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Linolenic Acid in Association with Malate or Fumarate Increased CLA Production and Reduced Methane Generation by Rumen Microbes*

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ABSTRACT : An *in vitro* study was conducted to investigate the effect of malate or fumarate on fermentation characteristics, and production of conjugated linoleic acid (CLA) and methane (CH_4) by rumen microbes when incubated with linolenic acid (α-$C_{18:3}$). Sixty milligrams of α-$C_{18:3}$ alone (LNA), or α-$C_{18:3}$ with 24 mM malic acid (M-LNA) or α-$C_{18:3}$ with 24 mM fumaric acid (F-LNA) were added to the 150 ml culture solution consisting of 75 ml strained rumen fluid and 75ml McDougall's artificial saliva. Culture solution for incubation was also made without malate, fumarate and α-$C_{18:3}$ (Control). Two grams of feed consisting of 70% concentrate and 30% ground alfalfa (DM basis) were also added to the culture solution of each treatment. *In vitro* incubation was made anaerobically in a shaking incubator up to 12 h at 39°C. Supplementation of malate (M-LNA) or fumarate (F-LNA) increased pH at 6 h ($p<0.01$) and 12 h ($p<0.001$) incubation times compared to control and linolenic acid (LNA) treatments. Both malate and fumarate did not influence the ammonia-N concentration. Concentration of total VFA in culture solution was higher for M-LNA and F-LNA supplementation than for control and LNA treatments from 6 h ($p<0.040$) to 12 h ($p<0.027$) incubation times, but was not different between malate and fumarate for all incubation times. Molar proportion of C_3 was increased by F-LNA and M-LNA supplementation from 6 h ($p<0.0001$) to 12 h ($p<0.004$) incubation times compared to control and LNA treatments. No differences in C_3 proportion, however, were observed between M-LNA and F-LNA treatments. Accumulated total gas production for 12h incubation was increased ($p<0.0002$) by M-LNA or F-LNA compared to control or LNA treatment. Accumulated CH_4 production for 12 h incubation, however, was greatly reduced ($p<0.0002$) by supplementing malate or fumarate compared to the control, and its production from M-LNA or F-LNA treatment was smaller than that from LNA treatment. Methane production from LNA, M-LNA or F-LNA treatment was steadily lower ($p<0.01$ - $p<0.001$) from 3 h incubation time than that from the control, and was also lower for M-LNA or F-LNA treatment at incubation times of 6 h ($p<0.01$) and 9 h ($p<0.001$) than for LNA treatment. Methane production from LNA, however, was reduced ($p<0.01$ - $p<0.001$) from 3 h to 9 h incubation times compared to the control. Both malate and fumarate increased concentration of *trans*11-$C_{18:1}$ from 3 h to 12 h incubation ($p<0.01$), *cis*9,*trans*11-CLA up to 6 h incubation ($p<0.01$ - $p<0.01$), *trans*10,*cis*12-CLA at 3 h ($p<0.05$) and 12 h ($p<0.01$), and total CLA for all incubation times ($p<0.05$) compared to corresponding values for the α-$C_{18:3}$ supplemented treatment (LNA). In conclusion, malate and fumarate rechanneled the metabolic H_2 pathway to production of propionate and CLA, and depressed the process of bio-hydrogenation and methane generation. Linolenic acid alone would also be one of the optimistic alternatives to suppress the CH_4 generation. (**Key Words :** Malate, Fumarate, Methane, Bio-hydrogenation, Linolenic Acid, Conjugated Linoleic Acid)

INTRODUCTION

Gas emission from ruminants is a direct result of fermentation performed by ruminal microbes (Makkar and Vercoe, 2007). Bryant (1979) indicated that the ruminal bacteria obtain energy for growth by catabolizing degradable organic matter to end-products such as CO_2 and methane (CH_4). Methanogens are the principal utilizers of hydrogen since methane is formed as a result of hydrogen (H_2) removal from the rumen (Asanuma et al., 1999; Lopez et al., 1999; Ungerfeld et al., 2003).

Both fumarate and malate, which are propionate precursors in the succinate to propionate pathway, act as H_2 acceptors (Callaway and Martin, 1996; Castillo et al., 2004). In this pathway, malate (Martin and Streeter, 1995; Lopez et al. 1999) or fumarate (Callaway and Martin, 1996; Lopez et

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al., 1999) accepts one pair of electrons in their conversion into propionate. Thus, H_2 is also required for propionate formation. Addition of fumarate and malate to the diet reduced methane production (Ungerfeld et al., 2003). Therefore, it would be energetically beneficial in the ruminant animal if H_2 use was shifted to propionate production at the cost of ruminal CH_4 formation (Bergen and Bates, 1984; Russell and Strobel, 1989). Reduction in CH_4 production in the rumen will even be beneficial to the environment (Van Nevel and Demeyer, 1996).

