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Combination Effects of Nitrocompounds, Pyromellitic Diimide, and 2-Bromoethanesulfonate on in Vitro Ruminal Methane Production and Fermentation of a Grain-Rich Feed

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ABSTRACT: An L_{16} (4^5) orthogonal experimental design was used to evaluate combination effects of nitroethane (0−15 mM), 2-nitroethanol (0−15 mM), 2-nitro-1-propanol (0−15 mM), pyromellitic diimide (0−0.07 mM), and 2-bromoethanesulfonate (0−0.05 mM) on in vitro ruminal fermentation of a grain-rich feed. In vitro dry matter disappearance was adversely affected by these inhibitors, while cumulative gas production was not affected. Volatile fatty acid production was increased by nitroethane and 2-bromoethanesulfonate in a dose-dependent manner and was decreased by 2-nitroethanol and pyromellitic diimide. All inhibitor treatments increased the molar acetate proportion, while decreasing proportions of propionate and butyrate; hydrogen recovery was decreased by 36.9−45.2%; and methane production was reduced by 95.2−99.2%. The methanogenesis inhibition ranked: nitroethane > 2-nitroethanol > 2-nitro-1-propanol > 2-bromoethanesulfonate > pyromellitic diimide; combined concentrations of 5, 5, 5, 0.02, and 0.03 mM, respectively, gave the optimal inhibiting efficiency. These results may provide a reference to develop effective mitigation of methane emission from ruminants.

KEYWORDS: *rumen, nitrocompounds, cumulative gas production, methane, volatile fatty acids, batch culture*

INTRODUCTION

Methane eructation from ruminants represents a loss of 2−15% of gross energy intake. At the same time, methane release to the atmosphere contributes to global warming.[1] For these reasons, and in response to the growing concern for the global environment, recent research in animal science is now focusing on the reduction of overall methane emissions. Strategies for reducing methane emissions from ruminants involve altering the patterns of rumen fermentation.[2] One strategy is to provide feed additives, such as nitrocompounds and halogenated compounds, that directly inhibit the growth of methanogens or that suppress the biochemical reactions involved in the production of methane.[3] Nitrocompounds such as nitroethane, 2-nitroethanol, and 2-nitro-1-propanol inhibit ruminal methanogenesis by as much as 90% in vitro at a dose level of 12 mM.[4−6] Pyromellitic diimide also decreases the amount of methane formed in vitro by more than 90% at the level of 10 ppm,[1,7] while 2-bromoethanesulfonic acid, a structural analogue of coenzyme M, is a specific methanogen inhibitor,[1,8] and 2-bromoethanesulfonic acid in in vitro studies could reduce 76−94% of methane production ranging from the level of 0.01−0.05 mM.[1,7,9]

Orthogonal experimental design by the Taguchi method is one way to qualitatively analyze the correlations among the relevant variables at different levels, which enable us to get the parameters optimized, to achieve the predetermined features, and to uncover the statistic principle based on the hidden or equivocal factors. Our previous study[10] with a hay-rich fermentation substrate showed a reduction in methane production of 81.4−99.1%; and the optimized combination was nitroethane, 2-nitroethanol, 2-nitro-1-propanol, pyromellitic diimide, and 2-bromoethanesulfonic acid, at concentrations of 15, 10, 5, 0.07, and 0.01 mM, respectively, based on methane inhibition and total volatile fatty acid production. However, the type of diet could affect the methane production, as the structural carbohydrate gave rise to more acetate and butyrate in the course of digestion, resulting in the production of more H_2, a substrate for methane synthesis.[11] The objective of the present study was to study the combination effects of these inhibitors on ruminal methane production and fermentation characteristics, and to identify the optimal in vitro combination of these inhibitors under the supply of a starch-rich substrate.

