

Effects of Malic Acid and Unsaturated Fatty Acids on Methanogenesis and Fermentation by Ruminal Microbiota *in vitro*

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Abstract: Methanogens and protozoa in the rumen negatively affect rumen function by wasting ingested energy. It is desirable to modulate rumen fermentation by cost-effective dietary intervention. The objective of this study was to evaluate effects of unsaturated C18 fatty acids (oleic, linoleic and linolenic acids), either alone or in combination with malic acid on *in vitro* ruminal fermentation, protozoa and *Methanobacterium formicicum*. Rumen fluid collected from ruminally fistulated lactating Chinese Holstein cows served as the inoculum and the diet consisted of alfalfa hay and corn (50:50). The results showed addition of unsaturated fatty acids tended to increase fermentation pH ($p>0.05$) and degree of unsaturation of fatty acids tended to affect such effect on the pH ($p>0.05$). Acetate, butyrate, total VFA, total gas production and methane production decreased ($p<0.01$) with increasing degree of unsaturation of C18 fatty acids but addition of malic acid did not have any additive or synergistic effect except for propionate which was decreased by the addition of malic acid ($p<0.01$). Both malate and the unsaturated fatty acids, either alone or in combination, decreased methane production ($p<0.01$) with combination of these two types of acid further decreasing methane production. Except for oleic acid that decreased population of *M. formicicum* decreased ($p<0.01$), either protozoa or *M. formicicum* was affected by these fatty acid and malic acid. It was concluded that when used together, malic acid and fatty acids could reduce methane emission without negative impairing fermentation *in vitro*.

Key words: Malic acid, unsaturated fatty acid, ruminal methanogenesis, synergistic effect, Holstein cows

INTRODUCTION

One goal in improving ruminant nutrition is to reduce the methane emission from the host for global environment protection (Moss *et al.*, 2000) and improved feed utilization. A number of dietary additives have been evaluated for their potency particularly ionophores (Guan *et al.*, 2006). Although, ionophores were shown effective in reducing methane emissions from the rumen, concerns arose over the food safety associated with the use of antimicrobials in dairy cows (Russell and Mantovani, 2002). Also, the effects of antibiotics seems to reduce over time. Non-antimicrobial alternatives including organic acids and oils have attracted much research interest (McCrabb *et al.*, 1998; Carro and Ranilla, 2003).

Malic acid is a four carbon dicarboxylic acid that is found in biological tissues as an intermediate of the citric acid cycle. Malate is also a key intermediate in of conversion of succinate to propionate by some ruminal bacteria including the predominant *Selenomonas ruminantium* (Gottschalk, 1986). Some previous studies

suggested that organic acids can stimulate the growth of *S. ruminantium* and decrease the methane emission from the rumen (Martin, 1998; Martin *et al.*, 1999; Lopez *et al.*, 1999; Mohammed *et al.*, 2004a). Similarly, fatty acids which have been known to be antagonistic against bacteria, yeasts, tumor cells and viruses (Ababouch *et al.*, 1992) have also been shown to be effective in reducing methane production in the rumen when supplied as dietary fats (Van Nevel and Demeyer, 1995; Wettstein *et al.*, 2000). The anti-methanogen activities of fatty acids were attributed to their direct toxic effects on methanogens and competition with methanogens for hydrogen for biohydrogenation of unsaturated fatty acids.

However, it remains to be determined how supplementation levels and degree of unsaturation of fatty acids as well as possible interactions with other anti-methanogen affect the efficacy of methane production in the rumen.

The objective of this study was to determine the effect of fatty acids at different levels and saturations either alone or in combination with malic acid on

methanogenesis, fermentation and growth of ruminal protozoa and *Methanobacterium formicicum* *in vitro*.

MATERIALS AND METHODS

In vitro experiment design: The C18 Fatty Acid (FA) used in this study included Oleic Acid (OA), Linoleic Acid (LA) and Linolenic Acid (LNA) and Malic Acid (MA). A 4×3 factorial experiment was designed with three concentrations of MA (0 mM, MA0; 5 mM, MA5; 10 mM, MA10) and three C18 FA (OA, LA, LNA) 15 mg. Each of these treatments was conducted in triplicate. The experiment was repeated two times with a 3 weeks interval. Rumen fluid was obtained 3 h after the morning feeding from four ruminally fistulated lactating Chinese Holstein dairy cows.

Samples were transported to the laboratory in a sealed container at room temperature and were squeezed through four layers of cheesecloth into fermenters. Each 120 mL fermenter was filled with 20 mL of rumen fluid and 40 mL of prewarmed (39°C) McDougall buffer.

Each fermenter received 0.5 g of substrate consisting of corn and alfalfa meal (1:1, w/w based on DM), the later of which had a particle size <40 mesh. The fermenters were purged with CO₂ then maintained at 39°C with their content being mixed periodically. Each experimental period lasted for 24 h (Mohammed *et al.*, 2004b).

