Short Communication: Malic Acid Does Not Promote Vaccenic Acid Accumulation in Mixed Ruminal Fluid with Fractionated Fish Oil by a Rumen-Simulation Technique

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ABSTRACT

The objective of this study was to determine whether malic acid could promote the accumulation of vaccenic acid in the rumen. The control diet was composed of a 65:35 ratio of forage to concentrate with 1% (dry matter basis) added fractionated fish oil (rich in docosahexaenoic acid), and treatment diets consisted of the control diet with added malic acid to achieve final concentrations of 10 mM (treatment 1) and 20 mM (treatment 2), respectively. The experiment was conducted with rumen-simulation equipment (Rusitec) consisting of 9 fermenters. Each treatment included 3 fermenters as replicates. After 7 d of incubation, concentrations of vaccenic acid from treatment 1 (4.38% fatty acids) and treatment 2 (4.46% fatty acids) were similar to that of the control treatment (4.51% fatty acids). The disappearance of docosahexaenoic acid was not different among the control, treatment 1, or treatment 2. These data indicated that malic acid did not promote the accumulation of vaccenic acid in ruminal fluid.

Key words: biohydrogenation, fractionated fish oil, malic acid, rumen-simulation technique

Most of the *cis*-9, *trans*-11 isomer of conjugated linoleic acid (CLA) in milk fat, which is well documented to be beneficial to human health (Belury, 1995; Parodi, 1997), is derived from vaccenic acid (VA; *trans*-11 C18:1) via Δ^9 -desaturase in the ruminant mammary gland (Piperova et al., 2002). Turpeinen et al. (2002) further suggested that VA has potential benefits for human health because it could form *cis*-9, *trans*-11 CLA by the action of Δ^9 -desaturase in the tissues of humans. Many researchers have been interested in how to increase the content of VA in the rumen, because it could enhance both the *cis*-9, *trans*-11 CLA and VA content in the ruminant product, both of which benefit human health. Some research has shown that the ad-

dition of fish oil, which is rich in docosahexaenoic acid (DHA), in diets could increase the content of VA in the rumen more than could plant oils (Scollan et al., 2001; Shingfield et al., 2003). The present consensus on the steps in the biohydrogenation (BH) of α -linolenic acid, linoleic acid, and γ -linolenic acid is that the *cis*-12 double bond will first undergo isomerization, then the *cis*-9 double bond will be hydrogenated, and finally, the *cis*-15 and *cis*-6 double bonds will be hydrogenated to VA, respectively. This leaves VA as the penultimate product subsequent to the formation of stearic acid, a metabolic step that involves the transfer of 2 H (hydrogen ions) to the *trans* double bond. In this pathway, flavin acts as an endogenous donor, transferring H to α -tocopherolquinol in the final reduction step of VA to stearic acid by using the reduced nicotinamide adenine dinucleotide (NADH) pathway (Harfoot and Hazlewood, 1997). Furthermore, the extent of BH of polyunsaturated fatty acids (PUFA) in the rumen is correlative with the rumen environment (e.g., pH; AbuGhazaleh et al., 2005). AbuGhazaleh and Jacobson (2007) observed a greater concentration of *trans* C18:1 under a higher pH condition. Similarly, Martin and Jenkins (2002) noticed less *trans* C18:1 FA formation at culture pH 5.5 compared with culture pH 6.5.

Malic acid is a key intermediate in the succinate-propionate pathway. In this pathway, malic acid might act as an electron sink for H before the dehydration of malic acid to fumarate (Newbold et al., 2005). Previous studies showed that the addition of some organic acids, including aspartate, malate, and fumarate, in the diet could decrease methane production in the rumen (García-Martínez et al., 2005; Newbold et al., 2005). These organic acids may act as electron sinks, competing with methanogens for the available H (Callaway and Martin, 1996; Ungerfeld et al., 2003). Newbold et al. (2005) calculated that approximately 44% of the H was used by fumarate in the rumen before being used for CH₄ formation. Many studies have been conducted to evaluate the effect of malic acid addition on rumen fermentation and rumen microbial populations. Addition of malate in the diet could affect rumen fermenta-

