No interactions among three methane inhibitors on \textit{in vitro} methane production

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\section*{ABSTRACT}

Two experiments (Exps.) were conducted to examine the interactions among methane inhibitors on 24 h \textit{in vitro} methane production using monensin (MON), fumaric acid (FA), and bromochloromethane (BCM). Donor goats and \textit{in vitro} fermentation incubations were both treated with a 50:50 concentrate:forage diet (DM basis). In Exp. 1, MON (0, 60, or 120 mg/kg DM) and FA (0, 3.44, or 6.89 mM) were used to examine their two-way interaction, and no interaction was found on methane production ($P > 0.05$). In Exp. 2, MON (0 or 60 mg/kg DM), FA (0 or 6.89 mM), and BCM (0 or 3.08 $\mu$M) were used to examine their three-way interaction, and no three-way interaction was observed ($P > 0.05$). The correlation was significant under the MON and FA treatments without BCM ($r = 0.55$, $P < 0.05$), and under the MON, FA, and BCM treatments, the correlation was also significant ($r = 0.59$, $P < 0.05$). Collectively, no two- or three-way interactions were observed among MON, FA, and BCM. Regression analyses showed that MON, FA, and BCM were independent from each other, and the inhibitory effects of the three methane inhibitors on methanogenesis to a certain range are additive.

\section*{Introduction}

Methane, a greenhouse gas, has contributed mainly to climate warming. During the past few decades, the contribution of ruminants to methane emissions has increased rapidly, accounting for half of all noncarbon dioxide greenhouse gas emissions from the agriculture (Dangal et al. 2017). In addition, methane produced in the rumen accounts for, on average, 6.4% of gross energy loss to the ruminants (Ranga Niroshan Appuhanny et al. 2013). As a consequence, reducing methanogenesis in ruminants is of great significance and interest and is accompanied by the application of many methane inhibitors (Hristov et al. 2013).

Owing to different inhibitory mechanisms on methanogenesis, all methane inhibitors can be divided into three types: microflora modifying inhibitors (MMIs), hydrogen receptors (HRs), and enzymatic inhibitors (EIs). MMIs, which can inhibit gram-positive bacteria and protozoa, decrease substrates for methanogenesis (Hook et al. 2010; Kumar et al. 2013). Monensin (MON), saponins, and tannins are considered MMIs. HRs are well known as a hydrogen sink in the rumen (Hristov et al. 2013), and compete with methanogens for the utilization of metabolic hydrogen (Castillo et al. 2004; Li et al. 2009), resulting in a decrease in the proportion of acetate and an increase in the proportion of propionate in the rumen (Hook et al. 2010). Unsaturated fats, nitrates, and organic acids such as fumaric acid (FA) are HRs (Castillo et al. 2004). As EIs, halogenated methane analogs, including bromochloromethane (BCM) (Shima et al. 2002), 2-bromoethanesulfonate (Lee et al. 2009), and 3-nitroxypropanol (Pitta et al. 2018), inhibit the enzymatic activities needed in methanogenesis and decrease methane emissions. An increasing number of studies have focused on the combination of these inhibitors owing to their different modes of action in the rumen to maximize methane mitigation ability (Patra and Yu 2015; Capelari et al. 2018; Vyas et al. 2018). Some binary combination studies have been performed among these three types of inhibitors: MMIs and HRs (Callaway and Martin 1996; Capelari et al. 2018), HRs and EIs (Choi et al. 2004; Ebrahimii et al. 2011), and MMIs and EIs (Romero-Pérez et al. 2017; Vyas et al. 2018).

However, no study has investigated the impacts of the three-way interaction of these three types of methane inhibitors. The hypothesis of this research was that no interactions (two- and/or three-way) exist among the three types of methane inhibitors owing to the different inhibitory mechanisms. Accordingly, the objective of the present study was to examine the interactions among three typical and functionally well-defined methane inhibitors (MON, FA, and BCM) after 24 h of \textit{in vitro} methane production and fermentation.