MATERIALS AND METHODS

Substrate. Chinese wild ryegrass (*Leymus chinensis*) hay at the late-bloom stage was harvested in the Jilin province of China, and representative hay samples were chopped into 2−5 mm lengths, dried at 65 °C overnight in a forced air oven, ground in a Wiley mill to pass a 2.0-mm sieve, mixed, and stored prior to in vitro batch culture trials. Samples of the hay and maize meal, stored in our laboratory, were analyzed following the standard methods[12] for dry matter (ID 930.5), crude protein (ID 984.13), ether extract (ID 920.30), and ash (ID 942.05). Neutral detergent and acid detergent fibers were analyzed[13] and expressed without residual ash. Nonfiber carbohydrate content of the samples was calculated by subtracting neutral detergent fiber, crude protein, ether extract, and ash contents from the dry matter content of the feeds. Representative samples of the hay and the maize meal were mixed (1:4) to prepare a grain-rich substrate for later in vitro batch

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culture. The chemical compositions (per kg dry matter) of the substrate were 99.1 g of crude protein, 241.5 g of neutral detergent fiber, 87.3 g of acid detergent fiber, 542.6 g of nonfiber carbohydrate, and 26.3 g of ash.

Experimental Design and in Vitro Batch Culture. The media were prepared according to the description of Menke and Steingass.[14] The buffer was bubbled with CO_2 until saturated, and the pH was adjusted to 6.8 before being used. A 0.5 g sample of the grain-rich substrate was used for in vitro fermentation. The selected inhibitors, nitroethane (Alfa Aesar, Heysham, UK), 2-nitroethanol (Sigma Aldrich, St. Louis, USA), 2-nitro-1-propanol (Sigma Aldrich), pyromellitic diimide (Alfa Aesar), and 2-bromoethanesulfonic acid (Acros Organics, New Jersey, USA), were added according to an L_{16} (4^5) orthogonal table (Tables 1−6) to 512 bottles which contained 25 mL rumen liquor and 50 mL media. Structures of these inhibitors are shown in Figure 1. Three Holstein dairy cows (550 ± 25 kg) fed 3.3 kg

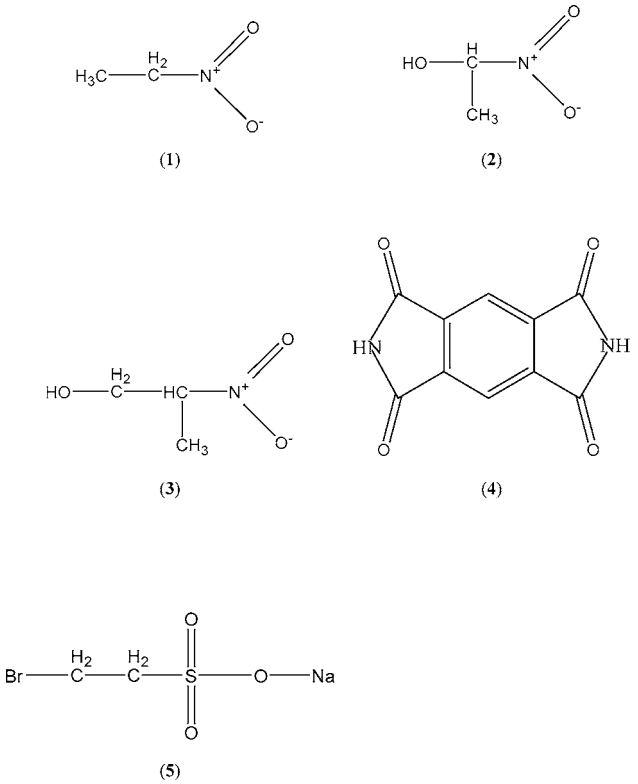

Figure 1. Chemical structures of nitroethane (**1**), 2-nitroethanol (**2**), 2-nitro-1-propanol (**3**), pyromellitic diimide (**4**), and 2-bromoethanesulfonate (**5**).

of maize silage, 3.5 kg of Chinese wildrye grass hay, 2.5 kg of alfalfa hay, and 8.7 kg of commercial concentrate supplement per day (dry matter basis) were chosen as the rumen fluid donators. The rumen liquor was collected 2 h after morning feeding from rumen fistula, the pH was determined, and the values were between 6.3−6.5. It was then filtered through four layers of cheesecloth under N_2 in a water bath at 39 °C. All bottles were arranged at random into 4 runs and incubated at 39 °C for 48 h. In each run, treatments were replicated 8 times. Four replicates of each treatment were connected to the gas channel inlets of an automated gas production recording system (AGRS, Beijing, China)[10] to determine cumulative gas production against the incubation time. The others were connected to pre-emptied airbags to collect the whole fermentation gas for later gas composition analysis of H_2, methane, and CO_2. Four substrate-free bottles, filled with 25 mL of rumen fluids and 50 mL of media, served as blanks in each run.