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Table 1. Ingredient and chemical composition content of the basal diet

Item	Content (% of DM)
Ingredient	
Alfalfa hay	23.0
Chinese wild rye	42.0
Corn	16.5
Wheat bran	6.0
Soybean meal	8.0
Cottonseed meal	2.0
Calcium carbonate	0.6
Calcium phosphate, dibasic	0.6
Sodium chloride	0.3
Fractionated fish oil ¹	1.0
Chemical composition of total diet (% of DM)	
CP	14.22
Ether extract	4.02
Net energy (MJ/kg)	4.98
NDF	49.75
ADF	30.43
Ca	1.2
P	0.8

¹Fractionated fish oil was dissolved in ethanol before being added directly to the fermenter.

tion by increasing VFA concentration, increasing pH, and changing the microbial populations and activity (Carro and Ranilla, 2003; Gomez et al., 2005).

Thus, we hypothesized that supplementing malic acid in the rumen might promote VA accumulation. We speculated that this might be via some potential mechanism if it was similar to what we expected. One potential mechanism is that malic acid might act as a competitive inhibitor for hydrogenation of PUFA or might act as an electron sink, disturbing the electron transfer in the BH of PUFA. Another is that malic acid might affect rumen fermentation, which then affects the BH of PUFA. Thus, the objective of this study was to evaluate whether malic acid could promote the accumulation of VA in the rumen.

Malic acid (99% purity) was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Fractionated fish oil (rich in DHA, 85% purity) was purchased from Hebei Haiyuan Chemical Company (Haiyuan City, Hebei Province, P. R. China). Before the experiment was conducted, 40 g of fractionated fish oil was dissolved in 200 mL of ethanol (0.2 g in 1 mL) and stored at 4°C. Two ruminally fistulated lactating Holstein dairy cows were fed a TMR composed of 50% concentrate mix [composed mainly of corn (26%), wheat bran (4.5%), soybean meal (11%), cottonseed meal (4%), and rapeseed meal (2%), with the other components being minerals and salt], 25% corn silage, and 25% alfalfa hay (on a DM basis), and inoculum fluid was drawn with a vacuum pump 2 h after the morning feeding and strained through 4 layers of cheesecloth. Inoculum fluid obtained from the 2 cows was mixed in

a bottle and transferred to the laboratory under anaerobic conditions at 39°C and then distributed to each fermenter within 20 min. The control diets consisted of a 65:35 ratio of forage to concentrate with 1% (on a DM basis) fractionated fish oil (high-DHA diet; Table 1). The treatment diets consisted of the control with malic acid added to achieve a final concentration of 10 mM (**Trt1**) and 20 mM (**Trt2**), respectively. Each treatment contained 3 fermenters as replicates. The experimental period consisted of 6 d for adaption and 1 d for sampling, and was repeated 2 times within a 3-wk interval.

Each fermenter, with an effective volume of 2,000 mL, was filled with 800 mL of inoculum fluid and 800 mL of prewarmed buffer according to the method of McDougall (1948). The fermenters, which were maintained at 39°C, were mixed constantly at 15 rpm via an impeller stirrer and purged with CO₂ gas (99% purity). The pH of each fermenter was regulated by addition of McDougall's buffer (9.8 g of NaHCO₃, 0.57 g of KCl, 9.3 g of Na₂HPO₄·12H₂O, 0.47 g of NaCl, 0.12 g of MgSO₄·7H₂O, and 0.04 g of CaCl₂ dissolved in 1,000 mL of distilled water and adjusted to pH 6.9 using CO₂; McDougall, 1948) through a tube connected to a pump at a continuous rate of 0.78 mL/min. A total of 40 g of substrate was placed into each fermenter daily in 2 equal portions, at 0800 and 2000 h. After replenishing diets in the fermenters daily, fractionated fish oil in 1 mL of ethanol was added directly to all the fermenters, and 2.68 and 5.36 g of malic acid was added to the Trt1 and Trt2 fermenters, respectively. To maintain anaerobic conditions, all fermenters were purged with CO₂ gas for 3 min.

