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Effects of Addition Level and Chemical Type of Propionate Precursors in Dicarboxylic Acid Pathway on Fermentation Characteristics and Methane Production by Rumen Microbes *In vitro**

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ABSTRACT : Two *in vitro* experiments were conducted to examine the effects of propionate precursors in the dicarboxylic acid pathway on ruminal fermentatation characteristics, CH_4 production and degradation of feed by rumen microbes. Fumarate or malate as sodium salts (Exp. 1) or acid type (Exp. 2) were added to the culture solution (150 ml, 50% strained rumen fluid and 50% artificial saliva) to achieve final concentrations of 0, 8, 16 and 24 mM, and incubated anaerobically for 0, 1, 3, 6, 9 and 12 h at 39°C. For both experiments, two grams of feed consisting of 70% concentrate and 30% ground alfalfa (DM basis) were prepared in a nylon bag, and were placed in a bottle containing the culture solution. Addition of fumarate or malate in both sodium salt and acid form increased ($p<0.0001$) pH of culture solution at 3, 6, 9 and 12 h incubations. The pH ($p<0.0001$) and total volatile fatty acids (VFA, $p<0.05$) were enhanced by these precursors as sodium salt at 3, 6 and 9 h incubations, and pH ($p<0.001$) and total VFA ($p<0.01$) from fumarate or malate in acid form were enhanced at a late stage of fermentation (9 h and 12 h) as the addition level increased. pH was higher ($p<0.001$) for fumarate than for malate as sodium salt at 3 h and 6 h incubations. Propionate (C_3) proportion was increased ($p<0.0001$) but those of C_2 ($p<0.05$) and C_4 ($p<0.01$ - $p<0.001$) were reduced by the addition of sodium salt precursors from 3 h to 12 incubation times while both precursors in acid form enhanced ($p<0.011$ - $p<0.0001$) proportion of C_3 from 6h but reduced ($p<0.018$ - $p<0.0005$) C_4 proportion at incubation times of 1, 3, 9 and 12 h. Proportion of C_3 was increased ($p<0.05$ - $p<0.0001$) at all incubation times by both precursors as sodium salt while that of C_3 was increased ($p<0.001$) from 6h but C_4 proportion was decreased by both precursors in acid form as the addition level increased. Proportion of C_3 was higher ($p<0.01$ - $p<0.001$) for fumarate than malate as sodium salt from 6 h incubation but was higher for malate than fumarate in acid form at 9 h ($p<0.05$) and 12 h ($p<0.01$) incubation times. Increased levels (16 and 24 mM) of fumarate or malate as sodium salt ($p<0.017$) and both precursors in acid form ($p<0.028$) increased the total gas production, but no differences were found between precursors in both chemical types. Propionate precursors in both chemical types clearly reduced ($p<0.0001$ - $p<0.0002$) CH_4 production, and the reduction ($p<0.001$ - $p<0.0001$) was dose dependent as the addition level of precursors increased. The CH_4 generated was smaller ($p<0.01$ - $p<0.0001$) for fumarate than for malate in both chemical types. Addition of fumarate or malate as sodium type reduced ($p<0.004$) dry matter degradation while both precursors in both chemical types slightly increased neutral detergent fiber degradability of feed in the nylon bag. (**Key Words** : Propionate Precursors, Chemical Type, Fumarate, Malate, Addition Level, Fermentation, Total Gas, Methane)

INTRODUCTION

Methane (CH_4) is one of the most important greenhouse gases and domestic ruminants are responsible for 25% of these emissions (Makkar and Vercoe, 2007). Since its release to the atmosphere represents an energy loss of between 2% and 15% of ingested gross energy (Van Nevel and Demeyer, 1996) reducing its emission would benefit both ruminant production and environment (Van Nevel and Demeyer, 1996). Ionophores and unsaturated fatty acids (Demeyer and Fievez, 2000), anthraquinones (Kung et al., 2003), sulphur-containing amino acids (Takahashi, 2001), inhibitors of methanogenic archaea (Miller and Wolin, 2001) and inhibitors of oxidative pyruvate decarboxylation (Ungerfeld et al., 2003b) have been studied as potential feed additives to suppress CH_4 production in the rumen.

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Although some chemicals are capable of decreasing the ruminal CH_4 production, most of them not only depress microbial activities or population but also depress fiber digestibility (Chen and Wolin, 1979; Hinto, 1981).

Since the metabolic process in CH_4 generation is principally a sink for metabolic H_2 in the rumen, a possible alternative to suppress it might be the use of compounds, such as propionate precursors (Ungerfeld et al., 2003a), which may act as an electron sink competing with methanogens for the available H_2. Both fumarate and malate, that may act as H_2 acceptors (Martin and Park, 1996), are key propionate precursors in the dicarboxylic acid pathway (Castillo et al., 2004). Additional effects of these precursors have been increased pH, total volatile fatty acid (VFA) production and concentration of propionate (Martin and Streeter, 1995; Carro and Ranilla, 2003) in the rumen. It would be ideal to shift the fermentation pathway to one that increases propionate (C_3) as well as reduces CH_4 production in order to improve the energetic efficiency of ruminant animals. Two different chemical types of fumarate and malate are commercially available, but only their sodium salts have been applied (Martin and Streeter, 1995; Carro and Ranilla, 2003; Ungerfeld et al., 2003a) in the examination of ruminal fermentation and CH_4 production.