After the incubation, a 1.0 mL gas sample was removed from the airbags in the manually operated system, and gas composition of methane, CO_2, and H_2 was analyzed by a gas chromatographic method.[10]

The pH values in culture fluids from all 512 bottles were measured, and 1.0 mL of samples were mixed with 0.3 mL of 25 mg/mL *meta*-phosphoric acid solution for 30 min at 4 °C and centrifuged at 10,000*g* for 15 min at 4 °C. Concentrations of volatile fatty acids in the supernatants were measured by a gas chromatographic method[15] using 2-ethylbutyric acid (Sigma Aldrich) as the internal standard. The remaining incubation mixtures in all bottles were transferred to preweighed centrifuge tubes and centrifuged at 1,000*g* for 10 min at room temperature.[16] The pellets were dried at 105 °C to a constant weight, and in vitro dry matter disappearance (IVDMD) was calculated as the dry matter loss, represented as the difference between the original incubated dry matter and the residual dry matter, corrected by blanks.

Biometric Analysis. Concentrations of acetate, propionate, butyrate, valerate, iso-butyrate, and iso-valerate were summed to give a total volatile fatty acid concentration. After incubation, total gas production, net methane production, and total volatile fatty acid yield per g fermented dry matter were calculated by total cumulative gas production, methane production, and total volatile fatty acid production, respectively, divided by the dry matter loss and corrected by blanks.

The ratio of nonglucogenic to glucogenic acids (NGR)[17] was calculated as shown below:

$$\mathrm{NGR} = \frac{\text{acetate} + 2 \times \text{butyrate} + \text{valerate}}{\text{propionate} + \text{valerate}}$$

Hydrogen recovery (2Hrec)[18] was calculated as follows:

$$\begin{aligned}\mathrm{2Hrec} = {} & (2 \times \text{propionate} + 2 \times \text{butyrate} \\ & + 4 \times \text{methane} + \text{hydrogen}) \\ & /(2 \times \text{acetate} + \text{propionate} + 4 \times \text{butyrate})\end{aligned}$$

Volatile fatty acids in these equations were expressed in molar proportions (mmol/mol) of total volatile fatty acid production. Methane and hydrogen in the second equation were expressed as molar proportions of total gas production (mmol/mol).

Effects of 5 inhibitors at 4 dose levels on the IVDMD, total gas production, total volatile fatty acid yield, NGR, net methane production, and 2Hrec were investigated according to an L_{16} (4^5) orthogonal design. The boundary values for the levels were set, based on the results in the literature, to ensure inhibitory effects. Data were collected for 16 treatment combinations at 4 separate incubations (runs). In total, 16 (treatments) × 8 (replicates) × 4 (runs) = 512 bottles were used, and each was considered as a statistical unit. Dose level effects of nitroethane, 2-nitroethanol, 2-nitro-1-propanol, pyromellitic diimide, or 2-bromoethanesulfonic acid were included as fixed effects in the model using the ANOVA procedure. The model applied was

$$Y_{ij} = \mu + R_i + I_j + \varepsilon_{ij}$$

where Y_{ij} is the dependent variable, μ is the overall mean, R_i is the run effect of ($i = 4$), I_j is the dose level effect ($j = 4$; I−IV), and ε_{ij} is the error term.

The responses of IVDMD, total gas production, total volatile fatty acid yield, NGR, net methane production, and 2Hrec to 5 dose levels were averages for each dose level (I−IV) and inhibitor (Tables 1−6). In the case of, e.g., 2-bromoethanesulfonic acid at level IV, combinations 2, 7, 9, and 16 were averaged. Least square means of the averaged variables of 16 combination treatments and standard errors of least-squares means were calculated. The partial correlation coefficients and P values between inhibitors and fermentation parameters were tested by the CORR program. Significance was declared at $P < 0.05$ unless otherwise noted.

RESULTS

In Vitro Dry Matter Disappearance and Total Gas. As shown in Table 1, the IVDMD was decreased by 11.1−25.3% by the addition of inhibitors ($P < 0.01$). The inhibitor combination

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had a minimal impact on IVDMD ($P < 0.05$) when nitroethane, 2-nitroethanol, 2-nitro-1-propanol, pyromellitic diimide, and 2-bromoethanesulfonic acid were supplied at 5, 5, 5, 0.02, and 0.05 mM, respectively. Table 2 shows that total gas production was not affected by any inhibitor.