\section*{Materials and methods}

\section*{Experimental design}

Exp. 1. Effect of MON and FA on \textit{in vitro} methane production and fermentation

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In this experiment (Exp. 1), MON (Monensin Premix, 20%; Shandong Shengli Co., Ltd, Jinan, Shandong Province, China) and FA (F19353, 99%; Sigma–Aldrich Corp., St. Louis, Missouri, USA) were added to a medium grain diet (concentrate and ground alfalfa hay; 50:50) to examine the two-way interaction between MON and FA on in vitro methane production and fermentation. The MON at levels of 0, 60, and 120 mg/kg dry matter (DM) and FA at levels of 0, 3.44, and 6.89 mmol were used in the present experiment, and each experimental run was carried out with eight duplicates.

Exp. 2. Effect of MON, FA, and BCM on in vitro methane production and fermentation

The aim of the present experiment was to examine the three-way interaction among MON, FA, and BCM on in vitro methanogenesis and fermentation. The levels of 0 and 60 mg/kg DM for MON and the levels of 0 and 6.89 mmol for FA were used. The BCM (457493, 99%; Sigma–Aldrich Corp., St. Louis, Missouri, USA) was added at 0 and 3.08 mg/kg DM for MON and the levels of 0 and 6.89 mmol for FA respectively. In the present experiment, the same medium grain diet as Exp. 1 was used and carried out with six duplicates in each experimental run.

**Fermentation preparation**

All procedures in this work were reviewed and approved by the Northwest A&F University Animal Care and Use Committee. Ruminal inocula were collected from three rumen-fistulated goats before morning feeding, and the inocula were used for the in vitro incubations. These goats were offered a diet of a total of 2.4 kg DM daily (provided 1.2 kg twice at 08:00 and 20:00). The diet contained 50% pelleted concentrate and 50% pelleted alfalfa hay, and the concentrate was composed of 25% soybean meal, 70% ground corn, and 5% premix. All goats had been adapted to this diet prior to the experiment for 14 days.

Ruminal contents were moved to an insulated container and then transported to the laboratory immediately (within 5 min). The samples from the three donor goats were mixed (1:1:1, v/v) and then blended using a Waring blender (Fisher 14-509-1) under a carbon dioxide atmosphere (20 s) to detach the bacteria and protozoa from the feed particles. The blended samples were then strained through glass wool and 4 layers of cheesecloth and used as ruminal inocula for subsequent fermentation, followed by combination with a buffer solution. The buffer solution, reducing solution, and macromineral solutions were prepared as previously described by Goering and Van Soest (1970) and as a ruminal buffer solution. These ruminal buffer solutions (1100 mL) and the ruminal inocula (200 mL) were combined and warmed, and then 50 mL was added to fermentation vessels (260 mL) to ferment at 39°C for 24 h. Feed samples (600 mg) were weighed and placed in the sealed fermentation vessels equipped with a gas sampling port and automatic pressure transducers (Ankom Technology, Macedon, New York, USA). The control fermentation vessels (without additives) and blank fermentation vessels (without feed or additives) in Exps. 1 and 2, were both supplemented with 50 mL of the ruminal buffer solution.

**Collection and analysis of samples**

For each incubation in the vessel, gas pressure was monitored using the previously mentioned automated pressure transducer system (Xu et al. 2010). At termination of the incubation, gas samples were drawn into sampling syringes and transferred into a vacuum test tube (Vacutainer, Becton Dickinson, Franklin Lakes, New Jersey, USA), and then gas chromatography (7820A, Agilent Technologies, Santa Clara, California, USA) was used to analyze the methane content (Xu et al. 2010). The fermentation substrates were then analyzed for pH immediately with a pH meter (Model PH5-2F, Shanghai Precision and Scientific Instrument Co., LTD, Shanghai, China). An aliquot of the fermentation substrates (1.5 mL) was combined with 25% metaphosphoric acid (0.3 mL) and then centrifuged for 15 min (39,000 × g, 4°C). The supernatant (1 mL) was transferred into vials and then capped and analyzed for total volatile fatty acid (VFA) concentrations (including acetate, butyrate, propionate, valerate, isobutyrate, and isovalerate) by gas chromatography. The gas chromatography was fitted with an autosampler, a flame-ionization detector, and a 30 m × 0.32 mm × 0.25 μm capillary column (DB-FFAP, Agilent Technologies, Santa Clara, California, USA). The concentrations of individual VFAs were summed to obtain the total VFA (TVFA) concentration.