Determination of methane production: Gas production was measured continuously in an automated cumulative gas production estimation system (APES-IGER, Aberystwyth, Wales) for 24 h. At the end of the incubation, 0.2 mL fermentation gas was analyzed for methane content using a gas chromatograph equipped with a flame ionization detector and a column packed with Carboxen 1000 (Supelco, Madrid, Spain). Helium gas was used as the carrier gas.

The methane peak was identified by comparison with that of a methane standard. The yield of methane gas was calculated from the methane concentrations determined and the volume of the fermentation gas from each fermenter.

Analyses of pH, NH₃-N and VFA: The pH of the fermenter fluid was measured with a pH meter. About 10 mL of the fermentation fluid was collected for analysis of both NH₃-N and VFA. For NH₃-N analysis, 3 mL of fermentation fluid was mixed with 1 mL of 25% metaphosphoric acid, centrifuged at 10,000×g for 20 min at 4°C and the concentration of NH₃-N was determined using the Modified Colorimetric Method (Weatherburn, 1967).

Table 1: Primers for real-time PCR assay

Target species	Forward/Reverse		Amplicon (base pairs)
	Reverse	Primer sequence	
<i>Ciliate protozoa</i>	F	GCTTTCGWTGGTAGTGTATT	223
	R	CTTGCCCTCYAATCGTWCT	
<i>Methanobacterium formicicum</i>	F	CACCCCGTTAAGAGTGGCAC	182
	R	GCAGCAGGCGCGAAAC	

The concentration of acetic, propionic and butyric acids was subsequently determined by a gas chromatograph (Mohammed *et al.*, 2004a, b).

Real-time PCR: The fermentation fluid samples for real-time PCR analysis were collected also after the incubation. Total microbiota DNA was extracted using the Bead-Beating Method (Burgmann *et al.*, 2003). The primer sets specific ciliate protozoa (Sylvester *et al.*, 2004) and *Methanobacterium formicicum* (Zhao and Wang, 2006) are shown in Table 1 (the universal archaeal primers was not provided). The species-specific real-time PCR was performed using an Applied Biosystems 7500 System Real-time PCR Detection System (Applied Biosystems, Carlsbad, California, USA).

Statistical analyses: The data were analyzed using a mixed model procedure of SAS (1999) for a 3×4 factorial arrangement with three levels of MA (MA0, MA5, MA10) of and 3 types of AF (OA, LA and LNA) plus no-FA control. For the statistical analysis of ruminal characteristics (pH, VFA, NH₃-N, gas production, methane production) and abundance of protozoa and *M. formicicum*, orthogonal contrasts were used to test for linear and quadratic effects of MA levels. The statistical significance was declared at the p<0.05 unless otherwise noted while trends for significance were declared at p = 0.05-0.10.

RESULTS AND DISCUSSION

Effect of malic acid on rumen fermentation and methanogenesis: The effects of malic acid were compared among the three concentrations tested within each fatty acid supplementation treatment. In the absence of any of the unsaturated fatty acids, the final pH decreased slightly but significantly (p<0.01) as MA concentration increased (Table 2). However, the final pH within the OA group increased by 0.02 pH unit (p<0.01) when 5 mM of MA was added.

Malic acid concentration did not affect the pH in either LA or LNA group. Dicarboxylic acids such as malate, aspartate and fumarate have been tested as feed additives for ruminants (Callaway and Martin, 1996; Martin and Park, 1996). Malate and other dicarboxylic acids were shown to promote lactate utilization by *S. ruminantium*,