Meanwhile, unsaturated fatty acids (UFA) released as a result of the hydrolysis of dietary lipids by microbes in the rumen are subjected to a bio-hydrogenation process which also requires H_2 (Bauman et al., 1999; Wang et al., 2002b; Jin et al., 2008). Linoleic acid ($C_{18:2}$) supplementation reduced fermentation rate (Prins et al., 1972) and thus depressed CH_4 generation in the rumen (Broudiscou et al., 1990). One of the major intermediates of bio-hydrogenation of C_{18}-poly unsaturated fatty acids (PUFA) by the rumen bacteria is conjugated linoleic acid (CLA) (Harfoot and Hazlewood, 1988; Wang et al., 2005). The CLA is derived from both $C_{18:2}$ (Kelly et al., 1998; Bessa et al., 2000; Wang et al., 2003; Chantaprasarn and Wanapat, 2008) and linolenic acid (α-$C_{18:3}$) (Dhiman, 2000; Wang et al., 2002a, b; Wang et al., 2003; Choi and Song, 2005).

A possible alternative to suppress the CH_4 in the rumen is the use of various electron sinks competing with methanogens for the available H_2. However, no information was found on the effect of propionate enhancers on production of CLA and methane by rumen microbes when incubated with α-$C_{18:3}$. Objective of the present study, therefore, was to examine the effect of malate or fumarate on rumen fermentation characteristics and production of CH_4 and CLA by rumen microbes when incubated with α-$C_{18:3}$ *in vitro*.

MATERIALS AND METHODS

Preparation of culture solution and its incubation

Rumen contents were obtained 2 h after the morning feeding (08:00) from three ruminally-cannulated Holstein cows fed 7 kg/d of concentrate (70%) and rice straw (30%) on a dry matter (DM) basis in equal portions twice daily. The rumen contents were blended 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 protozoa and feed particles. Gaseous CO_2 was flushed into the strained rumen fluid for 30 seconds.

Table 1. Chemical composition of feed added to the culture solution (%, DM basis)

Compound	Concentrate	Alfalfa
Dry matter (DM)	92.23	93.67
Crude protein (CP)	15.18	13.51
Ether extract (EE)	4.18	3.12
Neutral detergent fiber (NDF)	38.73	56.23
Ash	6.71	8.71

Solution for the culture was prepared by mixing 75 ml strained rumen fluid with 75 ml McDougall's artificial saliva (1948) under flushing with CO_2. Sixty milligrams of α-$C_{18:3}$ (Sigma, L-2376, cis,cis,cis-9,12,15-Octadecatrienoic acid, ≥99%) dissolved in a solution of albumin fragments (0.24 g) in distilled water (1 ml) alone (LNA), or α-$C_{18:3}$ with 24 mM malic acid (Sigma-Aldrich, M0875, 98%, M-LNA) or α-$C_{18:3}$ with 24 mM fumaric acid (Sigma-Aldrich, F19353, 99%, F-LNA) was added to the solution in a 250 ml flask. Culture solution for incubation was also made without malate, fumarate and α-$C_{18:3}$ (Control). Two grams of feed consisting of 70% concentrate and 30% ground alfalfa (DM basis) were also added to the culture solution of each treatment. The flasks were then sealed with butyl rubber stoppers fitted with a 3-way stopcock, and were incubated anaerobically in a shaking incubator (VS-8480SR) at 135 rpm/min for up to 12 h at 39°C. The *in vitro* incubation was made 3 times in duplicate, each time under similar conditions. Chemical composition of feed added to the culture solution (%, DM basis) is shown in Table 1, and concentrations of C_{18}-fatty acids in rumen fluid and mixed feed are shown in Table 2.

