pH Affects the *In vitro* Formation of *cis*-9, *trans*-11 CLA and *trans*-11 Octadecenoic Acid by Ruminal Bacteria When Incubated with Oilseeds*

J. H. Wang and M. K. Song**

Department of Animal Science, Chungbuk National University, Chungbuk 361-763, Korea

ABSTRACT : The effect of pH on the fermentation characteristics and the formation of *cis*-9, *trans*-11 conjugated linoleic acid (CLA) and *trans*-11 octadecenoic acid by mixed ruminal bacteria was examined *in vitro* when incubated with linseed or rapeseed. Concentrate (1%, w/v) with ground linseed (0.6%, w/v) or rapeseed (0.5%, w/v) was added to 600 ml mixed solution of strained rumen fluid with artificial saliva (1:1, v/v), and was incubated anaerobically for 12 h at 39°C. The pH of culture solution was maintained at level close to 4.5, 5.3, 6.1 and 6.9 with 30% H_2SO_4 or 30% NaOH solution. pH increment resulted in increases of ammonia and total volatile fatty acid (VFA) concentration in culture solutions containing both oilseeds. Fermentation did not proceeded at pH 4.5. Molar proportion of acetate decreased but that of propionate increased as pH increased when incubated with oilseeds. While the hydrogenating process was very slow at the pH range of 4.5 to 5.3, rapid hydrogenation was found from the culture solutions of pH 6.1 and 6.9 when incubated with linseed or rapeseed. As pH in culture solution of linseed or rapeseed increases proportions of oleic acid (*cis*-9 $C_{18:1}$) and *trans*-11 octadecenoic acid increased but those of linoleic acid and linolenic acid decreased. The CLA proportion increased with pH in culture solution containing rapeseed but CLA was mostly not detected from the incubation of linseed. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 12 : 1743-1748)***

Key Words : pH, Oilseed, Biohydrogenation, *cis*-9, *trans*-11 CLA, *trans*-11 Octadecenoic Acid, *In vitro*, Mixed Ruminal Bacteria

INTRODUCTION

Conjugated linoleic acids (CLA) and *trans*-11 octadecenoic acid isomers are the two major intermediate products of biohydrogenation of C_{18}-polyunsaturated acids by the rumen bacteria (Harfoot and Hazlewood, 1988), and changes in substrate supply and extent of biohydrogenation influenced on the production of those intermediates (Kelly et al., 1998; Dhiman et al., 1999). CLA acts as an effective anticarcinogen and exhibits other important physiological effects (Ha et al., 1987; Ip et al., 1995). While *trans*-11 ocdecenoic acid may have the negative effects, not only on animal productivity but also on human health (Wahle and James, 1993).

In an early study, decreased lipolysis in the rumen was observed in cows with low ruminal pH (Latham et al., 1972). Kalscheur et al. (1997) also reported that altered ruminal function in lipolysis and hydrogenation resulting from low ruminal pH for cows fed the low forage diet. Kepler et al. (1970) reported the requirements of free carboxyl radical which occurs more at high pH for the production of CLA. Romo (1995) found that lowering the pH below 6 caused an accumulation of *trans*-11 ocdecenoic acid *in vitro*. A previous *in vitro* study (Wang et al., 2002a) indicated that *trans*-11 ocdecenoic acid and CLA proportions decreased as pH became lower with increasing concentrate addition level. Thus, we postulate that pH may be a critical factor in the regulation of hydrogenating process. But the changes in pH were certainly influenced by the amount of concentrate added. An effect of various pH levels on the hydrogenation of unsaturated fatty acids at fixed concentrate level, however, has not been examined since designated pH may regulate the microbial growth and hydrolytic activity.

Therefore, the present *in vitro* study was conducted to examine the effect of pH on fermentation characteristics and formation of CLA and *trans*-11 ocdecenoic acid by ruminal bacteria when used two lipid sources which differ in fatty acid composition.

MATERIALS AND METHODS

Preparation of rumen fluid

Rumen contents were collected at 3 h after morning feeding (0600) from a ruminally cannulated Holstein cow fed 5 kg of corn silage (60%) and concentrate (40%) on a dry matter (DM) basis twice daily. The rumen contents were brought to the laboratory and were blended in a Waring blender (Fisher Co.) for 20 seconds at high speed to detach the bacteria from the feed particles, and were strained through 12 layers of cheesecloth to remove the feed particles and large protozoa. CO_2 was flushed into the strained rumen fluid.

