentrations were obtained using gas chromatography equipped with a flame ionization detector (FID; GC-2014; Shimadzu Scientific Instruments, Inc., Addison, IL), and nitrous oxide (N₂O) concentrations were obtained using an electron capture detector (GC-2014; Shimadzu Scientific Instruments, Inc.). Carrier gas was ultra-purity N₂ gas, with a total flow of 40 mL/min and a purge flow of 0.5 mL/min. The column oven was maintained at 75°C, FID at 250°C, and electron capture detector at 325°C. The gas chromatograph was equipped with an automatic headspace sampler (Combi PAL; LEAP Technologies, Carrboro, NC).

Gas production at each time point was calculated as follows:

$$V_{\text{GAS}} = [(V_{\text{BT}} - V_{\text{R}})/P_{\text{EL}}] \times P_{\text{BT}} \quad [1]$$

in which V_{GAS} is the volume of gas production at each time point (mL); V_{BT} is the volume of serum bottle, 165 mL; V_{R} is the volume of buffered rumen fluid, 50 mL; P_{EL} is the atmospheric pressure in East Lansing, MI, adjusted for temperature and humidity, 14.69 kPa; and P_{BT} are the pressure measurements from the gauge (kPa). Total gas production and CH_4 and N_2O production at each time point (4, 8, 12, 24, and 48 h) were added to obtain total gas production over the 48-h incubation.

After the last gas sampling at 48 h, fermentation was stopped by placing serum bottles in ice-water bath for 15 min. Bottles were then opened, and pH was measured with a pH meter (HQ40d Portable pH meter; Hach Co., Reno, NV) by submerging the probe approximately 3 cm in the fermentation media. Fermentation media (7 mL) was then collected and transferred to 15-mL plastic centrifuge tubes. The tubes were initially centrifuged with $15,000 \times g$ for 15 min at 4°C . Five milliliters of the supernatant was transferred to a second set of 15-mL tubes and 1 mL of 25% *m*-phosphoric acid was added. After 15 min, samples were recentrifuged under the same conditions and 1 mL of supernatant was collected and transferred to gas chromatography vials for VFA analysis. Sample VFA profile was obtained using a gas chromatograph equipped with a FID (GC-2010; Shimadzu Scientific Instruments, Inc.). The carrier gas was ultra-high-purity He, the flow rate was set at 62 mL/min, and the purge flow was 3 mL/min. The oven temperature ramp was set to 150°C for 2 min with further increments of $15^\circ\text{C}/\text{min}$ until the temperature reached 220°C .

Samples of ground corn, mixed grass hay, and ingredients used in the total mixed ration fed to donor cows of Exp. 2 were analyzed for DM, CP, NDF, nonfiber carbohydrates, ether extract, Ca, and P by wet chemistry (Dairy One Forage Analysis Laboratory, Ithaca, NY).

Fermentation Balance Calculations

All equations used here are described in detail by Wolin (1960), Demeyer and Tamminga (1987), and Demeyer (1991). In summary, the H_2 recovery (%) Eq. [4]) was calculated from the ratio of H_2 utilized and H_2 produced ($\mu\text{mol}/\text{mL}$; Eq. [2 and 3], respectively). The H_2 produced as fermentation end products and H_2 consumed to form CH_4 and VFA were determined from molar concentration of acetate (C_2), propionate (C_3), butyrate (C_4), isovalerate (C_{i5}), valerate (C_5), and CH_4 . The equations do not account for H_2 released in the gaseous form, lactate, microbial mass, and potential acetate produced via reductive acetogenesis and were calculated as follows:

$$\text{H}_2 \text{ utilized} = (2 \times \text{C}_3) + (2 \times \text{C}_4) + (4 \times \text{CH}_4) + \text{C}_{i5}, \quad [2]$$

in which H_2 utilized is expressed in micromoles per milliliter;

$$\text{H}_2 \text{ produced} = (2 \times \text{C}_2) + \text{C}_3 + (4 \times \text{C}_4) + (2 \times \text{C}_5) + (2 \times \text{C}_{i5}), \quad [3]$$

in which H_2 produced is expressed in micromoles per milliliter; and

$$\text{H}_2 \text{ recovery} = (\text{H}_2 \text{ utilized} / \text{H}_2 \text{ produced}) \times 100, [4]$$

in which H_2 recovery is expressed as a percent.

The amount of hexose (C_6) fermented was calculated as follows:

$$\text{hexose fermented} = (0.5 \times \text{C}_2) + (0.5 \times \text{C}_3) + \text{C}_4 + \text{C}_5, \quad [5]$$

in which hexose fermented is expressed in micromoles per milliliter.

Fermentation efficiency was calculated considering the heat of combustion of glucose, acetate, propionate, and butyrate and their molar concentration (mM), as follows:

$$\text{fermentation efficiency} = \{[(0.62 \times \text{C}_2) + (1.09 \times \text{C}_3) + (0.78 \times \text{C}_4)] / (\text{C}_2 + \text{C}_3 + \text{C}_4)\} \times 100, \quad [6]$$

in which fermentation efficiency is expressed as a percent.

In Vitro Dry Matter Disappearance

Three extra bottles per treatment were processed and incubated as previously described to determine IVDMD. The bottles were fitted with a 16-gauge needle for gas release, and at the end of the 48-h incubation, fermentation media with undegraded substrate was filtered using the same apparatus and procedure described by Goering and Van Soest (1970). Hot water (20 mL; $90\text{--}100^\circ\text{C}$) was used to clean all serum bottles and avoid unaccounted for undegraded material. The substrate DM content was determined by drying at 55°C in a forced-air oven for 48 h followed by hot weighing, and the fraction of IVDMD for each bottle was calculated by subtracting the dry residue weight (corrected for the blank) from the dry substrate weight and dividing by the dry weight of substrate.