Table 2: Effects of combination of fat acid and malic acid on rumen fermentation

Type	Malate ¹ (mM)	pH	mM					Total VFA	Acetate: Propionate	Total gas production (mL g ⁻¹)	Methane production (mmol)	<i>Ciliate</i> <i>protozoa</i> (×10 ⁵ L ⁻¹)	<i>Methano bacterium</i> <i>formicum</i> (×10 ⁸ L ⁻¹)
			NH ₃ -N (mg mL ⁻¹)	Acetrate	Propionate	Butyrate	VFA						
Control	MA0 ¹	6.51	17.3	62.9	16.7	13.7	96.80	3.77	144.80	0.57	0.340	0.24	
	MA5 ²	6.46	17.2	64.0	20.1	14.1	101.8	3.18	150.30	0.44	0.770	1.20	
	MA10 ³	6.42	15.9	65.4	23.0	14.3	106.2	2.84	152.40	0.29	2.520	0.55	
Oleic acid	MA0	6.39	16.0	64.0	18.7	14.5	101.0	3.42	126.1	0.44	44.800	0.29	
	MA5	6.41	15.7	62.8	22.3	14.5	103.3	2.80	140.5	0.38	46.600	0.91	
	MA10	6.38	15.4	63.2	27.0	14.6	108.6	2.33	151.6	0.28	41.300	0.05	
Linoleic acid	MA0	6.49	15.1	59.4	23.6	12.7	99.80	2.62	97.20	0.29	21.800	12.3	
	MA5	6.42	15.2	56.3	25.8	13.3	104.8	2.38	97.10	0.23	5.500	6.38	
	MA10	6.39	14.2	61.8	28.0	12.3	105.5	2.01	107.8	0.18	4.120	3.71	
Linolenic acid	MA0	6.46	13.9	58.2	20.4	11.6	93.50	2.84	104.2	0.30	8.330	4.06	
	MA5	6.45	14.6	54.9	25.8	9.85	93.70	2.13	93.10	0.24	1.770	4.71	
	MA10	6.45	14.8	57.9	32.7	9.63	103.8	1.76	104.5	0.11	4.300	7.43	
SEM		0.013	0.395	1.064	0.951	0.391	1.537	0.100	3.284	0.012	0.951	0.327	
p-values	MA	**	0.373	0.829	**	0.371	**	0.254	0.068	**	0.310	0.205	
	FA	**	**	**	**	**	0.108	**	**	**	0.145	**	
	L ¹	***	0.779	0.548	***	0.171	***	0.217	*	***	0.705	0.555	
	Q	***	0.293	0.815	**	0.592	***	0.725	0.388	***	0.721	0.286	
	FA×MA	0.081	0.211	*	0.074	0.099	*	**	**	**	0.969	0.112	

¹MA0, MA5, MA10: 0, 5 and 10 mM Malate; ¹L: Linear effects of level of malic acid concentration; Q: Quadratic effects of malic acid concentration; *p<0.05; **p<0.01; ***p<0.001

a predominant lactate-utilizing bacterium in the rumen (Nisbet and Martin, 1990). Theoretically, the pH of the fermentation fluid should increase when the utilization of lactate, a much stronger acid is improved by malic acid. The pH decrease observed in the malic acid-supplemented fermenters might be attributed to the acidity of malic acid (pKa = 3.4-5.05; Segel, 1976) itself.

The increased VFA production in the presence of malic acid might also contribute to the observed pH decrease. Malic concentration did not affect the concentration of ammonia in the fermenter liquid irrespective of fatty acid supplementation. The malic acid addition increased the concentration of propionate and total VFA (p<0.01) but not acetate was similar. However, the increase in propionate concentration was relatively small and thus malic acid did not affect acetate:propionate ratio (p>0.05). The observed increases in total VFA confirm the findings of several previous studies where organic acids when added to batch rumen cultures fed concentrate-rich diets, increases total VFA concentration (Carro and Ranilla, 2003; Beauchemin and McGinn, 2006). Organic acids including malate, fumarate and aspartate are intermediates of the citric acid cycle and have similar chemical characteristics.

Giraldo *et al.* (2007) suggested that when fumarate was added to rumen cultures, the observed increase in VFA concentrations mainly results from fermentation of fumarate itself as fumarate can be converted into propionate and acetate by a number of different rumen microorganisms. Nisbet and Martin reported that fumarate increased lactate uptake by *S. ruminantium* by >4 fold while malate stimulated lactate uptake by >10 fold.

Irrespective of the fatty acid added, the total gas production was not affected by the addition of malic acid (p>0.05).

On the contrary, methane production decreased significantly (p<0.01) by 19.05 and 42.86% for addition of 5 and 10 mM malic acid, respectively. The observed change in VFA profiles (increased propionate production) appeared to be consistent with the lowered methane production observed in the malic acid-supplemented fermenters. One possible explanation was that shift from acetate and butyrate to propionate production reduces production of H₂, the primary energy source used by methanogens to form methane (Stewart *et al.*, 1997). Malic acid could be utilized by *S. ruminantium* to synthesize succinate and propionate in the rumen. In this pathway, malic acid might act as an electron sink for hydrogen, competing with methanogens for the available hydrogen (Callaway and Martin, 1996; Martin and Park, 1996; Ungerfeld *et al.*, 2003).

Newbold *et al.* (2005) calculated that about 44% of the hydrogen was used for fumarate reduction in rumen. Malate has been tested as alternative of antibiotic feed additive and addition of malate could promote protozoa and bacteria growth (Carro and Ranilla, 2003; Martin and Streeter, 1995). Mohammed also reported that addition of malate at 10 mM increased protozoa population by 11.36%. No such stimulatory effect was observed in the current study.

Effect of fatty acids on rumen fermentation and methanogenesis: The final pH in all the treatments increased (p<0.01) with the degree of unsaturation of the C18 fatty acids evaluated (Table 2). Ammonia N

concentration was greater ($p < 0.01$) in the control receiving FA supplementation than in the OA-, LA- or LNA-supplemented treatments. The molar proportion of acetate was reduced ($p < 0.01$) but the propionate proportion was increased ($p < 0.01$) markedly with increasing degree of unsaturation of C18 FA.