The objective of the present study, therefore, was to examine the effect of the addition level and type of propionate precursors in the dicarboxylic acid pathway on ruminal fermentation characteristics, methane generation and feed degradation by rumen microbes *in vitro*.

MATERIALS AND METHODS

Preparation of culture solution and its incubation

In vitro experiment 1. Influence of sodium fumarate or sodium malate : *In vitro* incubation was carried out with rumen fluid obtained 2 h after the morning feeding (08:00) from three ruminally cannulated Holstein cows fed the diet of 5 kg daily consisting of concentrate for non-lactating dairy cows (60%) and rice straw (40%) on a dry matter (DM) basis twice daily in an equal amount. The rumen contents collected from 3 cows were blended in equal volume in a Waring blender (Fisher 14-509-1) for 20 seconds to detach the bacteria from the feed particles, and were strained through 12 layers of muslin to remove the feed particles and large protozoa. CO_2 was flushed into the strained rumen fluid for 30 seconds.

Under flushing of CO_2, 75 ml strained rumen fluid was mixed with 75 ml artificial saliva (1:1, v/v) consisting of 2.0 g NaCl, 0.5 g $(NH_4)_2SO_4$, 1.0 g K_2HPO_4, 1.0 g KH_2PO_4, 0.265 g $CaCl_2 \cdot 2H_2O$, 0.409 g $MgSO_4 \cdot 7H_2O$ per L. Sodium salts of fumarate (Sigma, Sodium fumarate dibasic, F1506, 98%) or malate (Sigma, DL-malic acid disodium salts, M6773, 99%) were then added to the mixed solution (150 ml) to achieve a final concentration of 0, 8, 16 or 24 mM. Two grams of feed consisting of 70% concentrate and 30% ground alfalfa on a DM basis were prepared in a nylon bag, and were placed in the bottle containing the mixed solution. The bottles were then sealed with butyl rubber stoppers fitted with 3-way stopcocks and were incubated anaerobically for 12 h at 39°C. Incubation was also made without malate or fumarate (Control) and incubation for each chemical type was made three times with duplicates under similar conditions.

In vitro experiment 2. Influence of fumaric acid or malic acid : Preparation and incubation of the culture solution was similar to experiment 1 except that fumarate (Sigma-Aldrich, Fumaric acid, F19353, 99%) or malate (Sigma-Aldrich, DL-malic acid, M0875, 98%) as the acid form of propionate precursors were added to the mixed solution (150 ml) to achieve a final concentration of 0, 8, 16 or 24 mM.

Measurements and analyses

In both experiments 1 and 2, incubation was stopped by removing the bottles from the shaking incubator at 0, 1, 3, 6 and 12 h, and pH of culture solution was immediately measured. At the same time an aliquot of culture solution (0.8 ml) was collected from each bottle for ammonia and VFA analysis. All samples collected were kept frozen at -20°C until analyzed. Ammonia concentration was determined by the method of Fawcett and Scott (1960) using a spectrophotometer (DU-650). Culture solution (0.8 ml) was mixed with 0.2 ml 25% phosphoric acid and 0.2 ml pivalic acid solution (2%, w/v) as the internal standard. VFA were determined by gas chromatograph (HP5890 series II, Hewlett Packard Co.) equipped with flame ionization detector (FID). The total gas production from the incubation bottle was measured at the incubation times of 1, 3, 6, 9 and 12 h through the 3-way stopcock using a 50 ml glass syringe connected to a needle. A gas sample was transferred to a vacuum tube from each bottle and analyzed for CH_4 by the same gas chromatograph as for VFA. The oven temperature was 40°C and flow rate of carrier gas (He) was adjusted to 30 ml/min. The CH_4 peak was identified with standard gas. DM degradation of feed in the nylon bag was measured after the experiment was terminated. The nylon bag containing feed residue was washed with tap water and dried at 110°C for 24 h in a forced-air drying oven to measure DM degradation. Neutral detergent fiber (NDF) content of feed prior to the experiment and that in the nylon bag after 12 h incubation was analyzed by the method of Van Soest et al. (1991).

Statistical analysis

The results obtained were subjected to least squares analysis of variance according to the general linear models procedure of SAS (1985) and significances were compared by S-N-K's Test (Steel and Torrie, 1980). Direct comparison

of data between chemical type of propionate precursor was not possible because the two experiments were conducted one after another. Thus, statistical analysis of the data was made within precursors only. Contrast analysis was applied to compare the effects of source (precursors) and addition level of each precursor, and interaction between source and level was also examined.