Table 2. Effect of calcium nitrate (NIT; 0, 1.25, and 2.5 g/100 g of DM), monensin sodium (MON; 0, 3, and 6 mg/L), and their combination on in vitro gas and CH₄ production in Exp. 1 (100:0 roughage:concentrate ratio)

Item	0 g NIT/100 g DM			1.25 g NIT/100 g DM			2.5 g NIT/100 g DM			Pooled SEM	P-value		
	0 mg MON/L	3 mg MON/L	6 mg MON/L	0 mg MON/L	3 mg MON/L	6 mg MON/L	0 mg MON/L	3 mg MON/L	6 mg MON/L		NIT	MON	NIT × MON
pH	6.2	6.2	6.2	6.2	6.2	6.2	6.3	6.3	6.2	0.2	NS ¹	NS	NS
IVDMD, %	37.5 ^{abc}	40.0 ^a	39.3 ^{ab}	37.3 ^{abc}	35.6 ^{bcd}	34.7 ^{cd}	38.4 ^{abc}	35.6 ^{cd}	31.7 ^d	0.7	NS	NS	*
Gas production, mL													
Gas (0–4 h)	21.69 ^a	18.60 ^b	19.23 ^b	21.28 ^a	17.79 ^b	18.2 ^b	21.8 ^a	18.53 ^b	19.54 ^b	0.9	NS	*	NS
Gas (4–8 h)	16.35 ^a	14.03 ^{bc}	13.04 ^c	15.06 ^b	13.2 ^c	13.48 ^c	15.24 ^b	13.89 ^c	13.73 ^c	0.3	NS	*	NS
Gas (8–12 h)	12.18 ^a	10.46 ^b	9.72 ^c	10.62 ^b	10.02 ^{bc}	9.16 ^c	10.51 ^b	9.6 ^c	9.35 ^c	0.5	*	*	*
Gas (12–24 h)	22.5 ^a	17.71 ^b	15.83 ^{bc}	21.11 ^a	13.56 ^c	12.57 ^c	20.42 ^a	12.48 ^c	11.45 ^c	0.4	*	*	*
Gas (12–48 h)	21.94 ^a	20.48 ^a	18.32 ^b	20.73 ^a	18.46 ^b	17.3 ^b	19.07 ^{ab}	18.13 ^b	12.7 ^d	0.6	*	NS	*
Total gas	94.66 ^a	81.26 ^c	76.15 ^d	88.81 ^b	73.04 ^d	70.72 ^{de}	87.04 ^b	72.43 ^{de}	66.77 ^e	0.8	*	*	NS
CH ₄ production, mL													
CH ₄ (0–4 h)	0.1 ^a	0.08 ^b	0.08 ^b	0.08 ^b	0.06 ^c	0.07 ^b	0.1 ^a	0.09 ^a	0.07 ^b	0.02	NS	*	NS
CH ₄ (4–8 h)	0.85 ^a	0.45 ^b	0.23 ^c	0.45 ^b	0.16 ^d	0.16 ^d	0.4 ^b	0.13 ^d	0.19 ^c	0.06	*	*	*
CH ₄ (8–12 h)	1.41 ^a	0.48 ^b	0.57 ^b	0.5 ^b	0.21 ^c	0.21 ^c	0.57 ^b	0.18 ^c	0.22 ^c	0.06	*	*	*
CH ₄ (12–24 h)	2.99 ^a	1.61 ^b	1.24 ^b	0.66 ^c	0.33 ^d	0.21 ^d	0.73 ^c	0.22 ^d	0.15 ^d	0.2	*	*	*
CH ₄ (24–48 h)	3.8 ^a	1.34 ^c	2.24 ^b	2.24 ^b	0.82 ^d	0.67 ^d	1.22 ^c	0.31 ^d	0.17 ^e	0.15	*	*	*
Total CH ₄	9.17 ^a	3.98 ^b	4.38 ^b	3.95 ^b	1.58 ^{cd}	1.33 ^{cd}	3.03 ^{bc}	0.95 ^d	0.81 ^d	0.2	*	*	*
CH ₄ , μM	346.7 ^a	150.6 ^b	165.4 ^b	149.4 ^b	59.8 ^d	50.1 ^d	114.7 ^c	35.9 ^e	30.5 ^e	13.8	*	*	*
CH ₄ , mL/g DM	18.3 ^a	7.9 ^b	8.7 ^b	7.9 ^b	3.1 ^{cd}	2.6 ^{cd}	6.0 ^{bc}	1.9 ^d	1.6 ^d	0.5	*	*	*
CH ₄ , mL/g DMD ²	38.4 ^a	20.6 ^c	17.3 ^c	31.6 ^b	4.5 ^e	3.3 ^e	11.4 ^d	3.1 ^e	4.3 ^e	0.7	*	*	*
N ₂ O, mL × 10 ⁻⁴	1.2 ^c	1.2 ^c	1.2 ^c	12.6 ^b	17.0 ^b	16.7 ^b	23.3 ^a	26.2 ^a	25.1 ^a	0.8	*	NS	NS

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹NS = non significant ($P > 0.10$).

²DMD = DM digestibility.

* $P < 0.05$.

Statistical Analysis

All statistical analyses used the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC), using bottle as the experimental unit. Means for all variables were obtained using the LSMEANS statement. The model was as follows:

$$y_{ij} = \mu + \beta_i + T_j + e_{ij},$$

in which μ is the overall mean, β is the fixed effect of the i th bottle ($\beta = 45$ for gas variables, pH, VFA, and fermentation balance parameters and 27 for IVDMD), T is the fixed effect of the j th treatment ($T = 1$ to 9), and e is the residual term [$e_{ij} \sim N(0, \Sigma)$]. A level of 0.05 was used to determine significance, with tendencies associated with P -values between 0.05 and 0.10.