**Regression analysis between the predicted methane and observed methane production**

Regression analysis between the predicted methane production (calculated based on the production of VFAs) and the observed methane production was conducted in the current study to investigate whether the MON-, FA- and BCM-induced reduction in methanogenesis was caused by changes in VFA production. The predicted methane production relative to VFA concentration (mmol/mol VFA) was estimated using the following equation (Castro Montoya et al. 2011):

\[
\text{Methane (mmol/mol VFA)} = 0.45 \times \text{acetate} + 0.4 \times \text{butyrate} - 0.275 \times \text{propionate}
\]

The units of methane, acetate, butyrate, and propionate are all mmol relative to VFA (mol), and VFAs include production of acetate, butyrate, and propionate. The observed methane production relative to VFA concentration (mmol/mol VFA) was presented in the current study and calculated from: methane production (mL)/22.4/(TVFA × 50 mL/1000).

**Statistical analysis**

The data were analyzed by a two-factor complete random test design, and the GLM of SPSS13.0 was selected in Exp. 1. The experimental factors included the main effects of MON and FA and their interactions. In Exp. 2, the data were analyzed by a three-factor complete random test design, and the GLM of SPSS13.0 was selected. The main effects of MON, FA, and BCM and their interactions were investigated. Regression analysis was performed by a linear regression model in SPSS13.0. The factors that predicted methane and observed methane production were included in the linear regression model.
TVFA concentration, VFA production, pH, or TVFA concentration (P > 0.05) on total gas production, fermentation pH, TVFA concentrations or methane percentage in total gas production, TVFA concentration, or VFA profiles as shown in Table 1.

MON had no effect on the total gas production, fermentation pH, or TVFA concentration (P > 0.05) and tended to decrease methane production (P = 0.100) and the methane percentage in total gas production (P = 0.059). FA increased gas production (P < 0.05), decreased fermentation pH and methane percentage in total gas production (P < 0.05), and did not affect TVFA concentrations and methane production (P > 0.05). MON decreased the acetate proportion (P < 0.05), increased the propionate proportion (P < 0.01), and decreased the acetate:propionate ratio (A:P ratio) (P < 0.01). FA decreased the acetate proportion and A:P ratio (P < 0.01), and increased the propionate proportion (P < 0.05). Neither MON nor FA affected the proportion of butyrate, valerate, isobutyrate, or isovalerate (P > 0.05).

Exp. 2. Effect of MON, FA, and BCM on in vitro methane production and fermentation

For all fermentation parameters, there were no two-way interactions among MON, FA, and BCM (P > 0.05) or three-way interactions (P > 0.05) on in vitro methane production and fermentation (Table 2).

In Table 2, MON tended to decrease fermentation pH (P = 0.081) and did not decrease the methane percentage in total gas production or methane production (P > 0.05). MON had the same effects on other parameters (total gas production, TVFA concentration, VFA profiles, and A:P ratio) as Exp. 1. For FA, all fermentation parameters were affected similarly to those in Exp. 1, except for the methane percentage in the total gas production, which only tended to decrease with FA (P = 0.055). BCM decreased methane production and methane percentage in total gas production (P < 0.05) and had no effect on total gas production, pH, TVFA concentrations or the A:P ratio (P > 0.05). BCM did not affect VFA profiles but increased the valerate proportion (P < 0.01).

Significance was declared at P values of ≤ 0.05, and tendencies were declared at P values of ≤ 0.10.

### Results

**Exp. 1. Effect of MON and FA on in vitro methane production and fermentation**

There were no interactions (P > 0.05) between MON and FA on fermentation pH, total gas production, methane production, methane percentage in total gas production, TVFA concentration, or VFA profiles as shown in Table 1.