RESULTS

In vitro experiment 1. Effect of sodium fumarate and DL-malic acid disodium on fermentation characteristics,

Table 1. pH, ammonia-N concentration, and concentration and proportions of major volatile fatty acids (VFA) in culture solution as influenced by addition of fumarate or malate as sodium salt

	Treatments (Sodium salts, mM)[1]								SEM[2]	Pr<F[3]	Effects[4]		
	Fumarate				Malate						Source	Level	S×L
	0	8	16	24	0	8	16	24			(S)	(L)	
	1 h												
pH	6.25	6.30	6.26	6.27	6.25	6.24	6.12	6.34	0.054	0.637	NS	NS	NS
Ammonia-N (mg/100 ml)	9.14	10.25	10.70	10.93	9.14	11.42	11.28	11.45	1.473	0.748	NS	NS	NS
Total VFAs (mmoles/100 ml)	45.15	41.96	44.82	43.21	45.15	40.04	40.62	35.12	4.984	0.529	NS	NS	NS
Molar proportion (mmoles/100 mmoles)													
Acetate (C_2)	53.06	53.98	54.09	55.23	53.06	53.06	54.05	55.64	2.10	0.725	NS	NS	NS
Propionate (C_3)	23.79	24.55	23.18	23.05	23.79	24.52	23.57	22.81	0.532	0.055	NS	*	NS
Butyrate	15.51	15.44	16.06	15.73	15.51	16.04	16.11	15.61	0.771	0.937	NS	NS	NS
C_2/C_3	2.23^{ab}	2.20^{ab}	2.33^{ab}	2.40^{ab}	2.23^{ab}	2.16^{b}	2.29^{ab}	2.44^{a}	0.059	0.021	NS	**	NS
	3 h												
pH	5.97^{d}	6.14^{b}	6.25^{a}	6.21^{a}	5.97^{d}	6.08^{c}	6.14^{bc}	6.12^{bc}	0.015	<0.0001	***	***	**
Ammonia-N (mg/100 ml)	12.79	12.61	13.70	12.10	12.79	12.78	12.73	13.76	1.101	0.766	NS	NS	NS
Total VFAs (mmoles/100 ml)	59.33	62.43	58.81	63.68	59.33	65.77	60.33	62.76	3.942	0.724	NS	NS	NS
Molar proportion (mmoles/100 mmoles)													
Acetate (C_2)	50.77	48.30	50.11	51.05	50.77	49.23	49.61	50.83	0.807	0.037	NS	*	NS
Propionate (C_3)	25.99^{e}	30.45^{a}	29.44^{ab}	27.94^{d}	25.99^{e}	29.02^{bc}	29.64^{ab}	28.20^{cd}	0.301	0.0001	NS	***	*
Butyrate	16.88^{a}	15.37^{b}	14.70^{b}	15.18^{b}	16.88^{a}	15.41^{b}	15.08^{b}	15.28^{b}	0.406	0.011	NS	**	NS
C_2/C_3	1.95^{a}	1.59^{d}	1.70^{bcd}	1.83^{b}	1.95^{a}	1.70^{bcd}	1.67^{cd}	1.80^{bc}	0.042	0.001	NS	***	NS
	6 h												
pH	5.73^{d}	5.98^{c}	6.19^{a}	6.23^{a}	5.73^{d}	5.94^{c}	6.09^{b}	6.12^{b}	0.020	<0.0001	***	***	*
Ammonia-N (mg/100 ml)	12.91	13.38	12.46	13.29	12.91	13.39	13.71	12.58	1.879	0.982	NS	NS	NS
Total VFAs (mmoles/100 ml)	70.89	75.72	78.68	80.14	70.89	73.5	81.4	83.3	3.728	0.093	NS	*	NS
Molar proportion (mmoles/100 mmoles)													
Acetate (C_2)	48.82	47.76	46.99	46.99	48.82	48.32	47.72	47.68	0.600	0.161	NS	*	NS
Propionate (C_3)	25.47^{a}	29.42^{b}	32.29^{c}	33.70^{d}	25.47^{a}	28.60^{e}	30.75^{f}	31.57^{g}	0.321	<0.0001	***	***	**
Butyrate	19.07^{a}	16.86^{ab}	15.33^{b}	15.25^{b}	19.07^{a}	16.91^{b}	15.87^{b}	15.32^{b}	0.448	0.001	NS	***	NS
C_2/C_3	1.92a	1.62^{b}	1.46^{de}	1.39^{e}	1.92a	1.69^{b}	1.55^{b}	1.51^{cd}	0.029	<0.0001	***	***	NS
	9 h												
pH	5.60^{d}	5.85^{c}	6.04^{b}	6.16^{a}	5.60^{d}	5.81^{c}	5.99^{b}	6.08^{b}	0.030	<0.0001	NS	***	NS
Ammonia-N (mg/100 ml)	14.45	13.94	12.09	13.46	14.45	14.03	13.21	13.98	0.801	0.256	NS	NS	NS
Total VFAs (mmoles/100 ml)	76.70	82.51	84.4	90.23	76.70	79.37	82.58	81.53	3.790	0.157	NS	*	*
Molar proportion (mmoles/100 mmoles)													
Acetate (C_2)	48.99	48.30	48.51	46.63	48.99	48.41	47.44	47.38	0.766	0.090	NS	*	NS
Propionate (C_3)	24.93^{d}	28.38^{c}	30.43^{b}	33.24^{a}	24.93^{d}	27.99^{b}	30.22^{a}	31.21^{a}	0.235	<0.0001	***	***	***
Butyrate	19.74^{a}	17.54^{b}	15.85^{bc}	15.24^{c}	19.74^{a}	17.78^{b}	16.69^{bc}	15.89^{bc}	0.685	0.002	NS	***	NS
C_2/C_3	1.96^{a}	1.70^{b}	1.59^{c}	1.40^{d}	1.96^{a}	1.73^{b}	1.57^{c}	1.52^{c}	0.036	<0.0001	NS	***	NS
	12 h												
pH	5.54^{d}	5.78^{c}	5.95^{b}	6.10^{a}	5.54^{d}	5.75^{c}	5.92^{b}	6.06^{a}	0.040	<0.0001	NS	***	NS
Ammonia-N (mg/100 ml)	15.09	13.28	13.95	14.81	15.09	16.32	16.39	16.98	1.250	0.169	*	NS	NS
Total VFAs (mmoles/100 ml)	81.67	84.71	92.34	89.07	81.67	85.63	88.43	99.78	7.806	0.485	NS	NS	NS
Molar proportion (mmoles/100 mmoles)													
Acetate (C_2)	48.21^{a}	48.17^{a}	48.01^{a}	46.47^{b}	48.21^{ab}	49.19^{a}	48.05^{b}	48.37^{a}	0.464	0.022	*	*	NS
Propionate (C_3)	24.52^{c}	27.49^{d}	29.93^{b}	32.59^{a}	24.52^{d}	26.82^{c}	28.89^{b}	30.01^{a}	0.321	<0.0001	**	***	**
Butyrate	20.59^{a}	18.36^{b}	16.65^{c}	15.85^{c}	20.59^{a}	18.11^{b}	17.29^{bc}	16.19^{cd}	0.471	0.0001	NS	***	NS
C_2/C_3	1.97^{a}	1.75^{b}	1.60^{b}	1.43^{c}	1.97^{a}	1.83^{b}	1.66^{c}	1.61^{c}	0.032	<0.0001	**	***	NS