RESULTS

Experiment 1 (100:0 Roughage:Concentrate Ratio)

The addition of NIT and MON reduced total gas production over the 48-h incubation compared with the control (87.9 and 78.6 vs. 94.6 mL, respectively; $P < 0.01$; Table 2). Compared with the control, NIT and MON significantly reduced CH₄ production (mL,

mL/g DM, and mL/g DM digestibility; $P < 0.01$). The combination of NIT + MON further reduced CH₄ compared with the control (1.1 vs. 9.1 mL; $P < 0.01$). A significant increase in N₂O production was observed when NIT was added ($P < 0.01$). There was no effect of NIT and MON on IVDMD compared with the control ($P > 0.10$); however, the combination of both additives at higher doses reduced IVDMD compared with the control (31.7 vs. 37.5%; $P < 0.01$). Treatments had no effect ($P > 0.10$) on pH levels.

Total VFA production tended to decrease due to MON (46.8 vs. 48.5; $P = 0.08$; Table 3). Molar proportions of acetate and butyrate were reduced by MON ($P < 0.01$) whereas propionate was increased ($P < 0.01$). The acetate:propionate (A:P) ratio was reduced by MON compared with the control (1.2 vs. 2.8; $P < 0.01$) and tended to increase due to NIT ($P = 0.06$). Isobutyrate and isovalerate were not affected by treatments in Exp. 1 ($P > 0.10$), but valerate was reduced by MON (1.7 vs. 2.5 mol/100 mol; $P < 0.01$) and increased by NIT (2.9 vs. 2.5 mol/100 mol; $P < 0.05$).

The metabolic H₂ balance, especially H₂ recovery, was affected by both NIT and MON and reflected mainly the decrease in CH₄ output by NIT and the change in A:P ratio caused by MON addition. Total

Table 3. Effect of calcium nitrate (NIT; 0, 1.25, and 2.5 g/100 g of DM), monensin sodium (MON; 0, 3, and 6 mg/L), and their combination on in vitro VFA profile and fermentation balance in Exp. 1 (100:0 roughage:concentrate ratio)

Item	0 g NIT/100 g DM			1.25 g NIT/100 g DM			2.5 g NIT/100 g DM			Pooled SEM	P-value		
	0 mg MON/L	3 mg MON/L	6 mg MON/L	0 mg MON/L	3 mg MON/L	6 mg MON/L	0 mg MON/L	3 mg MON/L	6 mg MON/L		NIT	MON	NIT × MON
Total VFA, ¹ mM	48.5 ^a	47.5 ^{ab}	46.1 ^{ab}	49.2 ^a	46.4 ^b	45.3 ^b	47.1 ^{ab}	45.9 ^b	44.6 ^b	4.6	NS ²	0.08	NS
VFA composition, mol/100 mol													
Acetate	62.6 ^a	48.6 ^b	48.2 ^b	61.4 ^a	49.3 ^b	48.2 ^b	63.3 ^a	48.5 ^b	49.3 ^b	4.7	NS	*	NS
Propionate	22.5 ^b	40.2 ^a	40.5 ^a	21.1 ^b	39.4 ^a	40.8 ^a	18.6 ^b	41.5 ^a	39.4 ^a	2.1	NS	*	NS
Isobutyrate	1.4	1.2	1.5	1.6	1.1	1.0	1.7	1.4	1.4	0.8	NS	NS	NS
Butyrate	9.2 ^b	5.8 ^c	6.1 ^c	10.4 ^a	6.3 ^c	5.8 ^c	10.6 ^a	5.1 ^c	4.7 ^c	0.3	NS	*	NS
Isovalerate	0.9	1.2	1.1	0.8	0.6	0.7	1.2	1.1	0.8	0.06	NS	NS	NS
Valerate	2.5	1.8	1.5	2.8	2.1	2.2	3.1	1.5	2.7	0.5	*	*	NS
Caproate	1.2	1.5	1.4	2.1	1.3	1.4	2.2	1.2	1.4	0.1	†	NS	NS
Acetate:propionate ratio	2.8 ^a	1.2 ^b	1.1 ^b	2.9 ^a	1.2 ^b	1.1 ^b	3.4 ^a	1.1 ^b	1.2 ^b	0.05	†	*	NS
Metabolic hydrogen balance, ³ µmol/mL of fermentation media													
Produced	191.1 ^a	166.6 ^b	166.2 ^b	192.7 ^a	168.6 ^b	166.2 ^b	196.2 ^a	164.1 ^b	163.8 ^b	12.1	NS	*	NS
Utilized	102.3 ^{ab}	109.4 ^a	111.9 ^a	81.4 ^c	99.5 ^b	100.6 ^b	73.5 ^d	98.3 ^b	94.1 ^b	7.3	*	*	*
Recovered, %	53.5 ^{bc}	65.6 ^a	67.3 ^a	42.4 ^d	59.0 ^b	60.5 ^{ab}	37.4 ^d	59.9 ^b	57.4 ^b	6.4	*	*	*
Hexose fermented	27.1	26.0	25.9	27.2	26.3	26.2	27.3	25.8	25.8	2.5	NS	NS	NS
Fermentation efficiency, %	70.9 ^b	78.7 ^a	79.0 ^a	69.7 ^b	78.6 ^a	79.1 ^a	68.4 ^b	79.4 ^a	77.4 ^a	6.5	NS	*	NS

^{a-d}Means within a row with different superscripts differ ($P < 0.05$).