Preparation and incubation of culture

Strained rumen fluid was mixed with McDougall's artificial saliva (1948) at the ratio of 1:1 under flushing of

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** Corresponding Author: Man K. Song. Tel: +82-43-261-2545, E-mail: mksong@cbucc.chungbuk.ac.kr

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Table 1. Lipid contents and C_{18}-fatty acid composition of oilseeds and concentrate

	Lipid (%, DM)	Composition of C_{18}-fatty acid (%)			
		Stearic acid ($C_{18:0}$)	Oleic acid ($C_{18:1}$)	Linoleic acid ($C_{18:2}$)	Linolenic acid ($C_{18:3}$)
Linseed	33.9	2.79	21.8	14.3	52.5
Rapeseed	40.0	3.55	44.2	29.3	13.3
Concentrate	3.04	4.39	28.2	31.9	1.50

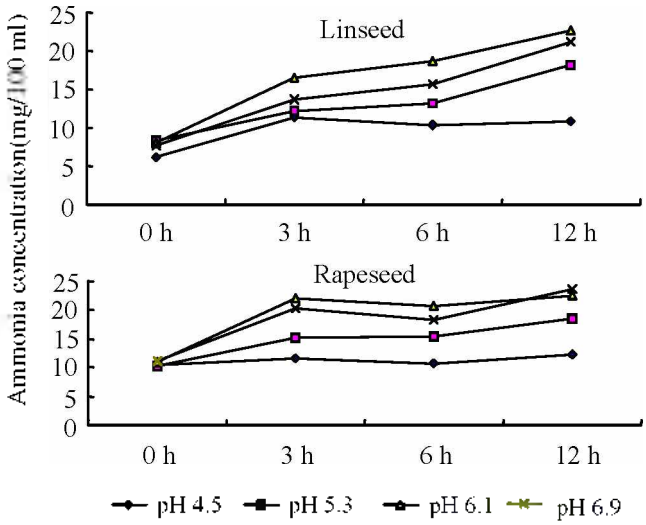

Figure 1. Ammonia concentration in culture solution.

CO_2. Six grams of concentrate (1% of culture solution, w/v, as-fed basis) with 3.6 g of ground (1 mm screen) linseed (*L. usitatissimus*, 0.6% of culture solution, w/v, DM basis) or 3.0 g of rapeseed (*B. napus*, 0.5% of culture solution, w/v, DM basis) were added to 600 ml mixed solution in the glass culture jar, and CO_2 was flushed into the culture solution for 3 minutes. The amount of oilseed added was based on the oil content of each oilseed to make the oil quantity in culture solution be similar between treatments. The culture jar was covered with a glass lid equipped with stirrer and was placed into a water-bath maintaining at 39°C. Culture solution was again flushed with CO_2 through glass tube connected to the jars for the infusion purpose for 3 min., and was incubated up to 12 h. The pH of culture solution was adjusted to 4.5, 5.3, 6.1 and 6.9 for each treatment throughout the incubation by infusing 30% H_2SO_4 or NaOH solution at 20 min interval into culture jar. Stirring speed during incubation was 120 times/min. Compositions of C_{18}-fatty acids in oilseeds and concentrate are presented in Table 1. The *in vitro* study was conducted three times under similar condition.

Sampling and analysis

At the incubation times of 3, 6 and 12 h, 5 ml culture solution was collected for ammonia (1 ml) and VFA analysis (4 ml). 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, Beckman). Four ml culture solution was mixed with 1 ml 25% phosphoric acid and 0.5 ml pivalic acid solution (2%, w/v) was added as an internal standard. The mixed solution was centrifuged at 15,000×g for 15 min, and the supernatant was used to determine the concentration and composition of VFA using gas chromatograph (GC. HP 5890 Ⅱ. Hewlett Packard Co.). One hundred ml incubation solution was also collected at the incubation times of 3, 6 and 12 h, and freeze dried and lipids were extracted using Folch's solution (Folch et al., 1957). Methylation of the lipids was followed the method of Lepage and Roy (1986) prior to injecting into the GC. A fused silica capillary column (100 m×0.25 mm, i.d.× 0.20 μm thickness, SPTM-2560, Supelco) was used.

Statistical analysis

The results obtained from these determinations 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 Test (Steel and Torrie, 1980).

RESULTS

Ammonia concentration in the culture solution slightly increased with pH, but relatively increased concentration was observed at pH 6.1 throughout the incubation times for both linseed and rapeseed (Figure 1). Total VFA concentrations increased greatly with incubation time and pH in incubation solution of both oilseeds except for pH 4.5 that small changes were observed (Table 2 and 3). While molar proportion of acetate (C_2) and ratio of C_2 to propionate (C_3) decreased those of C_3 increased with pH in both culture solutions of linseed and rapeseed. Molar proportion of butyrate at pH 5.3 tended to increase for both oilseeds compares to those at the other pH levels.