[1] Means in the same row with different letters differ. [2] Standard error of means. [3] Pr<F, significant level.

[4] Source, fumarate vs. malate; level, addition level of fumarate or malate; S×L, interaction between fumarate and malate.

* p<0.05; ** p<0.01; *** p<0.001; NS = Non significant.

methane production, and degradation of DM and NDF of feed : Fermentation characteristics (pH, ammonia-N and VFA) as influenced by type of propionate precursor as sodium salt and their addition level are shown in Table 1. Concentrations of ammonia-N and total VFA in the culture solution were increased but pH was decreased for all treatments as incubation time advanced. pH of the culture solution increased ($p<0.0001$) and total VFA tended to increase but ammonia-N content was not influenced by propionate precursors compared to the control. pH ($p<0.0001$) and total VFA ($p<0.05$) were enhanced as addition level of both precursors increased at 3, 6 and 9 h incubations, but ammonia-N content was not different among addition levels. pH was higher ($p<0.001$) for fumarate than for malate at incubation times of 3 h and 6 h, while ammonia-N content was higher ($p<0.05$) for malate than for fumarate. Total VFA was not different between precursors. Interactions between precursor and addition level were not observed in pH, contents of ammonia-N and total VFA in the culture solution.

Acetate (C_2) proportion was slightly reduced up to 6 h incubation regardless of precursor and addition levels. Propionate (C_3) proportion was increased ($p<0.0001$) while butyrate (C_4) was reduced by the addition of sodium salt propionate precursors at 3 ($p<0.011$), 6 ($p<0.001$), 9 ($p<0.002$) and 12 h ($p<0.0001$) incubations (Table 1) compared to those by control. Proportion of C_3 was increased ($p<0.05$ - $p<0.0001$) at all incubation times but those of C_2 ($p<0.05$) and C_4 ($p<0.051$ - $p<0.001$) at 3, 6, 9 and 12 h incubations were reduced as the addition level of both precursors as sodium salt increased. Proportions of C_2 ($p<0.05$) at 12 h was higher for malate than for fumarate while that of C_3 was higher for fumarate than malate at 6 ($p<0.001$), 9 ($p<0.001$) and 12 h ($p<0.01$) incubations. However, no differences were observed between precursors in C_4 proportion. The C_2/C_3 ratio from malate addition was higher ($p<0.0001$) than those from fumarate at 6, 9 and 12 h incubation.

Relatively high levels of fumarate (24 mM) and malate (16 and 24 mM) increased ($p<0.017$) the total gas production compared to control and low level treatments, but no difference was found between sodium salt precursors (Table 2). Both propionate precursors clearly reduced ($p<0.0002$) CH_4 production compared to the control, and CH_4 production was linearly decreased ($p<0.0001$) as the addition level of precursors increased (Table 2). Less CH_4 was generated ($p<0.0001$) for fumarate than for malate treatments. Interaction between precursor and addition level in CH_4 production was observed in which reduced rate of methane production was greater ($p<0.01$) for fumarate than for malate (Table 2).