Total Volatile Fatty Acid Production and Its Patterns. Total volatile fatty acid yield was positively affected by nitroethane and 2-bromoethanesulfonic acid in a dose-dependent manner and reached the highest yield for nitroethane and 2-bromoethanesulfonic acid 10 and 0.03 mM, respectively, and dropped with further increases in nitroethane and 2-bromoethanesulfonic acid. However, it was negatively affected by 2-nitroethanol and pyromellitic diimide ($P < 0.01$) and was not affected by the addition of 2-nitro-1-propanol. Total volatile fatty acid yield was increased when nitroethane, 2-nitroethanol, 2-nitro-1-propanol, pyromellitic diimide, and 2-bromoethanesulfonic acid were supplied at 5, 5, 5, 0.02, and 0.03 mM, respectively.

The volatile fatty acid production patterns were also shifted by the addition of inhibitors. Molar proportions of acetate were decreased by 8.0−14.1%, while molar proportions of propionate and butyrate were increased by 0.6−24.7% and 5.8−36.5%, respectively (data not shown). Addition of nitroethane reduced the molar proportion of acetate and raised that of propionate and butyrate ($P < 0.01$); 2-nitroethanol notably increased the molar proportion of acetate and decreased the molar proportion propionate ($P < 0.05$); 2-nitro-1-propanol markedly reduced the molar proportion of acetate and raised the molar proportion of propionate ($P < 0.01$); pyromellitic diimide had no effect on volatile fatty acid distributions; and 2-bromoethanesulfonic acid reduced the molar proportion of acetate and increased the molar proportion of propionate ($P < 0.05$). The NGR levels were reduced by nitroethane ($P < 0.01$) and pyromellitic diimide ($P < 0.05$) but were not affected by 2-nitroethanol, 2-nitro-1-propanol or 2-bromoethanesulfonic acid.

Methane Production and Hydrogen Recovery. The effects of inhibitors on net methane production and 2Hrec are shown in Tables 5−6, respectively. Net methane production was observed to be decreased by 95.2−99.2% with the addition of inhibitors compared to the control value. A 36.9−45.2% reduction was seen in 2Hrec compared to the control value ($P < 0.01$).

Net methane production was negatively correlated with the addition of nitroethane, 2-nitroethanol, 2-nitro-1-propanol, pyromellitic diimide, and 2-bromoethanesulfonic acid, with coefficients of −0.329, −0.318, −0.330, −0.302, and −0.309, respectively ($P < 0.05$); it was reduced significantly at the lowest level of each inhibitor and did not show further declines with increasing dose levels. A similar pattern was observed for 2Hrec. Hydrogen was accumulated to 3.2−5.5 μmol/mL incubation fluid with inhibitors and was affected by all of them ($P < 0.01$, data not shown).

For the control of net methane production, IVDMD, and total volatile fatty acid yield, the optimal combination of inhibitors was 5 mM nitroethane, 5 mM 2-nitroethanol, 5 mM 2-nitro-1-propanol, 0.02 mM pyromellitic diimide, and 0.03 mM 2-bromoethanesulfonic acid.

DISCUSSION

Total Gas and Methane Production. Methane production was inhibited by more than 95% in all incubations containing inhibitors, and the highest inhibition was 99.2%, of

Table 1. In Vitro Dry Matter Disappearance Affected by Nitroethane (1), 2-Nitroethanol (2), 2-Nitro-1-propanol (3), Pyromellitic Diimide (4), and 2-Bromoethanesulfonate (5) in an L_{16} (4^5) Orthogonal Test

	dose (mM) combination of different inhibitors																mean IVDMD[f]					
	1		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	I[a]	II[b]	III[c]	IV[d]	R[e]	*P*-values
1	0		0	0	0	5	5	5	5	10	10	10	10	15	15	15	15	0.69	0.66	0.64	0.64	0.05	[g]
2	0		5	10	15	0	5	10	15	0	5	10	15	0	5	10	15	0.70	0.67	0.65	0.61	0.06	[g]
3	0		5	10	15	5	0	15	10	10	15	0	5	15	10	5	0	0.70	0.67	0.64	0.62	0.05	[g]
4	0		0.02	0.05	0.07	0.05	0.07	0	0.02	0.07	0.05	0.02	0	0.02	0	0.07	0.05	0.67	0.65	0.65	0.65	0.02	[g]
5	0		0.01	0.03	0.05	0.05	0.03	0.01	0	0.01	0	0.05	0.03	0.03	0.05	0	0.01	0.67	0.65	0.65	0.65	0.02	[g]
IVDMD[f]	0.81		0.72	0.64	0.60	0.70	0.70	0.63	0.61	0.64	0.62	0.67	0.61	0.64	0.66	0.64	0.62						