¹VFA values reflect net production.

²NS = non significant; ($P > 0.10$).

³Stoichiometric calculations were based on balances applied to rumen fermentation (Wolin, 1960; Demeyer and Tamminga, 1987; Demeyer, 1991).

† $P < 0.1$; * $P < 0.05$.

recovered H_2 was significantly reduced by NIT compared with the control (39.9 vs. 53.5%; $P < 0.01$; Table 3) and increased by MON (66.4 vs. 53.5%; $P < 0.01$) and the combination of the additives (59.2 vs. 53.5%; $P < 0.01$). No effect of treatments was found on total hexose fermented ($P > 0.10$), but fermentation efficiency was increased by MON compared with the control (78.8 vs. 70.9%, respectively; $P < 0.01$).

Experiment 2 (10:90 Roughage:Concentrate Ratio)

Total gas production (mL), compared with the control, was reduced by NIT (181.7 vs. 196.9 mL; $P < 0.01$), MON (168.8 vs. 196.9 mL; $P < 0.01$) and further reduced by NIT + MON (161.2 vs. 196.9; $P < 0.01$; Table 4). Compared with the control, CH_4 (mL, mL/g DM, and mL/g DM digestibility) was decreased by NIT (8.3 vs. 15.3 mL; $P < 0.01$) and MON (7.6 vs. 15.3 mL; $P < 0.01$) and further reduced when the additives were combined (1.5 vs. 15.3 mL; $P < 0.01$). The addition of NIT increased N_2O production ($P < 0.01$) and the combination of NIT and MON further increased N_2O production ($P < 0.01$). The IVDMD, similarly to Exp. 1, was not affected by NIT or MON ($P > 0.10$) but was reduced when the additives were combined at higher doses (76.7 vs. 79.9%; $P < 0.01$). No treatment effect on pH levels ($P > 0.10$) was detected.

Total VFA production tended to decrease by NIT addition ($P = 0.07$), but no effect was found for MON or NIT + MON (Table 5). The molar proportion of acetate and butyrate was reduced by MON ($P < 0.01$), but only acetate was further reduced by the combination of the additives ($P < 0.01$), whereas butyrate was increased by NIT ($P < 0.01$). Propionate molar ratio was increased by MON compared with the control (43.2 vs. 23.8 mol/100 mol; $P < 0.01$) and further increased by NIT + MON (46.6 vs. 23.8 mol/100 mol; $P < 0.01$). Due to additives' effect on acetate and propionate molar proportions, A:P ratio was further reduced by NIT + MON ($P < 0.01$). Isovalerate and isobutyrate molar concentration was not affected by treatments ($P > 0.10$), but valerate was increased by NIT ($P < 0.01$) and reduced by MON ($P < 0.01$) and NIT + MON ($P < 0.01$). Caproate was significantly reduced by MON (0.4 vs. 2.3 mol/100 mol; $P < 0.01$) and NIT + MON (0.9 vs. 2.3 mol/100 mol; $P < 0.01$).

Additives affected H_2 balance; NIT and NIT + MON significantly reduced H_2 recovery compared with the control (58.5 and 68.5 vs. 74.1%, respectively; $P < 0.01$) and MON increased H_2 recovery (77.5 vs. 74.1%; $P < 0.01$). Metabolic H_2 production was reduced by MON ($P < 0.01$) and H_2 utilized was reduced by NIT and MON compared with the control ($P < 0.01$). Total hexose fermented was increased by NIT at the lower dose (45.2 vs. 41.1 µmol/mL; $P <$

0.01) and reduced by the combination of the additives (37.8 vs. 41.1 $\mu\text{mol/mL}$; $P = 0.02$). Fermentation efficiency was significantly increased by MON compared with the control (84.6 vs. 76.6%; $P < 0.01$), and a tendency for further increase was detected when the additives were combined (86.3 vs. 76.6%; $P = 0.07$).

DISCUSSION

Methane, N_2O , and VFA Production

The theoretical potential benefit of NIT and MON to interfere with the ruminal H_2 pool and decrease CH_4 production was confirmed in vitro by our studies. Lower and higher doses of NIT reduced CH_4 production compared with the control by 57 and 67% in Exp. 1, respectively, and by 54 and 71% in Exp. 2, respectively. The decrease in CH_4 found in both experiment can be explained by the effect of the additives on rumen metabolism. Nitrate reduction to NH_3 competes with methanogens in ruminal fluid for H_2 generated during fermentation of carbohydrates. When NIT is reduced to nitrite (NO_2) and then to NH_3 , the process captures 4 mol of H_2 per mole of NIT reduced (Asanuma et al., 2002). Thermodynamically, both reactions involved in NIT reduction to NH_3 are energetically more favorable than reduction of CO_2 by methanogens (Ungerfeld and Kohn, 2006). Decreased CH_4 formation as a result of NIT supplementation has been studied both in vitro (Guo et al., 2009; Patra and Yu, 2014) and in vivo (van Zijderveld et al., 2011; Newbold et al., 2014), suggesting a consistent effect of NIT as a CH_4 mitigation strategy. Moreover, additive CH_4 mitigation effects of NIT with other nutritional strategies that affect the ruminal H_2 pool, such as supplementation with lipids (Guyader et al., 2015), sulfate (van Zijderveld et al., 2010), and saponin (Patra and Yu, 2014), have been reported in the literature. In our trials, assuming that all NIT added was reduced to NH_3 , the CH_4 mitigation observed was explained by the NIT reduction pathway by 67 and 68% in Exp. 1 and Exp. 2, respectively (actual CH_4 mitigation in moles/theoretical mitigation potential, considering that 1 mol of NIT should decrease 1 mol of CH_4 and assuming all NIT was reduced to NH_3). This suggests that NIT or its reduction intermediates may affect the rumen metabolism and methanogenesis by an alternative mechanism.