Addition of fumarate or malate as sodium salt reduced ($p<0.004$) DM degradation of the incubated substrate compared to the control, and DM degradation was greater ($p<0.01$) for malate than for fumarate (Table 3). DM degradation with malate was not influenced but that with fumarate was reduced as addition level of precursor increased ($p<0.01$). Degradation of NDF in feed was not influenced by precursor or addition level.

In vitro experiment 2. Effect of fumaric acid and DL-malic acid on fermentation characteristics, methane production, and degradation of DM and NDF of feed : Fermentation characteristics (pH, ammonia-N and VFA) as influenced by propionate precursors in acid form and their addition level are shown in Table 4. Concentrations of ammonia-N and total VFA in the culture solution were

Table 2. Total gas and methane production as influenced by addition of fumarate and malate as sodium salt for 12 h incubation

Item	Treatments (Sodium salt)[1]								SEM[2]	Pr<F[3]	Effects[4]		
	Fumarate				Malate						Source	Level	S×L
	0	8	16	24	0	8	16	24			(S)	(L)	
Total gas (ml)	168.0^{b}	222.0^{ab}	226.7^{ab}	256.3^{a}	168.0^{b}	221.0^{ab}	242.8^{a}	253.0^{a}	18.117	0.017	NS	***	NS
CH_4 (ml)	15.58^{a}	14.56^{ab}	11.27^{c}	10.79^{c}	15.58^{a}	15.07^{a}	13.46^{b}	13.23^{b}	2.015	0.0002	***	***	**
CH_4 (μmol)	635.1^{a}	593.5^{ab}	459.5^{c}	439.6^{c}	635.1^{a}	614.5^{a}	548.7^{b}	539.2^{b}	20.212	0.0002	***	***	**

[1] Means in the same row with different letters differ. [2] Standard error of means. [3] Pr<F, significant level.
[4] Source, fumarate vs. malate; level, addition level of fumarate or malate; S×L, interaction between fumarate and malate.
** $p<0.01$; *** $p<0.001$; NS = Non significant.

Table 3. Degradation (%) of DM and NDF in supplemented substrate as influenced by addition of fumarate or malate as sodium salt for 12 h incubation

	Treatments (Sodium salts, mM)[1]								SEM[2]	Pr<F[3]	Effects[4]		
	Fumarate				Malate						Source	Level	S×L
	0	8	16	24	0	8	16	24			(S)	(L)	
DM	55.80^{a}	50.33^{b}	51.90^{b}	52.37^{b}	55.80^{a}	54.24^{a}	53.75^{ab}	52.3^{ab}	0.007	0.0037	**	NS	*
NDF	28.94	29.48	31.74	30.06	28.94	31.4	32.34	0.016	0.0917	0.074	NS	NS	NS

[1] Means in the same row with different letters differ. [2] Standard error of means. [3] Pr<F, significant level.
[4] Source, fumarate vs. malate; level, addition level of fumarate or malate; S×L, interaction between fumarate and malate.
** $p<0.01$; NS = Non significant.

increased as the incubation time advanced. Addition of fumarate slightly increased pH at 6, 9 and 12 h incubation while malate had a tendency to increase pH at all incubation times. Addition of both precursors decreased ($p<0.043$) ammonia-N content at 12 h incubation. pH of the culture solution was lowered ($p<0.001$) by fumarate at 3 h but both precursors in acid form increased ($p<0.001$) pH at 9, and 12 h incubation as the addition level increased. Ammonia-N content was decreased ($p<0.01$) while that of total VFA increased ($p<0.01$) at 12 h incubation only as the addition level increased.

Proportions of C_3 and C_4 had a tendency to be increased

Table 4. pH, ammonia-N concentration, and concentration and proportions of major volatile fatty acids (VFA) in culture solution as influenced by addition of fumarate or malate in acid form