[a]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 0 mM. [b]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 5, 5, 5, 0.02, and 0.01 mM, respectively. [c]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 10, 10, 10, 0.05, and 0.03 mM, respectively. [d]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 15, 15, 15, 0.07, and 0.05 mM, respectively. [e]The maximal difference among I–IV. [f]In vitro dry matter disappearance. [g]$P < 0.01$.

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Table 2. Total Gas Production Affected by Nitroethane (1), 2-Nitroethanol (2), 2-Nitro-1-propanol (3), Pyromellitic Diimide (4), and 2-Bromoethanesulfonate (5) in an L_{16} (4^5) Orthogonal Test

	dose (mM) combination of different inhibitors																mean TGP[g]					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	I[a]	II[b]	III[c]	IV[d]	R[e]	*P*-values
1	0	0	0	0	5	5	5	5	10	10	10	10	15	15	15	15	199	184	200	186	16.0	NS[f]
2	0	5	10	15	0	5	10	15	0	5	10	15	0	5	10	15	201	184	191	192	16.7	NS
3	0	5	10	15	5	0	15	10	10	15	0	5	15	10	5	0	167	202	196	200	35.3	NS
4	0	0.02	0.05	0.07	0.05	0.07	0	0.02	0.07	0.05	0.02	0	0.02	0	0.07	0.05	201	196	187	184	16.9	NS
5	0	0.01	0.03	0.05	0.05	0.03	0.01	0	0.01	0	0.05	0.03	0.03	0.05	0	0.01	199	180	193	197	18.9	NS
TGP[g]	202	193	198	204	203	148	189	199	192	204	182	218	207	197	194	148						

[a]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 0 mM. [b]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 5, 5, 5, 0.02, and 0.01 mM, respectively. [c]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 10, 10, 10, 0.05, and 0.03 mM, respectively. [d]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 15, 15, 15, 0.07, and 0.05 mM, respectively. [e]The maximal difference among I–IV. [f]NS, $P > 0.05$. [g]Total gas production (mL/g fermented dry matter).

Table 3. Total Volatile Fatty Acid Production Affected by Nitroethane (1), 2-Nitroethanol (2), 2-Nitro-1-propanol (3), Pyromellitic Diimide (4), and 2-Bromoethanesulfonate (5) in an L_{16} (4^5) Orthogonal Test

	dose (mM) combination of different inhibitors																mean tVFA[f]					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	I[a]	II[b]	III[c]	IV[d]	R[e]	*P*-values
1	0	0	0	0	5	5	5	5	10	10	10	10	15	15	15	15	119	150	147	133	30.2	*h*
2	0	5	10	15	0	5	10	15	0	5	10	15	0	5	10	15	158	144	127	121	36.2	*h*
3	0	5	10	15	5	0	15	10	10	15	0	5	15	10	5	0	142	143	132	134	10.8	*g*
4	0	0.02	0.05	0.07	0.05	0.07	0	0.02	0.07	0.05	0.02	0	0.02	0	0.07	0.05	149	139	129	134	19.5	*h*
5	0	0.01	0.03	0.05	0.05	0.03	0.01	0	0.01	0	0.05	0.03	0.03	0.05	0	0.01	134	133	155	128	27.7	*h*
tVFA[f]	152	130	107	90	157	167	144	125	152	140	134	163	170	136	120	106						

[a]The averages of the result of **1**, **2**, **3**, **4**, and **5** at dose levels of 0 mM. [b]The averages of the result of **1**, **2**, **3**, **4**, and **5** at dose levels of 5, 5, 5, 0.02, and 0.01 mM, respectively. [c]The averages of the result of **1**, **2**, **3**, **4**, and **5** at dose levels of 10, 10, 10, 0.05, and 0.03 mM, respectively. [d]The averages of the result of **1**, **2**, **3**, **4**, and **5** at dose levels of 15, 15, 15, 0.07, and 0.05 mM, respectively. [e]The maximal difference among I–IV. [f]Total volatile fatty acid production (mM/g fermented dry matter). [g]$P < 0.05$. [h]$P < 0.01$.