A possible alternative explanation for the effect of NIT on ruminal CH_4 decrease is that intermediates of NIT metabolism (NO_2 , nitric oxide [NO], and N_2O) can be toxic to rumen microorganisms. When NIT in the form of a potassium salt (KNO_3) was fed to goats at 6 and 9 g/d, the total number of methanogens and protozoa copy numbers estimated by real-time PCR was

significantly reduced (Asanuma et al., 2015). Protozoa are known to be important H_2 producers, and their decrease is often associated with a decrease in methanogenesis (Williams and Coleman, 1992; Morgavi et al., 2012). Feeding lactating cows 3% NIT (CaNO_3) in diet DM with or without linseed oil caused an increase in rumen dissolved H_2 up to 2 h after feeding (Guyader et al., 2015), which, according to the authors, could be caused by a toxic effect of NIT or its intermediates on H_2 utilizers such as methanogens.

Kaspar and Tiedje (1981) reported that in the rumen ecosystem, NIT is metabolized mainly by dissimilatory NIT reduction to ammonium, but depending on the balance of enzyme activities, N_2O can be formed through the denitrification pathway, which would involve the reduction of NIT to NO_2 followed by the conversion of NO_2 to N_2O or N_2 or a mixture of the 2 gases. Because the rumen inoculum used in our trials were derived from animals not adapted to NIT, it is possible that NO_2 may have accumulated in the system and, instead of being reduced to NH_3 , it diverged to the denitrification pathway, which is the primary source of N_2O in anaerobic conditions (Sorensen, 1978; Oremland et al., 1984; King and Nedwell, 1985). Wang (2012) also observed significant increases in N_2O levels when NIT was added at 2 and 3% of diet DM during 24-h in vitro incubations of ruminal mixed microorganisms. Increased N_2O production was also reported in vivo when NIT was supplemented to sheep at 2% of diet DM (de Raphélis-Soissan et al., 2014).

An interaction between NIT and MON resulted in higher levels of N_2O when the additives were combined in Exp. 2 (up to 75 times greater than the control). However, studies have shown that MON does not affect major species of NIT reducers in the rumen. *Selenomonas ruminantium* is a representative NIT- and nitrite-reducing ruminal bacterium present at much higher numbers than other nitrate and nitrite reducers, such as *Veillonella parvula* and *Wolinella succinogenes* (Asanuma et al., 2002; Iwamoto et al., 2002). However, concentrations of MON as high as 40 mg/L did not affect growth of pure cultures of *S. ruminantium*, and increases in propionate production commonly found in MON-fed animals may be related to selection of *Bacteroides* and *S. ruminantium* strains, given the ability of these bacteria to resist higher levels of MON (Chen and Wolin, 1979). *Veillonella parvula* required 14.3 mg/L of culture media of MON to have growth inhibited in pure cultures not adapted to MON (Newbold et al., 1993). This level was more than 2 times our highest dose of MON tested. However, it is possible that variations in animal microbial populations from inoculum donors (Kocherginskaya et al., 2001) could result in strains more susceptible to MON, and if MON at the levels tested in our experi-

Table 4. Effect of calcium nitrate (NIT; 0, 1.25, and 2.5 g/100g of DM), monensin sodium (MON; 0, 3, and 6 mg/L), and their combination on in vitro gas and CH₄ production in Exp. 2 (10:90 roughage:concentrate ratio)

