Effects of Halogenated Compounds, Organic Acids and Unsaturated Fatty Acids on *In vitro* Methane Production and Fermentation Characteristics*

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ABSTRACT : The objective of this study was to evaluate the effects of halogenated compounds, organic acids, unsaturated fatty acids and their mixtures on *in vitro* methane production and fermentative characteristics of mixed rumen microorganisms. Agents used in two *in vitro* experiments were bromoethanesulfonic acid (BES) and pyromellitic diimide (PMDI) as halogenated compound, fumarate and malate as organic acid, and linoleic acid and linolenic acid as unsaturated fatty acid sources. Ruminal fluid collected from a Holstein steer fed tall fescue and concentrate mixtures was incubated at 39°C for 48 h with addition of those materials. Single supplementation of halogenated compounds, organic acids or unsaturated fatty acids decreased *in vitro* methane production ($p<0.05$). The second experiment was designed to investigate effects of combination of one of halogenated compounds and either organic acids or fatty acids on methane production. Lower concentration of methane and lower A:P ratio were observed with PMDI compared with BES ($p<0.01$). In general medium pH, VFA, total gas and hydrogen production, and dry matter degradability were affected by addition of the same compounds. In addition, PMDI+malate treatment resulted in the highest molar proportion of propionate, and lowest A:P ratio and methane production ($p<0.01$). Hydrogen production was highest in PMDI+linolenic acid and lowest in BES+malate treatment ($p<0.01$). PMDI+malate combination was the most recommendable in reducing methane production without too much influence on digestibility under conditions of present studies. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 9 : 1255-1259)***

Key Words : Methane, Fermentation, Halogenated Compounds, Organic Acids, Unsaturated Fatty Acids

INTRODUCTION

Methane production during anaerobic fermentation in the rumen is a nutritionally wasteful process which represents 2 to 15% gross energy loss to the host animal (Moss, 1993). In addition, methane production by animals, principally ruminants, is estimated to constitute 15 to 20% of the global production of methane (Crutzen et al., 1986). Therefore, extensive research interests have been focused on developing methods of reducing methane production in the rumen. Several methods have been proposed as a means of reducing methane production in the rumen, and some of examples are: proportion of concentrates in the diet (Lee et al., 2003), processing of forages (Takahashi, 2001; Santoso et al., 2003), heat treatment of concentrates (Thomson, 1972), and some methane inhibitors such as halogenated compounds (Martin and Macy, 1985), ionophores (Van Nevel and Demeyer, 1988), organic acids (Martin, 1998), sarsaponin (Lila et al., 2003), and unsaturated fatty acids (Czerkawski et al., 1966).

Halogenated compounds such as bromoethanesulfonic acid (BES) and pyromellitic diimide (PMDI) are known to be the most effective methane inhibitor due to their direct inhibitory effects to methanogenic bacteria, but resulting hydrogen accumulation by halogenated compounds decreases microbial protein synthesis (Demeyer and Van Nevel, 1975). Organic acids such as malate and fumarate supplementation have been reported to increase propionate and decrease methane production (Martin and Streeter, 1995; Asanuma et al., 1999). Rumen microbes hydrogenate unsaturated fatty acids, and this process has been one time believed to inhibit methane production in the rumen (Demeyer et al., 1969), but their low affinity to hydrogen ion essentially eliminates such a possibility. While single supplementation of organic acids had little effect on methane production, mixed supplementation of organic acids and monensin decreased methane production (Callaway and Martin, 1996). It is not apparent why combinations of two compounds resulted in the reduction of methane production, but it may be possible that one compound such as monensin reduce the number of methanogens and the other compound serves as an alternative electron sink. The purpose of this experiment was to study effects of halogenated compounds, organic acids and fatty acids added singly or in combination on methane production and fermentation characteristics.