MON had no effect on the total gas production, fermentation pH, or TVFA concentration (P > 0.05) and tended to decrease methane production (P = 0.100) and the methane percentage in total gas production (P = 0.059). FA increased gas production (P < 0.05), decreased fermentation pH and methane percentage in total gas production (P < 0.05), and did not affect TVFA concentrations and methane production (P > 0.05). MON decreased the acetate proportion (P < 0.05), increased the propionate proportion (P < 0.01), and decreased the acetate:propionate ratio (A:P ratio) (P < 0.01). FA decreased the acetate proportion and A:P ratio (P < 0.01), and increased the propionate proportion (P < 0.05). Neither MON nor FA affected the proportion of butyrate, valerate, isobutyrate, or isovalerate (P > 0.05).

**Exp. 2. Effect of MON, FA, and BCM on in vitro methane production and fermentation**

For all fermentation parameters, there were no two-way interactions among MON, FA, and BCM (P > 0.05) or three-way interactions (P > 0.05) on in vitro methane production and fermentation (Table 2).

In Table 2, MON tended to decrease fermentation pH (P = 0.081) and did not decrease the methane percentage in total gas production or methane production (P > 0.05). MON had the same effects on other parameters (total gas production, TVFA concentration, VFA profiles, and A:P ratio) as Exp. 1. For FA, all fermentation parameters were affected similarly to those in Exp. 1, except for the methane percentage in the total gas production, which only tended to decrease with FA (P = 0.055). BCM decreased methane production and methane percentage in total gas production (P < 0.05) and had no effect on total gas production, pH, TVFA concentrations or the A:P ratio (P > 0.05). BCM did not affect VFA profiles but increased the valerate proportion (P < 0.01).

### Regression analysis between the predicted methane and observed methane production

Figure 1A and B shows the relationship between the predicted methane and observed methane production in Exp. 1 and 2, respectively. In Exp. 1, a significant correlation between the predicted methane and observed methane production under the MON and FA treatments was found (r = 0.46, P < 0.001). In Exp. 2, a significant correlation was shown for the predicted methane and observed methane production with the treatment of MON and FA without BCM (r = 0.55, P = 0.012), and a significant correlation between the predicted methane and observed methane production with the fermentation of MON, FA, and BCM treatment was also observed (r = 0.57, P = 0.006).

### Discussion

MON, FA, and BCM are different types of methane inhibitors and represent MMs, HRs, and EIs, respectively. In the present study, MON decreased methanogenesis and influenced the proportions of acetate and propionate. These results were in agreement with previous studies (Ponce et al. 2012; Wischer et al. 2013). FA has shown the effect of depressing methanogenesis, and the increasing propionate proportion at the expense of decreasing acetate proportion, so decreased the A:P ratio, both of which were in line with the results of previous studies (Newbold et al. 2005; Hook et al. 2010). In the current investigation, methane production decreased by 32.1% with the addition of BCM at 3.08 μmol, in contrast with the control, which was in line with the results of previous studies. Goel et al. (2009) reported that in vitro ruminal fermentation with the addition of BCM at 5 and 10 μmol decreased methane production by 77.9% and 93.0%, respectively. The results of our preliminary study showed that the addition of...
Table 2. The effects of MON, FA, and BCM on in vitro methane production and fermentation (Exp. 2).

| Treatment | Gas production, mL | Methane production, mL | VFA TVFA, mmol | A.P. ratio: acetate proportion (A): propionate proportion (P) | M: Monensin; F: Fumaric acid; BCM, μmol |
|-----------|--------------------|------------------------|----------------|-------------------------------------------------|----------------------------------|
| Control   | 90.3               | 13.20                  | 71.14          | 0.984                                           | 0.844 |
| Monensin  | 91.3               | 13.20                  | 71.14          | 0.984                                           | 0.844 |
| Fumaric acid | 92.3               | 13.30                  | 71.14          | 0.984                                           | 0.844 |
| Monensin + Fumaric acid | 92.3               | 13.30                  | 71.14          | 0.984                                           | 0.844 |
| BCM 3.08 μmol | 92.3               | 13.30                  | 71.14          | 0.984                                           | 0.844 |
| BCM 15.40 μmol | 92.3               | 13.30                  | 71.14          | 0.984                                           | 0.844 |