Item	0 g NIT/100 g DM			1.25 g NIT/100 g DM			2.5 g NIT/100 g DM			Pooled SEM	P-value		
	0 mg MON/L	3 mg MON/L	6 mg MON/L	0 mg MON/L	3 mg MON/L	6 mg MON/L	0 mg MON/L	3 mg MON/L	6 mg MON/L		NIT	MON	NIT × MON
pH	6.1	6.3	6.3	6.2	6.2	6.4	6.2	6.3	6.3	0.2	NS ¹	NS	NS
IVDMD, %	79.9 ^{ab}	80.4 ^{ab}	81.9 ^{ab}	77.4 ^{bc}	82.1 ^a	79.2 ^{abc}	78.9 ^{abc}	77.2 ^{bc}	76.6 ^c	0.6	NS	NS	*
Gas production, mL													
Gas (0–4 h)	23.54 ^b	18.82 ^c	18.62 ^c	26.09 ^a	22.99 ^b	20.25 ^{bc}	22.89 ^b	23.04 ^b	23.62 ^b	2.3	NS	*	†
Gas (4–8 h)	48.67 ^a	38.21 ^b	38.04 ^b	47.4 ^a	37.83 ^b	36.44 ^b	40.92 ^{bc}	38.57 ^b	41.11 ^c	4.5	*	*	*
Gas (8–12 h)	46.79 ^a	36.21 ^{bc}	34.77 ^c	43.94 ^{ab}	34.97 ^c	31.84 ^c	42.61 ^{ab}	28.44 ^d	28.85 ^d	3.7	*	*	*
Gas (12–24 h)	45.29 ^a	47.51 ^a	44.65 ^a	42.31 ^b	43.47 ^{ab}	42.55 ^b	40.51 ^b	44.8 ^a	38.45 ^c	3.8	*	NS	NS
Gas (12–48 h)	32.64 ^a	29.99 ^{ab}	31.02 ^a	31.05 ^a	24.92 ^c	27.72 ^b	24.82 ^c	27.56 ^b	27.58 ^b	3.0	*	NS	*
Total gas	196.92 ^a	170.74 ^{bc}	167.0 ^{bcd}	190.8 ^a	164.2 ^{bcd}	158.79 ^d	172.76 ^b	162.4 ^{bcd}	159.6 ^{cd}	6.1	*	*	*
CH ₄ production, mL													
CH ₄ (0–4 h)	0.46 ^a	0.29 ^b	0.18 ^c	0.55 ^a	0.21 ^{bc}	0.17 ^c	0.29 ^b	0.18 ^c	0.26 ^b	0.05	*	*	†
CH ₄ (4–8 h)	1.87 ^a	0.77 ^c	0.75 ^c	1.05 ^b	0.17 ^c	0.19 ^c	0.4 ^d	0.22 ^c	0.52 ^d	0.07	*	*	*
CH ₄ (8–12 h)	2.63 ^a	1.42 ^b	1.32 ^b	1.36 ^b	0.17 ^d	0.11 ^d	0.34 ^c	0.18 ^d	0.23 ^d	0.1	*	*	*
CH ₄ (12–24 h)	3.95 ^a	2.39 ^b	2.19 ^b	3.8 ^a	0.5 ^{cd}	0.29 ^d	0.88 ^c	0.21 ^d	0.29 ^d	0.14	*	*	*
CH ₄ (24–48 h)	6.42 ^a	3.42 ^c	2.48 ^d	5.5 ^b	0.8 ^c	0.54 ^c	2.6 ^d	0.49 ^c	0.33 ^c	0.2	*	*	*
Total CH ₄	15.3 ^a	8.3 ^c	6.9 ^c	12.2 ^b	1.8 ^e	1.3 ^e	4.5 ^d	1.2 ^e	1.6 ^e	0.2	*	*	*
CH ₄ , μM	580.1 ^a	314.3 ^c	262.2 ^c	463.8 ^b	70.3 ^e	49.5 ^e	170.9 ^d	48.4 ^c	62.3 ^c	19.2	*	*	*
CH ₄ , mL/g DM	30.7 ^a	16.6 ^c	13.8 ^c	24.5 ^b	3.7 ^e	2.6 ^e	9.0 ^d	2.5 ^c	3.3 ^c	0.5	*	*	*
CH ₄ , mL/g DMD ²	38.4 ^a	20.6 ^c	17.3 ^c	31.6 ^b	4.5 ^e	3.3 ^e	11.4 ^d	3.1 ^c	4.3 ^c	0.7	*	*	*
N ₂ O, mL × 10 ⁻⁴	0.8 ^d	0.7 ^d	0.7 ^d	9.7 ^d	28.5 ^c	30.7 ^{bc}	44 ^b	76.3 ^a	71.5 ^a	2.1	*	*	*

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹NS = non significant; ($P > 0.10$).

²DMD = DM digestibility.

† $P < 0.1$; * $P < 0.05$.

ments inhibited the growth of a NIT or NO₂ reducer, then NO₂ could accumulate and favor the formation of N₂O. Studies to measure NIT reduction intermediates and microbial population dynamics when MON is fed in combination with NIT are encouraged to clarify the role of different species in NIT metabolism in the rumen and potential effect of MON on growth of such species.

Monensin reduced CH₄ production by approximately 55% in Exp. 1 and 50% in Exp. 2. The most consistent and well-documented alteration caused by MON on ruminal fermentation is the increased molar proportion of propionate with a concurrent decrease in the molar proportion of acetate and butyrate. The increase in rumen propionate is accompanied by a decrease in the amount of CH₄ production because MON reduces the number of H₂-generating bacteria (Van Nevel and Demeyer, 1977; Wallace et al., 1981), which is supported by the inability of MON to reduce CH₄ production when H₂ and CO₂ are provided (Russell and Martin, 1984). Moreover, during propionate formation, fumarate is reduced to succinate and 1 mol of H₂ is consumed in this reaction, resulting in a net uptake of H₂ (Ungerfeld and Kohn, 2006). In Exp. 1 and Exp. 2, the addition of MON resulted in a 48 and 46% increase, respectively, in propionate molar proportion compared

with the control, partially explaining the decrease in CH₄ production observed when MON was added.

Hydrogen gradients exist in methanogenic ecosystems (Boone et al., 1989), and in the rumen, a major pathway for propionate synthesis involves the use of H₂ to reduce fumarate to propionate (Ungerfeld and Kohn, 2006). However, kinetic limitations such as availability of H₂ and/or propionate precursors (i.e., fumarate and succinate) may limit propionogenesis because the concentration of substrate (H₂) for half maximum reaction velocity from methanogens is lower than that of 5 major ruminal species of fumarate reducers (Asanuma et al., 1999). It is possible that NIT + MON coupled with a high concentrate diet in Exp. 2 further stimulates propionogenesis by reducing kinetic limitations of propionate formation, such as 1) greater H₂ concentration in liquid and gas phase due to NIT effect (van Zijderveld et al., 2011; Guyader et al., 2015), 2) increased proportion of propionate producing species due to MON effect (Van Nevel and Demeyer, 1977; Wallace et al., 1981), and 3) a diet consisting of 90% concentrate that could have supplied enough propionate precursors.