Measurements and analysis

Incubation was terminated by taking the flasks out of the incubator at indicated times of 1, 3, 6 and 12 h. pH of the culture solution was measured immediately after the incubation was terminated, and the culture solution, except for 5 ml which was retained for the determination of ammonia-N and volatile fatty acids (VFA), was kept frozen (-20°C) until analyzed. Ammonia-N concentration was determined by the method of Fawcett and Scott (1960) using a spectrophotometer (DU-650). The 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 for the VFA analysis. The VFA concentration was determined by gas chromatograph (GC, HP5890 series II, Hewlett Packard Co.) equipped with a flame ionization detector (FID). Total gas production was also measured at

Table 2. Concentration of C_{18} fatty acids of rumen fluid and feed added to the culture solution

Items	Stearic acid ($C_{18:0}$)	Oleic acid ($C_{18:1}$)	Linoleic acid ($C_{18:2}$)	Linolenic acid ($C_{18:3}$)	Total C_{18} fatty acid	Others
In 75 ml rumen fluid (mg)	18.28	0.89	3.89	0.08	23.46	17.26
Mixed feed (mg in2 g, DM)	8.32	11.55	12.59	0.14	32.54	27.02

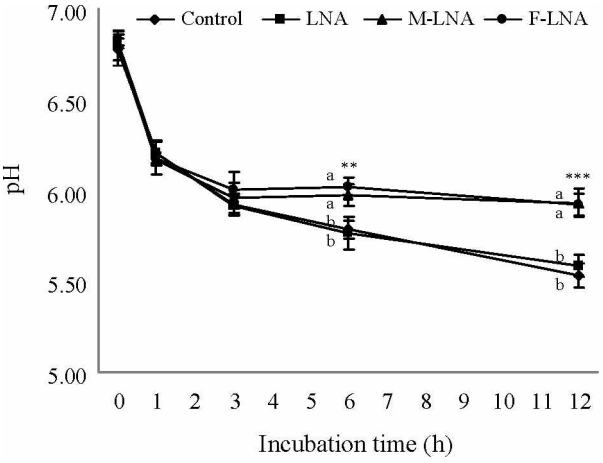


Figure 1. pH of culture solution by incubation time as influenced by fumarate or malate when incubated with linolenic acid. LNA = Linolenic acid; M-LNA = Malate+linolenic acid; F-LNA = Fumarate+linolenic acid. ** $p<0.01$; *** $p<0.001$.

the incubation times of 1, 3, 6, 9 and 12 h from the culture flasks through the 3-way stopcock using a 50 ml glass syringe connected to a needle. A gas sample was transferred to a 4 ml vacuum tube and analyzed for CH_4 by GC (HP5890 series II, Hewlett Packard Co.) equipped with FID. The oven temperature for CH_4 analysis was 40°C, and temperatures of injector and detector were maintained at 200°C. A 30 m fused silica capillary column (HP-MOLESIE, 19091P-MS4, 0.32 mm i.d. USA) was used. Ultra high purity Helium gas (He) was used at a flow rate of 30 ml/min. The methane peak was identified and quantified using standard CH_4 gas. The methane (umol) produced in the current study was determined by the following equation of Lopez and Newbold (2007) :

$$n_{CH4\ (umol)} = (A_{sample} \times n_{std(umol/100\ \mu l\ std)})/A_{std}$$
$$= (A_{sample} \times 40{,}632\ V_{(std,CH4,L\ /(100\ \mu l\ std))})/A_{std}$$

Where,

A_{sample}, peak area of CH_4 in sample; $A_{standard}$, peak area of CH_4 in standard mixed gas

$$n_{std}\ (\mu mol) = (1.01 \times 10^5 \times V_{std,CH4,L} \times 10^6)/8341.3 \times (273+25)$$
$$= 40{,}632\ V_{(std,CH4,L)/(100ul\ std)}$$

Here, 1.01×10^5, standard atmosphere pressure (Pa); V, gas volume (L); n, mole; 8341.3 ($Pa \cdot L \cdot mol^{-1} \cdot K$), gas constant (R) and (273+25), absolute temperature (T).

For the measurement of CLA production, lipids in culture solution were extracted using Folch's solution (Folch et al., 1957), and methylation of fatty acids followed the method of Lepage and Roy (1986) prior to injecting into a GC (Agilent 6890N) equipped with HP chemistation software for peak integration. A 100m fused silica capillary

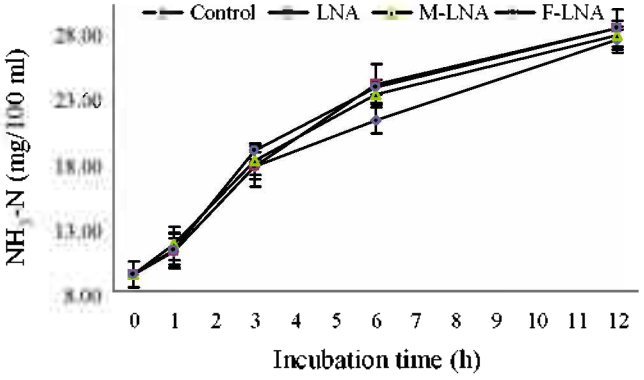


Figure 2. Ammonia-N concentration (mg/100 ml) of culture solution by incubation time as influenced by malate and fumarate when incubated with linolenic acid. LNA = Linolenic acid; M-LNA = Malate+linolenic acid; F-LNA = Fumarate+linolenic acid.