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Table 4. Ratio of Non-Glucogenic to Glucogenic Acids Affected by Nitroethane (1), 2-Nitroethanol (2), 2-Nitro-1-propanol (3), Pyromellitic Diimide (4), and 2-Bromoethanesulfonate (5) in an L_{16} (4^5) Orthogonal Test

	dose (mM) combination of different inhibitors																mean NGR[f]					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	I[a]	II[b]	III[c]	IV[d]	R[e]	*P*-values
1	0	0	0	0	5	5	5	5	10	10	10	10	15	15	15	15	3.5	3.2	3.1	3.2	0.42	*g*
2	0	5	10	15	0	5	10	15	0	5	10	15	0	5	10	15	3.3	3.2	3.2	3.4	0.28	*g*
3	0	5	10	15	5	0	15	10	10	15	0	5	15	10	5	0	3.3	3.1	3.3	3.4	0.22	*g*
4	0	0.02	0.05	0.07	0.05	0.07	0	0.02	0.07	0.05	0.02	0	0.02	0	0.07	0.05	3.4	3.2	3.1	3.2	0.28	*g*
5	0	0.01	0.03	0.05	0.05	0.03	0.01	0	0.01	0	0.05	0.03	0.03	0.05	0	0.01	3.4	3.2	3.2	3.3	0.27	*g*
NGR[f]	3.9	3.2	3.2	3.8	3.0	3.0	3.2	3.5	3.0	3.1	3.0	3.2	3.2	3.3	3.2	3.2						

[a]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 0 mM. [b]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 5, 5, 5, 0.02, and 0.01 mM, respectively. [c]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 10, 10, 10, 0.05, and 0.03 mM, respectively. [d]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 15, 15, 15, 0.07, and 0.05 mM, respectively. [e]The maximal difference among I–IV. [f]The ratio of nonglucogenic to glucogenic acids. [g]$P < 0.01$.

Table 5. Net Methane Production Affected by Nitroethane (1), 2-Nitroethanol (2), 2-Nitro-1-propanol (3), Pyromellitic Diimide (4), and 2-Bromoethanesulfonate (5) in an L_{16} (4^5) Orthogonal Test

	dose (mM) combination of different inhibitors																mean NMP[f]					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	I[a]	II[b]	III[c]	IV[d]	R[e]	*P*-values
1	0	0	0	0	5	5	5	5	10	10	10	10	15	15	15	15	14.1	1.0	0.9	1.2	13.17	*g*
2	0	5	10	15	0	5	10	15	0	5	10	15	0	5	10	15	15.5	0.5	1.2	1.5	15.01	*g*
3	0	5	10	15	5	0	15	10	10	15	0	5	15	10	5	0	13.8	1.3	1.2	0.9	12.90	*g*
4	0	0.02	0.05	0.07	0.05	0.07	0	0.02	0.07	0.05	0.02	0	0.02	0	0.07	0.05	11.3	0.9	1.0	1.2	10.33	*g*
5	0	0.01	0.03	0.05	0.05	0.03	0.01	0	0.01	0	0.05	0.03	0.03	0.05	0	0.01	15.7	0.8	1.1	1.0	14.89	*g*
NMP[f]	48.9	0.3	1.3	1.6	1.3	0.6	0.8	1.9	1.1	0.4	0.5	1.4	1.1	0.6	2.3	1.0						

[a]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 0 mM. [b]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 5, 5, 5, 0.02, and 0.01 mM, respectively. [c]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 10, 10, 10, 0.05, and 0.03 mM, respectively. [d]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 15, 15, 15, 0.07, and 0.05 mM, respectively. [e]The maximal difference among I–IV. [f]Net methane production (mL/g fermented dry matter). [g]$P < 0.01$.