Table 5. Effect of calcium nitrate (NIT; 0, 1.25, and 2.5 g/100 g of DM), monensin sodium (MON; 0, 3, and 6 mg/L), and their combination on in vitro VFA profile and fermentation balance in Exp. 2 (10:90 roughage:concentrate ratio)

Item	0 g NIT/100 g DM			1.25 g NIT/100 g DM			2.5 g NIT/100 g DM			Pooled SEM	P-value		
	0 mg MON/L	3 mg MON/L	6 mg MON/L	0 mg MON/L	3 mg MON/L	6 mg MON/L	0 mg MON/L	3 mg MON/L	6 mg MON/L		NIT	MON	NIT × MON
Total VFA, ¹ mM	66.7 ^{bc}	71.5 ^a	73.2 ^a	74.3 ^a	65.7 ^c	68.5 ^{bc}	63.2 ^d	68.6 ^{bc}	66.6 ^{bc}	2.2	†	NS ²	NS
VFA composition, mol/100 mol													
Acetate	54.2 ^a	43.6 ^c	44.9 ^c	54.9 ^a	39.2 ^e	38.6 ^c	48.9 ^b	41.4 ^d	42.3 ^d	0.5	*	*	*
Propionate	23.8 ^c	43.4 ^b	43.0 ^b	23.6 ^c	49.4 ^a	47.8 ^a	26.9 ^c	45.8 ^{ab}	43.4 ^b	1.1	*	*	*
Isobutyrate	2.8	3.4	1.6	3.0	0.6	2.4	3.3	1.4	3.5	0.8	NS	†	†
Butyrate	15.2 ^b	7.0 ^d	7.5 ^{cd}	14.7 ^b	7.9 ^c	7.8 ^c	17.4 ^a	8.3 ^c	7.7 ^c	0.3	*	*	NS
Isovalerate	3.1	2.8	2.6	3.0	2.5	2.7	2.5	2.7	2.7	0.1	NS	NS	NS
Valerate	6.6 ^b	4.2 ^c	4.0 ^c	6.2 ^b	4.2 ^c	4.5 ^c	9.1 ^a	4.7 ^c	4.3 ^c	0.3	*	*	*
Caproate	2.3 ^a	0.3 ^c	0.4 ^c	1.4 ^b	0.7 ^c	2.0 ^a	2.1 ^a	0.5 ^c	0.4 ^c	0.07	NS	*	*
Acetate:propionate ratio	2.3 ^a	1.0 ^c	1.0 ^c	2.3 ^a	0.8 ^d	0.8 ^d	1.8 ^b	0.9 ^{cd}	1.0 ^c	0.05	*	*	*
Metabolic hydrogen balance, ³ μmol/mL of fermentation media													
Produced	193.8 ^a	164.9 ^b	167.8 ^b	194.5 ^a	164.2 ^b	158.1 ^c	193.8 ^a	167.0 ^b	164.7 ^b	6.8	NS	*	NS
Utilized	143.7 ^a	130.6 ^b	127.3 ^b	125.8 ^b	119.0 ^c	111.0 ^c	101.9 ^d	110.9 ^c	107.2 ^c	6.1	*	*	*
Recovered, %	74.1 ^a	79.1 ^a	75.9 ^{ab}	64.6 ^c	72.4 ^b	70.2 ^{bc}	52.5 ^d	66.4 ^{bc}	65.0 ^c	3.4	*	*	*
Hexose fermented	41.1 ^b	39.4 ^b	40.9 ^b	45.2 ^a	37.3 ^c	38.3 ^{bc}	40.9 ^b	39.0 ^b	36.7 ^c	4.1	*	NS	*
Fermentation efficiency, %	76.6 ^c	84.8 ^b	84.4 ^b	76.4 ^c	87.3 ^a	87.1 ^a	78.5 ^c	85.9 ^{ab}	85.1 ^{ab}	6.7	NS	*	†

a–d Means within a row with different superscripts differ ($P < 0.05$).

¹VFA values reflect net production.

²NS = non significant; ($P > 0.10$).

³Stoichiometric calculations were based on balances applied to rumen fermentation (Wolin, 1960; Demeyer and Tamminga, 1987; Demeyer, 1991).

† $P < 0.1$; * $P < 0.05$.

Fermentation Balance

Stoichiometric calculations are helpful in describing alterations in fermentation, but they must be interpreted with caution because manipulating fermentation may invoke reactions not considered in the calculations and fermentation uncoupled from cell growth may occur (Chalupa, 1977). It should also be stressed that the approach used in our experiments involves a simplification of very complex systems of feed, animal, and microbial components in a closed, in vitro system. However, extensive empirical studies have been conducted in cocultures of several rumen anaerobic microorganisms and methanogens that validate the use of fermentation balance data to analyze treatment effects (Ørskov et al., 1968; Russell and Hespell, 1981; Demeyer, 1991).

A significant decrease in metabolic H₂ production compared to the control treatment, a significant decrease in metabolic H₂ production compared with the control was observed in EXP1 (166.4 vs. 191.1 μmol/mL) and EXP2 (166.3 vs. 193.8 μmol/mL). Because H₂ utilized was not significantly affected, recovered H₂ was increased by MON. Similar results were found when MON was tested at 0.1, 1, and 5 mg/L in 17-h in vitro incubations (Richardson et al., 1976). On the other hand, NIT reduced H₂ recovery compared with

the control because NIT did not affect production of major VFA although it significantly reduced utilization of metabolic H₂ through CH₄ formation.

In rumen studies, fermentation efficiency can be defined as a measure of the energy present in a fermentable substrate (i.e., hexoses and AA) that is recovered in usable forms, such as VFA and microbial protein, to the host animal. In this study, fermentation efficiency was increased by MON addition and tended to further increase in Exp. 2 due to the combination of the additives at lower doses. Production of propionate is energetically more efficient than production of other major VFA. Because of differences in the production and utilization of metabolic H₂, the efficiencies of fermenting hexose to acetate, propionate, and butyrate are 62, 109, and 78%, respectively (Ørskov and Ryle, 1990). Theoretically, if 1 mol of hexose is fermented to 2 mol of propionate, then no H₂ and CO₂ are lost (Wolin et al., 1997), whereas in the same process, 1 mol of H₂ is incorporated during propionate formation, as discussed before. Therefore, treatments that enhance propionate, and, to a lesser extent, butyrate, at the expense of acetate are likely to increase fermentation efficiency.