column (Supelco SP™-2560, 0.25 mm i.d. USA) was used and ultra high purity He was used as carrier gas at a flow rate of 45 ml/min. Injector and detector temperatures were 240 and 250°C, respectively. The split ratio to the FID detector was 1:100. The oven temperature was scheduled to maintain at 140°C for 2 min, then increased to 240°C at a rate of 4°C/min, and finally maintained at 240°C for 40 min. *Cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA isomers (Sigma, USA) were used to identify and quantify the relevant CLA isomer. Tridecanoic acid ($C_{13:0}$) was used as an internal standard and all CLA isomers and other FAs in rumen fluid were quantified using FA standards.

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 among treatments by incubation time were compared by S-N-K's Test (Steel and Torrie, 1980) with the following linear models:

$$y_{ij} = \mu + \tau_i + \varepsilon_{ij}$$

Where,

y_{ij} = Observation value

μ = Overall mean

τ_i = The fixed effect of the ith treatment

ε_{ij} = Random error

RESULTS

The pH of the culture solution decreased for all treatments as the incubation time advanced (Figure 1). Supplementation of malate (M-LNA) or fumarate (F-LNA) increased pH at 6 h ($p<0.01$) and 12 h ($p<0.001$) incubation times compared to the control and linolenic acid (LNA)

treatments. No difference in pH, however, was observed between M-LNA and F-LNA treatments for all incubation times. Concentration of ammonia-N in the culture solution for all treatments increased as the incubation time advanced but no differences were found among treatments (Figure 2). Malate and fumarate did not influence the ammonia-N concentration.

Concentration of total VFA in culture solution increased for all the treatments as the incubation time advanced, and was higher for M-LNA and F-LNA supplementations than for control and LNA treatments from 6 h ($p<0.040$) to 12 h ($p<0.027$) incubation times (Table 3). However, total VFA concentration was not different between malate and fumarate for all incubation times. Molar proportion of acetate (C_2) decreased but propionate (C_3) and butyrate (C_4) had increasing trends for all treatments as the incubation time advanced (Table 3). Molar proportion of C_2 was higher ($p<0.016$) for control and LNA treatments at 12 h incubation time than for M-LNA and F-LNA treatments. Molar proportion of C_3 was increased by F-LNA and M-LNA supplementation at 6 h ($p<0.0001$) to 12 h ($p<0.004$) incubation times compared to control and LNA treatments. No differences in C_3 proportion were observed between control and LNA treatments or between M-LNA and F-LNA treatments. Supplementation of M-LNA and F-LNA lowered molar proportion of C_4 at 6 h ($p<0.014$) and 12 h ($p<0.036$) compared to control and LNA treatments. No differences in C_4 proportion were observed between control and LNA treatments or between M-LNA and F-LNA treatments. The C_2/C_3 ratios were higher ($p<0.041$) at 1 h but were lower for M-LNA and F-LNA treatments at 6 h ($p<0.006$) and 12 h ($p<0.001$) incubation times.

Table 3. Concentration and composition of major VFAs in the culture solution as influenced by addition of malate or fumarate when incubated with linolenic acid

Incubation time (h)	Treatments[1]				SEM[2]	Pr<F[3]
	Control	LNA	M-LNA	F-LNA		
	Total VFA (mmoles/100 ml)					
1	58.3	55.82	51.31	53.28	7.113	0.783
3	57.09	54.21	53.71	56.1	3.456	0.37
6	70.43^{b}	64.60^{b}	82.61^{a}	74.04^{a}	4.018	0.04
12	81.77^{b}	86.49^{b}	99.79^{a}	96.45^{a}	4.148	0.027
	Acetic acid (C_2, mmols/100 mmoles)					
1	47.22	47.53	50.7	48.68	1.647	0.059
3	44.93	44.39	46.73	45.35	0.496	0.056
6	40.94	40.19	38.67	39.73	1.132	0.365
12	36.22^{a}	33.03^{a}	27.26^{b}	28.60^{b}	2.584	0.016
	Propionic acid (C_3, mmols/100 mmoles)					
1	28.55	28.49	25.22	26.33	0.933	0.054
3	28.77	28.75	27.59	28.99	0.628	0.255
6	28.03^{c}	30.03^{b}	34.32^{a}	35.83^{a}	0.496	0.0001
12	27.80^{b}	29.61^{b}	40.38^{a}	39.52^{a}	1.794	0.004
	Butyric acid (mmols/100 mmoles)					
1	18.77	18.42	18.6	19.02	0.107	0.051
3	20.45	21.67	20.5	20.49	0.286	0.052
6	24.90^{a}	24.42^{a}	21.06^{b}	20.12^{b}	0.841	0.014
12	29.16^{a}	30.65^{a}	24.15^{b}	23.52^{b}	1.999	0.036
	C_2/C_3					
1	1.65^{b}	1.67^{b}	2.01^{a}	1.85^{a}	0.089	0.041
3	1.56	1.54	1.69	1.56	0.051	0.117
6	1.46^{a}	1.34^{a}	1.13^{b}	1.11^{b}	0.051	0.006
12	1.31^{a}	1.12^{a}	0.68^{b}	0.77^{b}	0.152	0.001