P-value: Fumaric acid, mmol 0 6.89 0 6.89
SEM 1 μBCM, mol 0 3.08 0 3.08 0 3.08 0 3.08 M F B M×F M×B F×B M×F×B

pH 6.00 6.02 5.89 5.96 5.95 5.93 5.90 5.85 0.017 0.081 0.023 0.844 0.814 0.246 0.906 0.584

Gas production, mL 90.3 91.3 105.5 105.5 93.5 97.3 102.3 109.3 1.97 0.490 0.001 0.407 0.536 0.490 0.873 0.763

Methane Percentage, % 14.71 9.77 10.94 8.68 12.73 11.34 11.38 8.75 0.52 0.980 0.055 0.016 0.837 0.472 0.743 0.376

Production, mL 13.20 8.96 11.63 9.52 11.92 10.95 11.65 9.44 1.08 0.860 0.560 0.490 0.874 0.472 0.832 0.478

VFA TVFA, mmol 71.14 64.10 71.17 61.90 71.33 69.73 72.37 67.21 10.21 0.131 0.182 0.501 0.160 0.201 0.241 0.402

Acetate 59.15 59.43 56.47 56.56 57.68 57.10 56.29 54.46 3.11 0.038 0.004 0.272 0.327 0.206 0.336 0.377

Propionate 25.79 23.29 28.39 28.93 27.98 27.61 29.86 30.52 3.61 0.005 0.001 0.321 0.169 0.266 0.130 0.288

Butyrate 8.81 9.55 8.68 8.62 8.11 8.64 7.92 8.19 1.63 0.086 0.188 0.221 0.418 0.475 0.294 0.387

Valerate 3.33 4.34 3.43 3.44 3.27 3.82 3.30 4.17 0.75 0.498 0.301 0.002 0.071 0.311 0.199 0.101

Isobutyrate 1.28 1.37 1.26 1.26 1.23 1.37 1.20 1.57 0.355 0.372 0.204 0.199 0.444 0.444 0.266 0.178

Isovalerate 1.64 2.02 1.78 1.39 1.65 1.65 1.43 1.57 0.81 0.296 0.206 0.448 0.418 0.444 0.266 0.178
nitroxypropanol acted independently in animals fed high-forage or high-grain diets (Vyas et al. 2018). This study confirmed that in vivo research had the same results as the current study. Performing in vivo and in vitro experiments simultaneously with respect to methanogenesis inhibition gives that the in vitro results better resemble what happens in the rumen than in the whole animal (Hatew et al. 2015; Jonker et al. 2016). In vitro experimental approaches offer the opportunity to evaluate multiple additives alone or in combination over a wide range of supplementation levels (Yáñez-Ruiz et al. 2016), and they can eliminate the influence of the whole body on the rumen, such as the absorption of VFAs. Future research on in vivo binary or tertiary combinations, especially direct in vitro-in vivo comparisons, should be conducted. Furthermore, binary or tertiary combinations of the three types of methane inhibitors to inhibit methanogenesis should be widely used, since the combinations eliminate dietary interference from ruminant diets or other variations (Patra and Yu 2015).

Conclusions

In the present study, there were no two- or three-way interactions among MON, FA, and BCM in decreasing in vitro methane production. Thus, the inhibitory effects of the three types of methane inhibitors on methanogenesis to a certain range were additive. Correlations between the predicted and observed methane production under MON and FA treatment without or with BCM treatment were both significant. More practical strategies with binary and tertiary combinations of the three types of methane inhibitors might be promising.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Figure 1. Linear regression between the predicted methane and observed methane production. A (Exp. 1): y = 0.68x + 21.23 (r = 0.46, P < 0.001). B (Exp. 2): ‘NO’ indicates treatment without BCM (dotted line); y = 0.73x-0.82 (r = 0.55, P = 0.012); ‘BCM’ indicates treatment with BCM (solid line), y = 0.66x-4.22 (r = 0.59, P = 0.006).

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