On the last day of the experiment, 3 aliquots of a 10-mL sample (a mixture of liquid and solids) from each fermenter were collected with an injection syringe from the CO₂ airscoop at 0 h (before the morning feeding) and 3, 6, and 9 h later and immediately stored at -20°C until analysis. For extraction of fatty acids (FA) from the culture fluid, chloroform:methanol (2:1, vol/vol) was prepared (Kramer and Zhou, 2001). The chloroform solution containing the FA was dried under N₂, and the FA in the culture fluid were then methylated according to the method of Kramer et al. (1997). Separation of the individual FA in the samples was achieved by using a gas chromatograph (model 6890, Series II, Hewlett-Packard Co., Avondale, PA) fitted with a flame-ionization detector and a fused-silica SP2560 capillary column (100 m × 0.25 mm i.d., 0.20 µm film thickness; Supelco Inc., Bellefonte, PA). Detailed procedures and detection conditions for FA analysis were described previously (Bu et al., 2007). Heptadecanoic acid was used as a quantitative FFA internal standard. Each peak was identified by comparison with standard

Table 2. Composition of fatty acids of the basal diet and fractionated fish oil fed to the artificial rumen (Rusitec)

Fatty acid profile	Basal diet ¹ (%)	Fractionated fish oil (%)
C16:0	12.3	ND ²
C18:0	3.8	ND
<i>trans</i> -11 C18:1 (vaccenic acid)	ND	ND
<i>cis</i> -9 C18:1	10.5	ND
<i>cis</i> -9, <i>cis</i> -12 C18:2	25.2	0.17
C18:3	12.5	0.08
C20:0	0.7	ND
<i>cis</i> -9, <i>trans</i> -11 C18:2 (conjugated linoleic acid)	0.2	ND
C20:2	0.5	0.21
C20:5 (eicosapentaenoic acid)	ND	0.10
C24:0	ND	ND
C22:5	3.1	7.16
C22:6 (docosahexaenoic acid)	30.0	80.93
Other fatty acids	1.2	11.35

¹Basal diet included concentrate, forage, and fractionated fish oil.²ND = not detected or detected at <0.01.

FA methyl esters (**FAME**; Nu-Chek Prep, Elysian, MN; Matreya, Pleasant Gap, PA; and 37-Component FAME mix, Supelco, Bellefonte, PA). The percentage of each FA was calculated by dividing the area under the FA peak (minus the area under the peak for heptadecanoic acid) by the sum of the areas under all the reported peaks. All results were reported as the percentage of FAME. The data were analyzed by using PROC MIXED of SAS, version 8.2 (SAS Institute Inc., Cary, NC). Each fermenter was defined as a random effect, whereas treatments and sampling times were defined as fixed effects. For statistical analysis of the ruminal fluid, treatment, sampling time, and the interaction of sampling time $\times$ treatment were defined as fixed effects and analyzed by using repeated measures. Least squares means and pooled SEM were reported for all data. Significance was set at $P < 0.05$.

If malic acid were able to compete for H with BH of PUFA at the step from VA to stearic acid or if malic acid acted as an electron sink, disturbing the electron transfer of BH in the NADH pathway when incubated in mixed ruminal fluid with high-DHA diets, this would lead to a decreased extent of BH of FA, an increased accumulation of VA, and a decreased stearic acid concentration. However, after 6 d of adaption, the concentrations of VA among the control (4.51% FAME), Trt1 (4.38% FAME), and Trt2 (4.46% FAME) cultures were not different ($P > 0.05$). Addition of malic acid did not change the concentration of stearic acid in the cultures. Moreover, the disappearance of DHA was similar among the control (60.5%), Trt1 (62.7%), and Trt2 (56.9%) cultures by calculation based on Tables 2 and 3. In addition, concentrations of each FA in the culture fluid did not differ with sampling time, except for VA ($P < 0.01$), *cis*-9, *cis*-12 C18:2 ($P < 0.01$), and DHA

($P < 0.01$). The interaction of treatment and sampling time was not different ($P > 0.05$), except for C18:0 and DHA.