[1] Means in the same row with different superscripts differ. [2] Standard error of means. [3] Probability level.
LNA = Linolenic acid; M-LNA = Malate+linolenic acid; F-LNA = Fumarate+linolenic acid.

Table 4. Accumulated total gas and methane (CH_4) for 12 h incubation as influenced by addition of malate or fumarate when incubated with linolenic acid

	Treatments[1]				SEM[2]	Pr<F[3]
	Control	LNA	M-LNA	F-LNA		
Total gas (ml)	235.00^{b}	228.50^{b}	303.00^{a}	308.00^{a}	5.013	0.0002
CH_4 (μmol)	520.00^{a}	224.68^{b}	148.03^{c}	82.92^{c}	23.805	0.0002

[1] Means in the same row with different superscripts differ. [2] Standard error of means. [3] Probability level.
LNA = Linolenic acid; M-LNA = Malate+linolenic acid; F-LNA = Fumarate+linolenic acid.

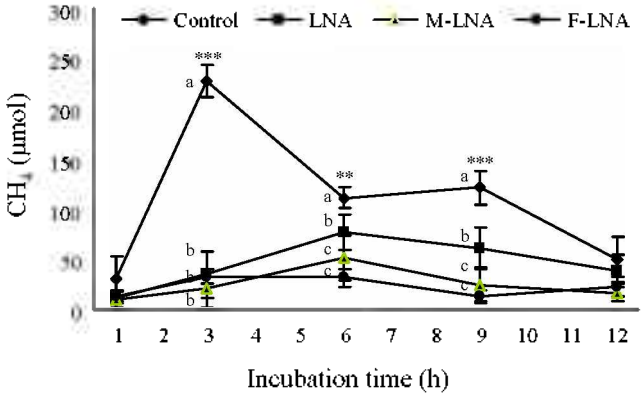


Figure 3. Methane production (µmol) by incubation time as influenced by addition of malate or fumarate when incubated with linolenic acid. LNA = Linolenic acid; M-LNA = Malate+linolenic acid; F-LNA = Fumarate+linolenic acid. ** p<0.01; *** p<0.001.

Accumulated total gas for 12 h incubation was increased (p<0.0002) by M-LNA or F-LNA but was not influenced by LNA supplementation compared to the control (Table 4). Accumulated total CH_4 for 12 h incubation was greatly reduced (p<0.0002) by supplementing malate or fumarate compared to the control, and CH_4 production from M-LNA or F-LNA treatment was smaller than from LNA treatment (Table 4). Methane production from LNA, M-LNA or F-LNA treatment was steadily lower (p<0.01 - p<0.001) from 3 h incubation time than from the control, and was also lower at incubation times of 6 h (p<0.01) and 9 h (p<0.001) for M-LNA and F-LNA treatments than for LNA treatment (Figure 3). Methane production from LNA was reduced (p<0.01 - p<0.001) from 3 h to 9 h incubation times compared to the control.

As the incubation time advanced, concentrations in culture solution of stearic acid ($C_{18:0}$, Figure 4) and vaccenic acid (*trans*11-$C_{18:1}$, Figure 6) tended to increase while those of oleic acid (*cis*9-$C_{18:1}$, Figure 5), linoleic acid (*cis*6-$C_{18:2}$, Figure 7), *cis*9,*trans*11-CLA (Figure 8), *trans*10,*cis*12-CLA (Figure 9), total CLA (Figure 10) and α-$C_{18:3}$ (Figure 11)

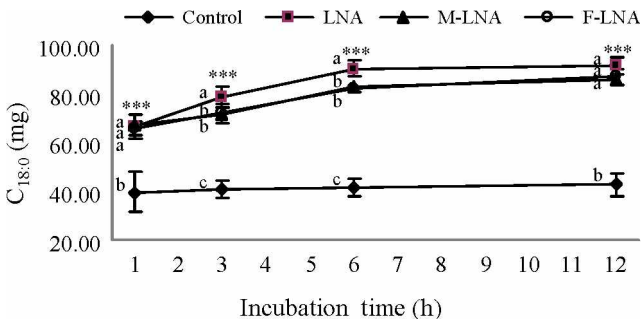


Figure 4. Concentration (mg) of stearic acid ($C_{18:0}$) in culture solution as influenced by addition of malate or fumarate when incubated with linolenic acid. Treatments: refer to Figure 3. *** p<0.001.