	Treatments (Free acid, mM)[1]								SEM[2]	Pr<F[3]	Effects[4]		
	Fumarate				Malate						Source	Level	S×L
	0	8	16	24	0	8	16	24			(S)	(L)	
	1h												
pH	5.77	5.78	5.72	5.65	5.77	5.87	5.87	5.87	0.030	0.001	***	NS	***
Ammonia-N (mg/100 ml)	11.27	8.86	8.64	10.69	11.27	11.2	11.45	11.37	0.924	0.132	*	NS	NS
Total VFAs (mmoles/100 ml)	48.03	44.72	41.20	40.64	48.03	41.60	40.41	40.67	2.068	0.062	NS	**	NS
Molar proportion (mmoles/100 mmoles)													
Acetate (C_2)	50.33	50.57	51.01	51.37	50.33	50.75	50.93	52.33	0.596	0.130	NS	*	NS
Propionate (C_3)	24.30^{a}	24.13^{a}	23.12^{ab}	22.83^{b}	24.30^{a}	24.16^{a}	23.11^{ab}	22.59^{b}	0.160	<0.0001	NS	***	NS
Butyrate	18.64	18.59	19.02	18.94	18.64	18.31	18.82	18.52	0.192	0.086	*	*	NS
C_2/C_3	2.07^{b}	2.10^{b}	2.21^{a}	2.25^{a}	2.07^{b}	2.10^{b}	2.20^{a}	2.32^{a}	0.034	0.001	NS	***	NS
	3 h												
pH	5.67^{b}	5.80^{a}	5.63^{b}	5.61^{b}	5.67^{b}	5.82^{a}	5.83^{a}	5.82^{a}	0.028	0.0003	***	***	***
Ammonia-N (mg/100 ml)	13.11	11.92	11.59	11.23	13.11	11.28	12.48	11.61	1.389	0.854	NS	NS	NS
Total VFAs (mmoles/100ml)	54.35	56.8	54.6	54.59	54.35	53.44	58.9	54.79	4.056	0.859	NS	NS	NS
Molar proportion (mmoles/100mmoles)													
Acetate (C_2)	48.94^{ab}	46.74^{b}	49.49^{ab}	50.33^{a}	48.94^{ab}	47.58^{b}	48.82^{ab}	50.24^{a}	0.590	0.004	NS	**	NS
Propionate (C_3)	25.49^{b}	29.50^{a}	25.86^{b}	24.91^{b}	25.49^{b}	28.39^{a}	27.02^{a}	25.47^{b}	0.254	<0.0001	NS	***	***
Butyrate	19.44^{a}	17.77^{b}	18.37^{b}	18.51^{b}	19.44^{a}	18.02^{b}	18.14^{b}	18.37^{b}	0.296	0.018	NS	***	NS
C_2/C_3	1.92^{a}	1.58^{b}	1.91^{a}	2.02^{a}	1.92^{a}	1.68^{c}	1.81^{b}	1.97^{a}	0.036	<0.0001	NS	***	*
	6 h												
pH	5.64^{b}	5.77^{a}	5.77^{a}	5.78^{ab}	5.64^{b}	5.86^{a}	5.89^{a}	5.94^{a}	0.046	0.007	**	***	NS
Ammonia-N (mg/100 ml)	13.19	12.06	11.03	12.00	13.19	12.33	13.75	12.64	1.032	0.428	NS	NS	NS
Total VFAs (mmoles/100 ml)	66.97	67.87	66.26	67.37	66.97	71.07	70.41	73.45	4.781	0.760	NS	NS	NS
Molar proportion (mmoles/100 mmoles)													
Acetate (C_2)	43.89	42.56	42.35	45.02	43.89	43.04	42.57	41.88	1.417	0.489	NS	NS	NS
Propionate (C_3)	25.41^{b}	29.69^{a}	32.12^{a}	33.77^{a}	25.41^{b}	29.77^{a}	31.69^{a}	31.80^{a}	1.096	0.011	NS	***	NS
Butyrate	23.37	21.00	19.21	19.04	23.37	20.39	19.58	22.69	2.075	0.410	NS	NS	NS
C_2/C_3	1.73^{a}	1.43^{b}	1.32^{b}	1.51^{b}	1.73^{a}	1.45^{b}	1.34^{b}	1.41^{b}	0.060	0.004	NS	***	NS
	9 h												
pH	5.66^{c}	5.80^{a}	5.83^{b}	5.90^{a}	5.66^{c}	5.85^{b}	5.95^{b}	6.10^{a}	0.054	0.003	**	***	NS
Ammonia-N (mg/100 ml)	16.55	15.42	14.73	12.58	16.55	15.84	15.09	13.72	1.322	0.256	NS	NS	NS
Total VFAs (mmoles/100 ml)	75.41	78.65	74.98	83.32	75.41	78.68	77.52	82.94	5.701	0.668	NS	NS	NS
Molar proportion (mmoles/100 mmoles)													
Acetate (C_2)	39.84	38.09	38.42	37.41	39.84	40.46	38.28	38.15	1.193	0.276	NS	NS	NS
Propionate (C_3)	24.47^{d}	28.03^{c}	31.15^{b}	34.04^{a}	24.47^{d}	28.74^{c}	32.42^{b}	34.85^{a}	0.492	<0.0001	*	***	NS
Butyrate	26.57^{a}	24.79^{b}	21.92^{c}	20.60^{c}	26.57^{a}	22.92^{b}	21.85^{bc}	19.99^{c}	0.715	0.0005	NS	***	NS
C_2/C_3	1.63^{a}	1.36^{b}	1.23^{bc}	1.10^{c}	1.63^{a}	1.41^{b}	1.18^{c}	1.09^{c}	0.064	0.0004	NS	***	NS
	12 h												
pH	5.63^{d}	5.77^{a}	5.79^{a}	5.89^{a}	5.63^{c}	5.80^{bc}	5.90^{b}	6.12^{a}	0.056	0.002	*	***	NS
Ammonia-N (mg/100 ml)	19.97^{a}	16.39^{b}	15.00^{c}	14.23^{c}	19.97^{a}	19.70^{a}	15.31^{b}	16.14^{b}	1.169	0.043	*	**	NS
Total VFAs (mmoles/100 ml)	73.15	83.44	82.46	90.11	73.15	83.69	91.46	94.49	5.853	0.064	NS	**	NS
Molar proportion (mmoles/100 mmoles)													
Acetate (C_2)	39.87	38.89	37.83	36.63	39.87	40.67	38.30	38.29	1.840	0.449	NS	NS	NS
Propionate (C_3)	23.92^{c}	27.16^{b}	29.98^{b}	33.13^{a}	23.92^{d}	27.99^{c}	31.04^{b}	34.50^{a}	0.354	<0.0001	**	***	NS
Butyrate	26.65^{a}	24.61^{ab}	22.50^{bc}	20.85^{c}	26.65^{a}	22.96^{bc}	22.23^{bc}	19.57^{c}	0.991	0.003	NS	***	NS
C_2/C_3	1.67^{a}	1.43^{b}	1.26^{bc}	1.11^{c}	1.67^{a}	1.45^{b}	1.23^{bc}	1.11^{c}	0.085	0.001	NS	***	NS