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Table 6. Hydrogen Recovery Affected by Nitroethane (1), 2-Nitroethanol (2), 2-Nitro-1-propanol (3), Pyromellitic Diimide (4), and 2-Bromoethanesulfonate (5) in an L_{16} (4^5) Orthogonal Test

	dose (mM) combination of different inhibitors																mean 2Hrec[f]					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	I[a]	II[b]	III[c]	IV[d]	R[e]	*P*-values
1	0	0	0	0	5	5	5	5	10	10	10	10	15	15	15	15	0.59	0.51	0.52	0.51	0.08	*g*
2	0	5	10	15	0	5	10	15	0	5	10	15	0	5	10	15	0.61	0.52	0.51	0.50	0.11	*g*
3	0	5	10	15	5	0	15	10	10	15	0	5	15	10	5	0	0.61	0.52	0.52	0.51	0.10	*g*
4	0	0.02	0.05	0.07	0.05	0.07	0	0.02	0.07	0.05	0.02	0	0.02	0	0.07	0.05	0.58	0.52	0.52	0.52	0.06	*g*
5	0	0.01	0.03	0.05	0.05	0.03	0.01	0	0.01	0	0.05	0.03	0.03	0.05	0	0.01	0.62	0.50	0.53	0.51	0.12	*g*
2Hrec[f]	0.84	0.51	0.53	0.48	0.53	0.53	0.49	0.51	0.53	0.53	0.51	0.52	0.52	0.51	0.52	0.46						

[a]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 0 mM. [b]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 5, 5, 5, 0.02, and 0.01 mM, respectively. [c]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 10, 10, 10, 0.05, and 0.03 mM, respectively. [d]The averages of the results of **1**, **2**, **3**, **4**, and **5** at dose levels of 15, 15, 15, 0.07, and 0.05 mM, respectively. [e]The maximal difference among I–IV. [f]Hydrogen recovery. [g]$P < 0.01$; NS, $P > 0.05$.

almost complete suppression. Further increases in dose levels of these inhibitors did not affect methane production, indicating that the combined doses could be further reduced below 5, 5, 5, 0.02, and 0.01 mM for nitroethane, 2-nitroethanol, 2-nitro-1-propanol, pyromellitic diimide, and 2-bromoethanesulfonic acid, respectively.

The inhibition of methane production sometimes results in depression of a series of rumen fermentative parameters associated with the digestive efficiency, including dry matter disappearance, gas production, and total volatile fatty acid production.[1,10,19] The nitrocompounds nitroethane, 2-nitroethanol, and 2-nitro-1-propanol caused a greater than 90% reduction in methane production.[4,6,20] These inhibitors were considered to be potent inhibitors of formate dehydrogenase and/or formate hydrogen lyase activity of both methanogens and nonmethanogens.[5] The present study indicated that 2-nitroethanol had a substantial negative influence on the rumen fermentation efficiency, as the IVDMD and total volatile fatty acid yield were markedly reduced. This indicated that the activity of microbes responsible for the degradation of plant material in the rumen was suppressed by this inhibitor. The negative effects of 2-bromoethanesulfonic acid and nitroethane on IVDMD and total volatile fatty acid yield did not appear to be as pronounced, in agreement with some previous studies.[6] 2-Bromoethanesulfonic acid, a specific inhibitor of methanogenesis, caused a 94% decrease of methane inhibition when supplied at 0.03 mM.[9] The growth of *Methanobrevibacter ruminantium*, which is regarded as the main ruminal methanogen,[21] was inhibited by 90% by 0.05 mM 2-bromoethanesulfonic acid.[22] However, methane production recovered 3 days after 2-bromoethanesulfonic acid addition to the rumen.[1] The organism *Entodinium caudatum* was preferentially affected by pyromellitic diimide, which led to a reduction in methane production.[23] However, this does not appear to be the main reason for the inhibition of methanogenesis by pyromellitic diimide, as this compound caused a 97% decrease in methane production at a concentration of 10 mg/kg, while methane production associated with protozoa accounts for only 37% of the rumen output.[24] Therefore, mechanism of pyromellitic diimide inhibition of methanogenesis remains unclear.[23]

Numerous methanogenesis inhibitors act by suppressing the oxidation of hydrogen.[1] Metabolic processes that consume hydrogen are thought to be beneficial to the animal and the microbial population because the accumulation of hydrogen adversely affects the digestive function and microbial cell yields.[19] The addition of these types of inhibitors decreased 2Hrec significantly, but the maximum accumulation of hydrogen in our incubations was not high enough to affect the synthesis of volatile fatty acids and microbes, ascribed to the electron-accepting effect of nitrocompounds.[4,19]