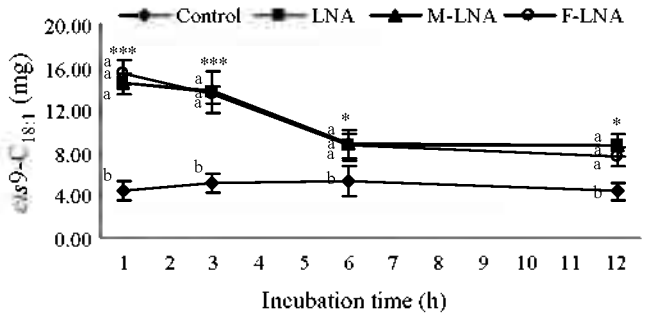


Figure 5. Concentration (mg) of oleic acid (*cis*9-$C_{18:1}$) in culture solution as influenced by addition of malate or fumarate when incubated with linolenic acid. Treatments: refer to Figure 3. * p<0.05; *** p<0.001.

were slightly decreased by the supplementation of α-$C_{18:3}$ with or without malate or fumarate. Both malate and fumarate increased concentration of *trans*11-$C_{18:1}$ from 3 h to 12 h incubation (p<0.01, Figure 6), *cis*9,*trans*11-CLA up to 6 h incubation (p<0.05 - p<0.001, Figure 8), *trans* 10,*cis*12-CLA (Figure 9) at 3 h (p<0.05) and 12 h (p<0.05), and total CLA for all incubation times (p<0.05, Figure 10)

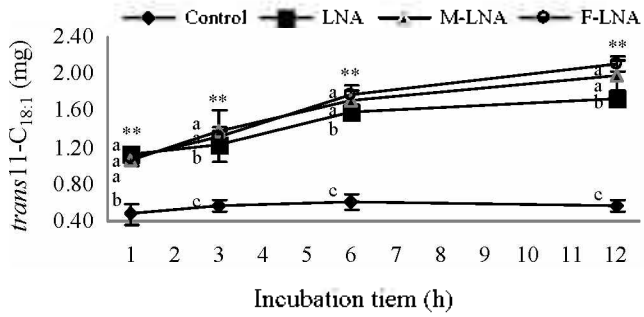


Figure 6. Concentration (mg) of vaccenic acid (*trans*11-$C_{18:1}$) in culture solution as influenced by addition of malate or fumarate when incubated with linolenic acid. Treatments: refer to Figure 3. ** p<0.01.

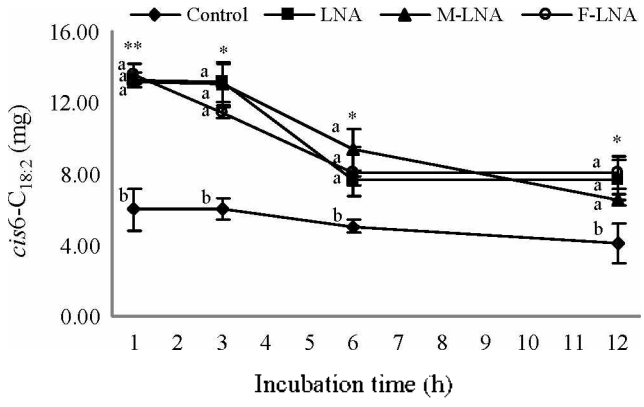


Figure 7. Concentration (mg/) of linoleic acid (*cis*6-$C_{18:2}$) in culture solution as influenced by addition of malate or fumarate when incubated with linolenic acid. Treatments: refer to Figure 3. * p<0.05; ** p<0.01.