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MATERIALS AND METHODS

Experimental materials and procedures

Three groups of potential methane inhibitors used in this experiment were halogenated compounds, organic acids

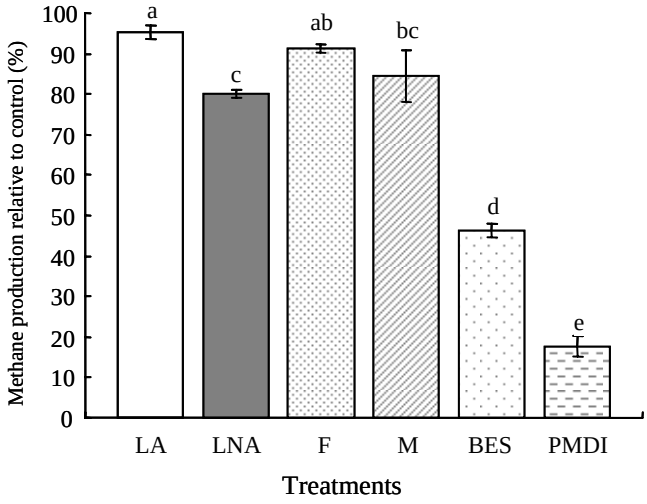

Figure 1. *In vitro* methane production as affected by linolenic acid (LA), linolenic acid (LNA), fumarate (F), malate (M), bromoethanesulfonic acid (BES) and pyromellitic diimide (PMDI) (Exp. 1). Methane concentration of control was 11.7 mM.
[a, b, c, d, e] Treatments with different letters are different at $p<0.05$.

and unsaturated fatty acids. The halogenated compounds were bromoethanesulfonic acid (BES) (ACROS ORGANICS, NJ, USA) and pyromellitic diimide (PMDI) (Aldrich Chem, WI, USA). Two unsaturated fatty acids were linoleic acid and linolenic acid (Sigma Chemical Co., St. Louis, MO, USA) and fumarate and malate were utilized as a source of organic acid (Sigma Chemical Co., St. Louis, MO, USA).

Two *in vitro* experiments were conducted in this study. The first experiment was designed to compare the efficacy of various compounds on methane production compared to control. There were three comparable tests, which were control vs. 30 μM of BES or 10 ppm of PMDI, control vs. 10 mM fumarate or malate, and control vs. 5% linoleic acid or linolenic acid, respectively. The second experiment had 8 treatments: BES+fumarate, BES+malate, BES+ linoleic acid, BES+linolenic acid, PMDI+fumarate, PMDI+ malate, PMDI+linoleic acid, PMDI+linolenic acid. Supplemental levels were the same as in the first experiment.

Rumen fluid was obtained from a ruminally fistulated Holstein steer fed twice a day (09:00 and 17:00 h) with a ration consisting of 60% of tall fescue and 40% of a commercial concentrate mixture (16% crude protein and 75% TDN). The collected rumen content was filtered through four layers of cheesecloth before mixing with buffer. The substrate was consisted of 0.3 g of tall fescue and 0.2 g of concentrate powder (60:40 ratio). The buffer used for *in vitro* incubation was the same as proposed by McDougall (1948). The anaerobic culture technique of Hungate (1969) was employed throughout the incubation, which were lasted for 48 h at 39°C with three replications.

Analysis

Total gas production was measured after 48 h incubation by the method of Theodorou et al. (1994). Then gas was sampled into 7 ml vacutainer for CH_4 and H_2 analysis. Both methane and hydrogen were measured with a chromatography (Varian 3800, USA). The medium pH was measured after 48 h incubation with a general-purpose pH meter (Mettler Delta 340), and then the medium was centrifuged at 3,000 rpm for 10 min. After centrifugation, 1ml from upper layer was transported into eppendorf tube and 0.2 ml of 25% metaphosphoric acid was added and left for 30 min. The VFA concentration was analyzed with a Gas Chromatography (HP 6890) by the method of Erwin et al. (1961). The medium was filtered with filter paper (Whatman No. 541), and dried in a drying oven for 48 h at 60°C to determine dry matter. Dry matter degradability was determined from the difference between substrate weight at the beginning and end of incubation.

Statistical analysis

The data of the first experiment was subjected to ANOVA (Analysis of Variance) (SAS, 1999) and the means were compared for significance by Tukey's test (Snedecor and Cochran, 1967) at $p<0.05$. In addition, the second experiment data was also subjected to general linear model (GLM) (SAS, 1999) and the differences among means were determined using single and multiple degree of freedom contrasts.