Previously, AbuGhazaleh and Jenkins (2004) concluded that DHA or its derivatives promoted VA accumulation, possibly by inhibiting the reductase enzymes from ruminal microorganisms that were responsible for the terminal hydrogenation of VA to stearic acid. However, results obtained from the present study indicated that addition of malic acid in ruminal fluid did not promote the accumulation of VA. Harfoot and Hazlewood (1997) considered that although unsaturated FA were known to inhibit methanogenesis in the rumen, they did so at millimolar concentrations, which are too low to serve as major competitors for H. We considered that the H used by malic acid may not affect the H that would be used by unsaturated FA, because the amount of H in the rumen used by unsaturated FA is very limited (Jenkins et al., 2008). Malic acid may possibly act as an electron sink that does not disturb the electron transfer of BH of FA in the NADH pathway. In the current study, addition of malic acid did not affect the concentration of FA. This was inconsistent with our theoretical deduction. The change in rumen pH has been shown to have a negative effect on microbial populations (Martin et al., 2002), particularly on the growth of cellulolytic bacteria, which are indeed the main rumen biohydrogenating bacteria (Harfoot and Hazlewood, 1997). Latham et al. (1972) found that switching lactating dairy cows from a high-forage to a low-forage diet, which promotes low rumen pH, resulted in lower levels of lipolytic activity and BH of unsaturated FA in ruminal fluid, as measured by an in vitro experiment. Accordingly, the addition of malic acid could have affected the concentration of FA.

Table 3. Fatty acid profiles (fatty acids as a % of fatty acid methyl esters) in the Rusitec fermenter content with or without addition of malic acid

Fatty acid (n = 6) ¹	Treatment ²				P-value		
	Control	Trt1	Trt2	SEM	Trt ³	samT ⁴	Trt × samT ⁵
C6:0	2.19	2.10	1.87	0.30	0.0729	0.5324	0.0078
C8:0	2.07	2.18	1.92	0.24	0.7338	0.0180	0.1937
C10:0	3.99	4.13	3.50	0.31	0.1459	0.0092	0.1830
C12:0	2.74	2.98	2.41	0.56	0.1351	0.0700	0.2724
C14:0	5.19	5.08	4.86	0.49	0.5283	0.3869	0.6745
C15:0	2.05	1.93	2.19	0.04	0.3862	0.0222	0.5994
C16:0	22.39	21.97	22.58	1.12	0.9260	0.7437	0.3115
C18:0	17.71	20.04	17.32	2.24	0.4441	0.0475	0.0017
<i>cis</i> -9 C18:1	4.92	4.76	4.85	0.26	0.9084	0.2522	0.1065
<i>trans</i> -11 C18:1 (vaccenic acid)	4.51	4.38	4.46	0.87	0.3231	0.0009	0.0816
<i>cis</i> -9, <i>trans</i> -11 conjugated linoleic acid	2.39	2.12	2.23	0.17	0.6543	0.1923	0.3149
C18:3	1.23	1.18	1.21	0.08	0.6593	0.0852	0.4726
<i>cis</i> -9, <i>cis</i> -12 C18:2	2.29	2.19	2.34	0.11	0.1741	0.0004	0.3881
C22:6 (docosahexaenoic acid)	11.85	11.20	12.91	1.12	0.1629	<0.0001	0.0334

¹n = number of samples in each mean.²Trt1 = control diet with 10 mM malic acid; Trt2 = control diet with 20 mM malic acid.³Trt = experimental treatment.⁴samT = sampling time.⁵Trt × samT = interaction of treatment and sampling time.

The reasons that the results from our study may be opposite our theoretical deductions are unclear. In the present experiment, the change in FA concentration in the fermenter contents across sampling times was consistent with the results of Váradyová et al. (2007). Interestingly, we found that the changes in some FA (C8:0, C10:0, *trans*-11 C18:1, *cis*-9, *cis*-12 C18:2, C22:6) across sampling times were different among treatments ($P < 0.05$), but the interaction of treatment and sampling time did not exert an obvious effect, except for C22:6 and C18:0, for which the reason was unclear. In summary, our results indicated that malic acid had no effect on VA accumulation and a decrease in stearic acid in the fermenter contents.

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