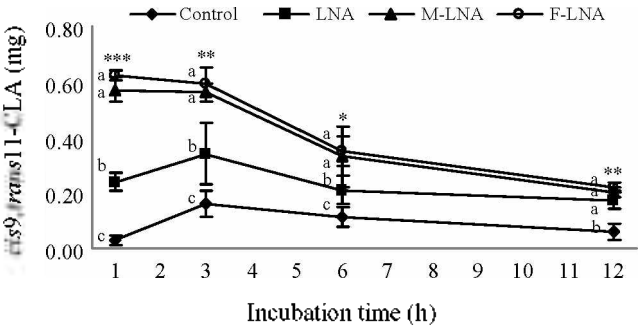


Figure 8. Concentration (mg) of *cis*9,*trans*11-CLA in culture solution as influenced by addition of malate or fumarate when incubated with linolenic acid. Treatments: refer to Figure 3. * p<0.05; ** p<0.01; *** p<0.001.

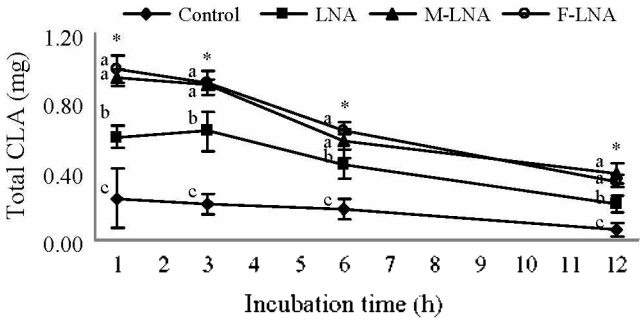


Figure 10. Concentration of total CLA (sum of *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA isomers, mg) in culture solution as influenced by addition of malate or fumarate when incubated with linolenic acid. Treatments: refer to Figure 3. * p<0.05.

compared to corresponding values for α-$C_{18:3}$ supplemented treatment (LNA). No differences were found in the concentrations of major C_{18}-fatty acids between malate and fumarate supplementation.

DISCUSSION

Major factors influencing ruminal fermentation are the type and amount of feed (García-López et al., 1996). In the current study, 2 g of feed consisting of 70% concentrate and 30% ground alfalfa as a forage (DM basis) was supplemented to every culture solution including the control treatment to avoid a shortage of nutrients for normal fermentation by rumen microbes. Feeding level to the cows and feeding time, collection time of rumen fluid and experimental procedures were kept accurately to minimize experimental errors.

The rumen microbes in the culture solution clearly responded to the supplements (α-$C_{18:3}$, malate or fumarate) in fermentation characteristics. The rumen microbial fermentation in the current *in vitro* study looked normal since pH (Figure 1) of the culture solution decreased while concentration of ammonia-N (Figure 2) and total VFA (Table 3) increased with incubation time. Both malate and fumarate increased pH of the culture solution at a late stage of fermentation (Figure 1) compared to control and LNA treatments. A similar result in pH was observed by Martin and Streeter (1995). One of the possible reasons for the increased pH of the culture solution in the present study might be due to the stimulation of lactate utilization by the predominant ruminal bacterium *S. ruminantium* (Nisbet and Martin, 1990, 1991, 1993), and thus decreased lactate concentration (Carro and Ranilla, 2003) by the supplementation of malate and fumarate.

Both malate and fumarate increased total VFA concentration at a late stage of fermentation (6-12 h, Table 3). Increased VFA concentration was observed as influenced by malate and fumarate (Martin and Streeter, 1995), and by fumarate (Mao et al., 2007). Malate or fumarate also increased C_3 proportion at a late stage of fermentation in the current study (Table 3). Malate with α-$C_{18:3}$ (M-LNA) increased C_3 proportion by 45.25% and 36.37%, and fumarate with α-$C_{18:3}$ (F-LNA) increased it by 42.15% and 33.47% compared to control and LNA treatments, respectively. Callaway and Martin (1996) observed increased C_3 proportion up to 82% with Na-

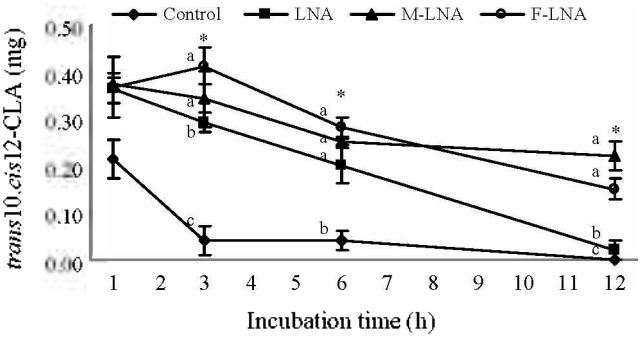
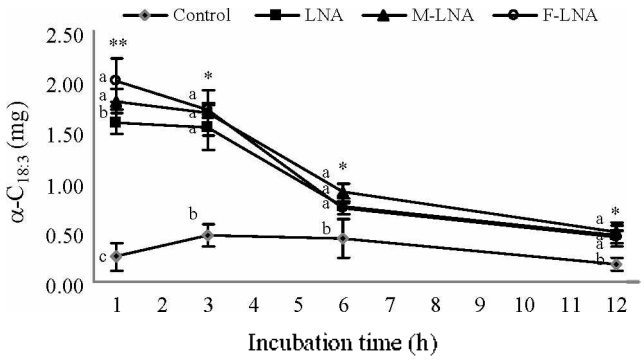