[1] Means in the same row with different letters differ. [2] Standard error of means. [3] Pr<F, significant level.

[4] Source, fumarate vs. malate; level, addition level of fumarate or malate; S×L, interaction between fumarate and malate.

* $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS = Non significant.

while that of C_2 tended to decrease as the incubation time advanced (Table 4). Addition of both precursors enhanced proportions of C_2 ($p<0.004$) at the highest level (24 mM) at 3 h and C_3 at 6 ($p<0.011$), 9 and 12 h incubations ($p<0.0001$), but reduced C_4 proportion at incubation of 3 ($p<0.018$), 9 ($p<0.0005$), and 12 h ($p<0.003$). Proportions of C_3 at 6, 9, and 12 h were increased ($p<0.001$) while those of C_4 at 9 and 12 h ($p<0.001$), and C_2/C_3 ratio at 6, 9 and 12 h ($p<0.001$) incubation time were decreased as the addition level increased. No differences were observed in the proportions of C_2 and C_4 between propionate precursors, but only malate increased C_3 proportion at 9 h ($p<0.05$) and 12 h ($p<0.01$).

Addition of both propionate precursors in acid form enhanced ($p<0.028$) the total gas production (Table 5), which was almost linearly increased ($p<0.01$) as the addition level of acid form precursors were increased. However, no difference was observed in total gas production between propionate precursors. Both propionate precursors clearly reduced ($p<0.0001$) CH_4 production in a dose dependent manner (Table 5). Acid form fumarate reduced ($p<0.001$) methane production more than malate. Addition of precursors and their level did not affect the DM degradation of feed *in vitro* but degradation of NDF in feed was slightly increased by the addition of both propionate precursors (Table 6).

DISCUSSION

Major factors influencing ruminal fermentation are the type and amount of feed (Garcı´a-Lo´pez et al., 1991). In the present studies, the same amount of feed consisting of 70% concentrate and 30% ground alfalfa (DM basis) was supplemented to all the culture solutions including the control to avoid a shortage of nutrients for normal fermentation by rumen microbes. It is considered, based on patterns of fermentation characteristics by incubation time that normal fermentation occurred in both *in vitro* 1 and 2 studies. The fermentation characteristics, in many cases, clearly responded to different propionate precursor (fumarate or malate) and their addition level rather than chemical type (sodium salt or acid) although the experiments were conducted at separate times. Feeding levels to the cows, collection time of rumen fluid and experimental procedures were kept accurately to avoid experimental error between chemical types of propionate precursor.

In the present studies, addition of fumarate and malate in both sodium salt and acid form increased pH, total VFA, and C_2 proportion in culture solution (Tables 1 and 4) and reduced methane (Tables 2 and 5). Major addition effects (C_2 proportion and generation of total gas and methane) of propionate precursors obtained from the two *in vitro* experiments were shown to be dose dependent. Both fumarate and malate in sodium salt form have been shown to increase final pH, total VFA production and concentration of propionate (Martin and Streeter, 1995; Carro and Ranilla, 2003) in the rumen fluid but decrease methane production (Carro and Ranilla, 2003). One of the possible reasons for the increased pH of the culture solution in the present studies could be due to an increase in lactate utilization by *S. ruminantium* when propionate precursors were added. Fumarate, malate and other dicarboxylic acids promote lactate utilization of *S. ruminantium* and its growth *in vitro* (Nisbet and Martin, 1990; Nisbet and Martin, 1993), resulting in stable pH of the ruminal fluid. In the present studies, sodium fumarate increased C_3 proportion up to 32.9% while sodium malate increased it up to 38.50% (Table 1), and acid form fumarate increased C_3 proportion up to 30.9% while acid type malate increased it up to 30.0%

Table 5. Methane production (μmol) as influenced by addition of fumarate or malate in acid form for 12 h incubation