In Vitro Dry Matter Disappearance

A significant effect of treatments compared with the control on IVDMD was detected only when NIT + MON were combined at higher doses in Exp. 1 (31.7 vs. 37.5%) and Exp. 2 (76.6 vs. 79.9%). The lack of effect of NIT on IVDMD is consistent with results found by Patra and Yu (2014), when NIT was added at 5 mM to rumen batch culture containing alfalfa hay and dairy concentrate feed (50:50) as substrate (higher and lower doses in our trials resulted in 4 and 2 mM of NIT, respectively). Similarly, Marais et al. (1988) observed that NIT (as KNO_3) affected the rate but not the extent of digestion in a 72-h fermentation, whereas NO_2 significantly reduced IVDMD and cellulolytic and xylanolytic microbial growth rates. In the latter study, the effect of NIT on digestion rate was suggested to be linked with accumulation of NO_2 in the media, which peaked at about 18 h. Therefore, NO_2 is likely the primary factor reducing digestibility of diets high in NIT. Adaptation of rumen microbes to NIT presence increases the activity of species able to reduce NIT to NH_3 , reducing negative effects of ruminal NO_2 accumulation on animal health and diet digestibility caused in vivo by NO_2 absorption into the bloodstream (Lee and Beauchemin, 2014) and by NO_2 effect on growth of major cellulolytic bacteria (Marais et al., 1988; Zhou et al., 2012), respectively.

The absence of effect of MON on IVDMD in Exp. 1 (39.5 vs. 37.5%) and Exp. 2 (81.1 vs. 79.9) is consistent with results of Duff et al. (1995), who found no effect of MON at 4 mg/L in 24-h IVDMD of a 90% concentrate substrate. Similarly, when studying the effect of MON (5 mg/L) on substrates containing different levels of S (0.2 to 0.8% of DM), no effect on IVDMD was detected by Quinn et al. (2009) and Smith et al. (2010). Van Nevel and Demeyer (1977) reported no effect of MON (5 mg/L) on the quantity of substrate fermented despite a decrease in microbial growth, suggesting that MON uncouples microbial growth from fermentation. Although we did not measure microbial growth, total VFA production (mM) was not affected by MON on Exp. 1 (46.8 vs. 48.5 mM) and Exp. 2 (72.3 vs. 66.7 mM), suggesting that total microbial growth was not affected, even though specific specie populations were likely changed (e.g., inhibition of acetate-producing bacteria and protozoa).

The Potential to Use Nitrate as a Feed Additive for Ruminants

Nitrates may be present in the diet of ruminants in a continuous basis, usually in the form of nitrate salts and ammonium nitrate. The most common source are forages with cultivars from the perennial ryegrass

and sorghum family (sorghum, sudan, pearl millet, and their crosses; Leng and Preston, 2010), and factors affecting NIT concentration in forage have been well demonstrated by Leng (2008) and include forage maturity, soil conditions (e.g., moisture content), and application of fertilizers to soil. The majority of research conducted in the past focused on understanding the metabolism of NIT in the rumen to avoid poisoning, which is caused when the rumen microbes are not adapted to NIT and NO_2 accumulates in the rumen, ultimately being absorbed into the blood (Lee and Beauchemin, 2014). However, recent studies have shown the potential of NIT to consistently reduce CH_4 levels (see previous discussion on CH_4) without resulting in toxicity. In addition, NIT serves as a valuable source of NPN to microorganisms, with recent studies reporting that NIT may increase microbial N output in isonitrogenous diets compared with urea (Guo et al., 2009; Guyader et al., 2017). An encapsulated form of NIT was also developed (El-Zaiat et al., 2014) to control the release rate of NIT in the rumen, allowing the microbes to adapt to higher NIT levels and therefore reducing potential toxicity in commercial sets. Despite the consistent results showing the benefits of supplementing ruminants with NIT as a CH_4 mitigation strategy and as a source of NPN, the authors are not aware of any regulations or recommendations for producers in the United States to use NIT as a feed additive.

A potential model to be used in the United States is the case of Australia, where carbon credits are now available for managed feeding of NIT for grazing ruminants (Department of Environment, Australian Government, 2015). Acceptance of NIT feeding by producers as a means of gaining carbon credits from reduced CH_4 output by their livestock will depend on whether they can achieve the nutritional benefits of NPN supplementation using NIT at a cost similar to that of urea supplementation and with a minimal risk of NO_2 poisoning or other adverse effects on animal production (Nolan et al., 2016). Because MON is a widely used additive in the United States, especially in finishing beef cattle, more in vivo studies looking at the interaction of both additives are needed before further recommending their combination.

Conclusion

Overall, the combination of NIT + MON in 2 contrasting diets showed beneficial effects on ruminal fermentation in vitro by reducing CH_4 formation while not affecting IVDMD and VFA production. However, NIT increased N_2O production, which partially offset the benefit of CH_4 decrease on the total greenhouse gas balance. The VFA profile was mainly affected by MON

presence as a consequence of its effect on ruminal microbiota, which favors more efficient fermentation pathways. The combination of the additives at lower doses further increased propionate levels in a high-concentrate diet, demonstrating a potential capacity of propionogenesis to take up additional H_2 present in ruminal fluid and convert it into a highly reduced VFA. The combination of NIT and MON may serve as a dietary means of reducing CH_4 generated during ruminants' foregut fermentation. More studies are encouraged to better understand the effect of the combination of the additives in ruminal fermentation, including the effect on NIT reduction intermediates, animal performance, and metabolism.

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