Figure 9. Concentration (mg) of *trans*10,*cis*12-CLA in culture solution as influenced by addition of malate or fumarate when incubated with linolenic acid. Treatments: refer to Figure 3. * p<0.05.



Fugure 11. Concentration (mg) of linolenic acid (α-$C_{18:3}$) in culture solution as influenced by addition of malate or fumarate when incubated with linolenic acid. Treatments: refer to Figure 3. * p<0.05; ** p<0.01.

fumarate supplementation (250 μmol) following 24 h incubation *in vitro*. Castillo et al. (2004) indicated that both fumarate and malate act as electron acceptors in the dicarboxylic acid pathway, in which malate is dehydrated to fumarate, and fumarate is reduced to succinate. Significant increase in C_3 by malate and fumarate supplementation in the current study demonstrates that these compounds affect the metabolic fate of H_2 and may stimulate the growth of microbes competing with methanogens for H_2.

Metabolic hydrogen (H_2) is of vital importance in the energy exchange within the rumen (Czerkawski, 1972). The current study was mainly focused on the fate of metabolic H_2 for methane generation and bio-hydrogenation of unsaturated fatty acids as well as propionate production. CH_4 generation was greatly decreased by the supplementation of malate or fumarate when incubated with α-$C_{18:3}$ compared to LNA and control treatments, despite the fact that accumulated total gas production was increased in the current study (Table 4). Bio-hydrogenation of α-$C_{18:3}$ by rumen microbes could be another process for the H_2 sink, and thus it should compete with methanogens for the available H_2. Researchers (Carro and Ranilla, 2003; Li et al., 2009) also observed a decreased methane production from malate or fumarate supplementation. The α-$C_{18:3}$ supplementation alone (LNA), however, generated more CH_4 than M-LNA and F-LNA treatments for 12 h incubation (Table 4) and at 6 h and 9 h incubations (Figure 4) in the current study. The M-LNA treatment decreased accumulated total CH_4 production by 34.12% and the F-LNA treatment decreased it by 63.09% compared to LNA alone (Table 4). Demeyer and Henderickx (1967) observed that the addition of 500 μM fumarate inhibited *in vitro* CH_4 production by 60%, but López et al. (1999) found only 6% reduction from fumarate addition. Asanuma et al. (1999) found the addition of 20 to 30 mM fumarate significantly decreased methane production and increased propionate production *in vitro*. Increased accumulated total gas production by addition of malate or fumarate (Table 4) might be simply due to the increased readily fermentable organic acid, and the increase in accumulated total gas could be accompanied by enhanced CO_2 production rather than CH_4. The CLA was mostly derived from the dietary $C_{18:2}$ in the rumen (Kelly et al., 1998), but Bessa et al. (2000) have revealed the possibility of an alternative pathway in the production of CLA from α-$C_{18:3}$. In fact, there have been reports that CLA was produced from α-$C_{18:3}$ or α-$C_{18:3}$ rich oil (Dhiman, 2000; Wang et al., 2002a, b; Wang et al., 2003; Choi and Song, 2005). Results of the current study were similar to previous reports. Higher concentrations of *cis*9,*trans*11-CLA (Figure 8) and *trans*10,*cis*12-CLA (Figure 9) for M-LNA and F-LNA treatments than for LNA treatment may indicate that malate and fumarate influence the bio-hydrogenation progress of α-$C_{18:3}$ in rumen. Furthermore, lower accumulation of $C_{18:0}$ (Figure 4) and higher concentration of *t*11-$C_{18:1}$ for M-LNA and F-LNA treatments than for LNA treatment (Figure 6) may indicate that malate and fumarate, to some extent, reduce the rate of bio-hydrogenation.

Overall data indicate that malate and fumarate act as alternative electron sinks, and may compete with CH_4 generation and bio-hydrogenation of α-$C_{18:3}$ for the utilization of metabolic H_2. Malate and fumarate rechanneled the metabolic H_2 pathway to production of propionate and CLA and depressed the process of bio-hydrogenation and methane generation. Linolenic acid alone would also be one of the optimistic alternatives to suppress CH_4 generation.

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