RESULTS AND DISCUSSION

Experiment 1- Single supplementation study

Supplementations of halogenated compounds, organic acids and unsaturated fatty acids reduced *in vitro* methane production compared with control ($p<0.05$) as depicted in Figure 1. Of the agents used, halogenated compounds were much more effective in reducing methane production than organic acids or unsaturated fatty acids. The average extent of decrease was 68.0%, 12.2% and 12.4% for halogenated compounds, organic acids and unsaturated fatty acids, respectively. The results of present study clearly indicate that halogenated compounds are a potent methane inhibitor, while efficacy of organic acids and unsaturated fatty acids are much lower, which confirms previous reports (Czerkawski et al., 1966; Martin and Macy, 1985; Carro and Ranilla, 2003). More reduction by PMDI over BES and linolenic over linoleic acid in methane production is also largely the same as previously reported (Czerkawski et al., 1966; Martin and Macy, 1985).

Experiment 2-Combination supplementation study

Halogenated compounds : Medium pH values were not significantly different between BES and PMDI treatments.

Total VFA production, and molar proportions of acetate, isobutyrate, and isovalerate, and acetate:propionate (A:P) ratio were significantly higher in BES compared with PMDI treatment ($p<0.01$). However, molar proportions of propionate and valerate were not significantly different between PMDI and BES, and that of butyrate was significantly higher in PMDI compared with BES ($p<0.01$). The decreased A:P ratio in PMDI treatment compared with BES treatment may suggest PMDI has more potential ability to reduce methane production despite molar proportion of propionate was similar between the treatments. Total gas and methane production were higher in BES compared with PMDI treatment ($p<0.01$), which is similar to that obtained in experiment 1. Hydrogen production was lower in BES compared with PMDI treatment ($p<0.01$). Degradability of dry matter was higher in BES compared with PMDI treatment ($p<0.01$).

The relationship between methane and hydrogen production in this study is supported by previous reports in that methane is formed by ruminal methanogenic bacteria using approximately 80% hydrogen as a substrate so that hydrogen concentration is decreased (Hungate et al., 1970), although different observation was reported by Russell and Strobel (1989) who noticed that ionophores simultaneously decreased both methane and hydrogen production in the rumen. In the present study hydrogen production was increased when methane production was decreased, which may indicate that halogenated compounds were not involved in hydrogen-consuming metabolic processes, but were toxic to either directly methanogenic bacteria or to some enzyme system of the bacteria. Lower dry matter degradability by PMDI than by BES supplementation is likely due to the accumulated hydrogen, which may interfere interspecies hydrogen transfer and alter NADH/NAD ratio in the microbes (Hino and Russell, 1985).

Organic acid and unsaturated fatty acid : Medium pH values were higher in the order of fumarate>linoleic or linolenic acid>malate ($p<0.01$). Molar proportions of acetate, butyrate, isobutyrate, and isovalerate, and A:P ratio were higher, but total VFA and molar proportion of propionate was lower in unsaturated fatty acids treatment compared with organic acids treatment ($p<0.01$). Total gas production was the highest in malate and lowest in linoleic acid treatment ($p<0.01$). Methane production was not significantly influenced by treatments, but hydrogen production was affected by treatments with highest value being obtained with linolenic acid and lowest value with malate ($p<0.01$). *In vitro* dry matter degradability was higher in the order of malate>fumarate>linoleic acid> linolenic acid treatment ($p<0.01$).

Although linolenic acid addition resulted in the highest accumulation of hydrogen, but that may not be the major factor responsible for the lower dry matter digestibility with this fatty acid. It is well known that fat supplementation has deleterious effects on protozoa and cellulolytic bacteria in the rumen (Hino and Nagatake, 1993). In addition, Zinn (1989) reported that decreased dry matter digestibility resulted from depressed fiber digestibility by fat supplementation.