Volatile Fatty Acid Production and Its Pattern. Total volatile fatty acid yield was improved by the addition of nitroethane, in agreement with the findings reported previously.[4,25] It also increased following 2-bromoethanesulfonic acid treatments not higher than 0.03 mM; however, a previous study observed that volatile fatty acid production was decreased by 17−19% by the addition of 0.01−0.03 mM 2-bromoethanesulfonic acid;[9] and other reports showed no obvious change in volatile fatty acid production following supplementation with 12 mM 2-bromoethanesulfonic acid.[25] Although total volatile fatty acid yield was strongly inhibited by pyromellitic diimide ($P < 0.01$), the effect was dose-dependent and not significant at 0.02 mM. Nevertheless, the adverse effects of pyromellitic

diimide on IVDMD would be expected to lead to a reduction in volatile fatty acid concentration during the incubation.

Methane synthesis in the rumen is usually associated with increased propionate production and a reduction in the acetate to propionate ratio.[9,26] This phenomenon was also observed in our study, indicating that H_2 was accumulating and promoting the production of larger amounts of reduced products such as propionate.[7] Different results for volatile fatty acid production in response to these inhibitors have been reported in different studies; for example, the production of acetate and propionate were reported to decrease in cultures treated with nitroethane;[6] however, in our study, propionate was increased by the addition of nitroethane, and according to a previous study,[4] nitroethane promoted both acetate and propionate productions. These discrepancies most likely arise due to differences in the experimental conditions. In particular, the feedstuffs fed to the ruminants and the substrates used for fermentation would be expected to affect the results, and the observed differences might reflect potential differences in sensitivity to the inhibitors by endogenous methanogens in different microorganism populations.[5,27]

The minimum net methane production occurred with a combination of 10 mM nitroethane, 5 mM 2-nitroethanol, 15 mM 2-nitro-1-propanol, 0.02 mM pyromellitic diimide, and 0.01 mM 2-bromoethanesulfonic acid; however, when the total volatile fatty acid yield, IVDMD, and 2Hrec values were taken into account, the optimal combination was nitroethane, 2-nitroethanol, 2-nitro-1-propanol, pyromellitic diimide, and 2-bromoethanesulfonic acid at 5, 5, 5, 0.02, and 0.03 mM, respectively. These data provide directions for choosing appropriate parameters that will provide optimal fermentation efficiency.

Though no evidence showed that these compounds had any interaction, the inhibitory effect might be strengthened by the combination due to differences in the sensitivities of methanogen species to various inhibitors.[22] The nitrocompounds and 2-bromoethanesulfonic acid acted on the different sites of the methane formation pathway; therefore, we hypothesized that a combination of nitroethane, 2-nitroethanol, 2-nitro-1-propanol, pyromellitic diimide, and 2-bromoethanesulfonic acid might result in a longer duration of the inhibition of methane production. This should be confirmed by further in vivo research on animals.

The use of these methanogensis inhibitors raises safety and public concerns with respect to animal health and animal products used for human consumption. However, in sheep, a daily oral dose of 72 mg nitroethane/kg body weight or 120 mg of 2-nitro-1-propanol/kg body weight did not cause any observable adverse physiologic or behavioral effects.[20] The addition of pyromellitic diimide, at the level of 400 mg/kg dry matter per day, also had no effect on apparent digestibility, degradation rate, or potential degradability of dry matter in the rumen of sheep.[28] However, a particularly attractive feature of these nitrocompounds is that they could potentially inhibit the growth of leading foodborne pathogens, such as *Listeria monocytogenes* and *Salmonella*.[29,30] No literature has been focused on testing the specific toxicity of these inhibitors on ruminants hitherto, and in vivo experiments need to be conducted for further research of side-effects on animals or on their products.

In summary, the present results contribute important new knowledge about mechanistic and dose dependent aspects of the tested compounds on ruminal methanogenesis and may guide animal studies to develop effective mitigation of methane emission from ruminants.

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ABBREVIATIONS USED

IVDMD, in vitro dry matter disappearance; NGR, ratio of nonglucogenic to glucogenic acids; 2Hrec, hydrogen recovery

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