Item	Treatments (Free acid, mM)[1]								SEM[2]	Pr<F[3]	Effects[4]		
	Fumarate				Malate						Source	Level	S×L
	0	8	16	24	0	8	16	24			(S)	(L)	
Total gas (ml)	125.0^{c}	149.0^{ab}	153.0^{ab}	171.0^{a}	125.0^{b}	153.5^{ab}	184.0^{a}	181.0^{a}	13.215	0.028	NS	**	NS
CH_4 (ml)	8.05^{a}	4.59^{b}	3.06^{c}	2.77^{d}	8.05^{a}	6.51^{b}	4.46^{c}	4.23^{c}	0.884	<0.0001	***	***	**
CH_4 (μmol)	328.1^{a}	187.0^{b}	148.2^{c}	112.8^{d}	328.1^{a}	265.5^{b}	181.8^{c}	172.3^{c}	9.665	<0.0001	***	***	**

[1] Means in the same row with different letters differ. [2] Standard error of means. [3] Pr<F, significant level.
[4] Source, fumarate vs. malate; level, addition level of fumarate or malate; S×L, interaction between fumarate and malate.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS = Non significant.

Table 6. Degradation (%) of DM and NDF in feed as influenced by addition of fumarate and malate in acid form for 12 h incubation

	Treatments (Acid, mM)								SEM[1]	Pr<F[2]	Effects[3]		
	Fumarate				Malate						Source	Level	S×L
	0	8	16	24	0	8	16	24			(S)	(L)	
DM	50.57	51.01	50.13	50.08	50.57	50.55	52.67	50.52	0.012	0.457	NS	NS	NS
NDF	25.39	25.99	29.22	29.93	25.39	28.69	31.39	33.42	0.041	0.6375	NS	NS	NS

[1] Standard error of means. [2] Pr<F, significant level. [3] Source, fumarate vs. malate; level, addition level of fumarate or malate.
S×L, interaction between fumarate and malate. NS = Non significant.

(Table 4) at 12 h incubation compared to corresponding values of the control. Callaway and Martin (1996) observed increased C_3 proportion up to 82% from sodium fumarate (250 μmol) for 24 h *in vitro* incubation. The significant increases in C_3 proportion by the addition of both fumarate and malate, regardless of their chemical type, demonstrates that they affect the metabolic fate of H_2 by being reduced to succinate then to propionate, and may stimulate the growth of bacteria competing with methanogens for H_2. Improved efficiency of fumarate in C_2 production over malate in both chemical types, however, requires further examination.

Methane generation is principally a sink for metabolic H_2 in the rumen and the quantity of methane generated is related to the end products produced from carbohydrate fermentation. Thus, the increased total gas production which occurred in a dose dependent manner from addition of malate or fumarate in both chemical forms in the present studies (Tables 2 and 5) simply might be due to the increased supplementation of readily fermentable organic acids in culture solution, and the increase in total gas production might be accompanied by increased CO_2 production rather than CH_4. Both fumarate and malate act as electron acceptors in the dicarboxylic acid pathway by which malate is dehydrated to fumarate, and fumarate reduced to succinate (Castillo et al., 2004). Thus, they are key propionate precursors (Castillo et al., 2004) and compete with methanogens for the available H_2, resulting in suppressed methane production in the rumen. In this pathway, malate (Callaway and Martin, 1996) and fumarate (López et al., 1999) accept one pair of electrons in their conversion into propionate. In the present study, sodium fumarate reduced CH_4 generation up to 30.8% while sodium malate reduced it up to 15.1%, and acid form fumarate reduced CH_4 up to 65.6% while acid form malate reduced it up to 47.5% after 12 h incubation *in vitro* compared to the control, respectively. Demeyer and Henderickx (1967) observed that the addition of 500 μM fumarate inhibited *in vitro* CH_4 production by 60%, whereas López et al. (1999) found only 6% reduction from fumarate addition. The reason for the greater reduced CH_4 generation by fumarate than by malate regardless of chemical type is not known.

Both fumarate and malate in both chemical types slightly increased *in vitro* NDF degradation, but propionate precursors as the sodium salt reduced DM degradation compared to the control (Tables 3 and 6). An explanation for these results was not evident, but it might be assumed that DM degradation of substrate would be reduced if the ruminal microbes under the present experimental conditions utilized the readily fermentable propionate precursors more preferably than supplemented substrate as feed. Carro et al. (1999), however, observed increased digestibility of major components in the diet when they supplemented both materials as sodium salt. It has been demonstrated that cellulolytic organisms benefited from the presence of methanogens and other H_2 utilizing bacteria because of interspecies H_2 transfer (Wolin and Miller, 1988).

Based on the results obtained from the present experiments, it is concluded that both fumarate and malate as sodium salt or acid form enhance total VFA production and C_3 proportion, and reduce the methane generation in a dose dependent manner under the present *in vitro* fermentation conditions. Fumarate in both chemical types could be a more efficient additive than malate in C_3 production and reduction of CH_4 in the rumen.

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