Previous studies observed that methane production was decreased by organic acids (Martin and Streeter, 1995; Asanuma et al., 1999) and unsaturated fatty acids (Van Nevel and Demeyer, 1996). Effects of fatty acids on methane production were originally attributed to partitioning of available hydrogen between methanogenesis and hydrogenation of unsaturated fatty acids (Lennarz, 1966). Considering lower affinity to hydrogen ion by unsaturated fatty acids compared to CO_2 (Czerkawski, 1986), this would not be a very likely mode of action for fatty acids in methane production. In addition, organic acids have also been claimed to serve as alternative electron acceptors, competing with methanogenesis for the hydrogen uptake with varying efficiencies (Martin, 1998). Current results indicate that effect of organic acids or unsaturated

Table 1. Effects of combinations of halogenated compounds with organic acids or unsaturated fatty acids on *in vitro* fermentation (Exp. 2)

Items	BES				PMDI				SEM	Contrasts		
	F	M	LA	LNA	F	M	LA	LNA		BES vs. PMDI	F vs. M vs. LA vs. LNA	Interaction
pH	6.48	6.39	6.47	6.47	6.58	6.36	6.47	6.47	0.05	NS	**	**
Total VFA (mM)	71.9	70.5	57.6	53.0	60.0	61.0	54.9	57.6	2.93	**	**	**
Individual VFA (molar proportion, %)												
Acetate	53.4	51.3	56.1	56.2	51.0	48.8	54.2	53.0	0.57	**	**	**
Propionate	28.1	29.2	22.1	22.9	29.0	29.4	22.9	22.8	0.86	NS	**	**
Butyrate	10.3	11.6	12.4	12.2	11.5	12.5	13.7	14.2	0.22	**	**	**
Iso-butyrate	1.4	1.3	1.5	1.5	1.3	1.2	1.4	1.6	0.03	**	**	**
Valerate	5.1	4.9	6.0	5.4	5.6	6.4	6.1	6.5	0.73	NS	NS	NS
Iso-valerate	1.7	1.7	1.9	1.8	1.6	1.7	1.7	1.9	0.06	*	**	**
A:P ratio	1.9	1.8	2.5	2.5	1.8	1.7	2.4	2.3	0.09	**	**	**

BES: Bromoethanesulfonic acid, PMDI: Pyromellitic diimide, F: Fumarate, M: Malate, LA: Linoleic acid, and LNA: Linolenic acid.
NS: Not significant, * $p<0.05$ and ** $p<0.01$.

Table 2. Effects of combinations of halogenated compounds with organic acids or unsaturated fatty acids *in vitro* gas production and dry matter degradability (Exp. 2).

Items	BES				PMDI				SEM	Contrasts		
	F	M	LA	LNA	F	M	LA	LNA		BES vs. PMDI	F vs. M vs. LA vs. LNA	Interaction
Total gas (ml)	132.0	146.9	133.7	132.5	127.3	137.3	124.2	140.7	2.36	**	**	**
CH_4 (mM)	5.0	5.4	5.2	5.3	2.6	2.4	2.4	3.2	0.32	**	NS	**
H_2 (mM)	1.6	1.4	2.1	1.7	3.2	3.3	4.0	5.1	0.25	**	**	**
DM degradability (%)	54.9	56.8	52.9	52.4	53.6	55.3	52.0	34.1	2.74	**	**	**

BES: Bromoethanesulfonic acid, PMDI: Pyromellitic diimide, F: Fumarate, M: Malate, LA: Linoleic acid, and LNA: Linolenic acid.
NS: Not significant, and ** $p<0.01$.

fatty aids on methane production is not very high. However, possibility can not be excluded completely that organic acids or unsaturated fatty acids can uptake more hydrogen ions and reduce methane production to a greater extent when methanogenesis is suppressed by halogenated compounds.

Effect of combination of halogenated compounds and organic acid or unsaturated fatty acid : Effects of combination of halogenated compounds and organic acids or unsaturated fatty acids on methane production and rumen fermentation characteristics are presented in Table 1 and 2. Judging from effects on A/P ratio, methane and hydrogen production, combination of halogenated compounds with organic acids were more effective than with unsaturated fatty acids. In terms of depression rate of methane, the best result was obtained when fumarate was combined with BES, but with PMDI malate or linoleic acid was the best combination. The negative relationship between methane and hydrogen production was also observed in this experiment, indicating that the role of organic acids and unsaturated fatty acids in diverting hydrogen ion was not appreciable under the conditions of present study.

Present results were obtained from *in vitro* culture studies for short period of time and therefore there is a possibility that effects of inhibitors and combinations under actual feeding conditions may be different from this study. Considering importance of methane production in terms of feed energy utilization and environmental pollution control, further research is warranted.

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