As a result, both total VFA concentrations ($p < 0.01$) and the acetate to propionate ratio decreased ($p < 0.01$). Compared to the control, total gas production decreased by 28.98 and 32.63% for the LA and LNA treatments, respectively.

Oleic acid did not affect methane production but both linoleic acid and linolenic acids significantly decreased ($p < 0.01$) methane production in the fermentation cultures (Table 2). This observation is consistent with the *in vitro* study by Cieslak *et al.* (2006) who reported that sunflower and linseed oil decreased methane production by 14 and 15%, respectively (Martin and Streeter, 1995).

Beauchenin and McGinn reported that oil supplementation could decrease feed intake due to decreased fiber digestibility in the rumen. The reduction of total tract fiber digestibility also indicates a possible reduction in ruminal digestion of fiber which was corroborated by a reduction in VFA concentration and decreased acetate to propionate ratio in the rumen. The reduced fiber digestibility was attributed, at least partially to inhibition of microbial adhesion to and subsequent degradation of feed fibers due to physical coating of fibers by lipids and to modification of the microbial population due to direct toxicity (Cieslak *et al.*, 2006). Another possible inhibition mechanism is that unsaturated fatty acids serve as a sink for hydrogen that would otherwise be used for methanogenesis.

Hypothetically, as the number of double bonds or degree of unsaturation of fatty acids increases, the inhibition to methanogenesis would increase. However, the relationship between degree of fatty acid unsaturation and inhibition to methanogenesis is inconsistent (Kreuzer and Kirchgessner, 1987). The results showed a positive relationship of potency and unsaturation degree between oleic acid (one double bonds) and linoleic acid (two double bonds) but a null relationship between linoleic acids and linolenic acid (three double bonds). The supplementation with unsaturated fatty acids significantly affected the abundance of *Methanobacterium formicicum* (Table 2). Compared to the control, supplementation with linoleic and linolenic acids significantly increased the abundance of *Methanobacterium formicicum* while oleic acid supplementation had not effect when malic acid was 5 mM or less. However, oleic acid appeared to decrease *Methanobacterium formicicum* in the fermenter that received 10 mM malate. The abundance of total archaeal

population was not effected. Selective inhibition of ruminal methanogens by dietary fats has been reported (Jalc and Ceresnakova, 2001) and this study corroborates that conclusion. The fatty acid supplementation did appear to affect ciliate protozoa.

Effect of fatty acids and malate in combination on rumen fermentation and methanogenesis: The effect of both fatty acids and malate was evaluated by comparison between the malate-only treatment and malate plus fatty treatments at each malate concentration. The results showed that there was no interaction between malate and each of the tested fatty acids with respect to pH, $\text{NH}_3\text{-N}$, propionate and butyrate. However, acetate was decrease ($p < 0.05$) when malate was added at 5 but not 10 mM. Total VFA increased ($p < 0.05$) while total gas and methane production decreased ($p < 0.01$) when both malate and fatty acid were present. These effects appeared to be dependent on the degree of unsaturation of the fatty acids.

The results suggest that under severe acidic conditions malate and the unsaturated fatty acids are not effective in decreasing ruminal pH. When both malate and a fatty acid are added, VFA concentration is expected to remain relatively stable because the fatty acid can decrease VFA while malate increased VFA production. The increase in VFA production observed in this study can not be explained by the data but the results suggest that the fatty acids and malate might not act synergistically in affecting VFA production in the rumen fermenters. However, they had more impact on the concentration of propionate and butyrate, acetate:propionate ratio and CH_4 emission in combination than alone. Fatty acids negated the effect of malate on acetate and $\text{NH}_3\text{-N}$.

With regard to *M. formicicum*, only oleic acid exhibited significant inhibition. Linoleic and linolenic acids tended to increase both *M. formicicum* and protozoa during the fermentation. It remains to be determined why *in vitro* these fatty acid failed to inhibit these two groups of microbes.

CONCLUSION

Collectively, the results suggest that a combination of both unsaturated fatty acid and malic acid might reduce methane emissions *in vitro* without negatively affecting VFA production as often observed when fatty acids were utilized alone. However, *in vivo* studies are needed to evaluate this conclusion and utility of this dietary manipulation approach.

ACKNOWLEDGEMENTS

This research was funded by Institute of Animal Science, Chinese Academy of Agricultural Sciences (2010jc-3-2) and National Key Basic Research Program of China No.: 2011CB100805. The contribution of each researcher to the present research was as follows: Dan Li and Jiaqi Wang designed the study and analysed the data; Fadi Li collected the data and Dan Li wrote the draft of the manuscript. There are no conflicts of interest in the present study.

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