The pH reflected clearly on the composition of C_{18} fatty acids in culture solution when incubated with ground oilseeds. While hydrogenating process was very slow at the pH range of 4.5 to 5.3, rapid hydrogenation was found from the culture solutions of pH 6.1 and 6.9 when incubated with linseed (Table 4). Similar trend was observed from the culture solution containing rapeseed (Table 5). The changes in the composition of most C_{18}-fatty acids as influenced by pH were clear for both oilseeds. As pH in culture solution

Table 2. Concentration and molar proportion of VFA in culture solution when incubated with ground linseed

Items	pH of culture solution [1), 2)]				SEM[3)]	Pr>F[4)]
	4.5	5.3	6.1	6.9		
	----------3 h----------					
Total VFA (mmoles/100 ml)	34.59^{c}	42.25^{b}	52.70^{a}	55.38^{a}	1.322	0.001
Molar proportion (mmoles/100 mmoles) :						
Acetate (C_2)	55.82^{a}	51.77^{ab}	49.05^{b}	49.79^{b}	1.060	0.035
Propionate (C_3)	27.65^{b}	29.14^{ab}	31.04^{a}	31.30^{a}	0.428	0.011
Butyrate	15.25	16.86	15.19	14.11	1.093	0.454
C_2/C_3	2.02^{a}	1.78^{b}	1.58^{b}	1.59^{b}	0.054	0.013
	----------6 h----------					
Total VFA (mmoles/100 ml)	34.44^{c}	49.68^{b}	64.89^{a}	68.45^{a}	1.590	0.0004
Molar proportion (mmoles/100 mmoles) :						
Acetate (C_2)	56.10^{a}	48.29^{b}	47.24^{b}	48.58^{b}	1.393	0.032
Propionate (C_3)	27.44^{b}	30.81^{ab}	31.84^{a}	32.05^{a}	0.851	0.044
Butyrate	15.18	17.92	15.76	14.11	1.104	0.241
C_2/C_3	2.05^{a}	1.57^{b}	1.49^{b}	1.52^{b}	0.075	0.017
	----------12 h----------					
Total VFA (mmoles/100 ml)	34.95^{c}	59.07^{b}	79.35^{a}	77.81^{a}	2.195	0.0004
Molar proportion (mmoles/100 mmoles) :						
Acetate (C_2)	56.87^{a}	41.83^{b}	45.82^{b}	47.94^{b}	1.342	0.006
Propionate (C_3)	27.08^{b}	33.68^{a}	31.57^{ab}	32.12^{ab}	1.142	0.056
Butyrate	14.77	21.49	16.86	14.00	1.608	0.094
C_2/C_3	2.10^{a}	1.24^{b}	1.45^{b}	1.49^{b}	0.053	0.001

[1)] pH of ruminal culture was adjusted with 30% of H_2SO_4 or NaOH solution. [2)] Means in the same row with different superscripts differ.
[3)] Standard error of the mean. [4)] Probability levels.

Table 3. Concentration and molar proportion of VFA in culture solution when incubated with ground rapeseed

Items	pH of culture solution [1), 2)]				SEM[3)]	Pr>F[4)]
	4.5	5.3	6.1	6.9		
	----------3 h----------					
Total VFA (mmoles/100 ml)	37.28^{c}	46.60^{b}	56.26^{a}	57.26^{a}	1.135	0.0007
Molar proportion (mmoles/100 mmoles)						
Acetate (C_2)	54.90	51.14	49.26	49.52	1.535	0.167
Propionate (C_3)	23.40^{c}	25.93^{b}	27.36^{a}	27.86^{a}	0.246	0.0007
Butyrate	19.39	19.68	18.09	16.96	1.167	0.429
C_2/C_3	2.35^{a}	1.97^{b}	1.80^{b}	1.78^{b}	0.051	0.004
	----------6 h----------					
Total VFA (mmoles/100 ml)	37.80^{c}	54.00^{b}	68.47^{a}	68.94^{a}	1.141	0.0001
Molar proportion (mmoes/100 mmoles)						
Acetate (C_2)	54.99	47.89	48.04	48.96	1.401	0.061
Propionate (C_3)	23.33^{b}	27.72^{a}	27.75^{a}	28.65^{a}	0.248	0.0004
Butyrate	19.40	20.44	18.89	16.92	1.329	0.407
C_2/C_3	2.36^{a}	1.73^{b}	1.73^{b}	1.71^{b}	0.044	0.001
	----------12 h----------					
Total VFA (mmoles/100 ml)	37.52^{c}	62.42^{b}	78.88^{a}	78.35^{a}	0.877	0.0001
Molar proportion (mmoles/100 mmoles)						
Acetate (C_2)	56.05^{a}	42.26^{c}	46.91^{b}	48.93^{b}	1.102	0.004
Propionate (C_3)	23.04^{b}	31.18^{a}	27.67^{a}	28.61^{a}	0.760	0.007
Butyrate	18.63	22.82	19.75	16.53	1.325	0.110
C_2/C_3	2.43^{a}	1.35^{c}	1.70^{b}	1.71^{b}	0.044	0.0003

[1)] pH of ruminal culture was adjusted with 30% of H_2SO_4 or NaOH solution. [2)] Means in the same row with different superscripts differ.
[3)] Standard error of the mean. [4)] Probability levels.

of linseed increases *trans*-11 ocdecenoic acid proportion increased while those of $C_{18:2}$ and $C_{18:3}$ decreased (Table 4). The trend in the proportional changes of oleic acid (*cis*-9 $C_{18:1}$), *trans*-11 ocdecenoic acid and $C_{18:2}$ from the rapeseed incubation was similar to those of linseed incubation (Table 5). However, $C_{18:3}$ proportion tended to increase as pH increases when ground rapeseed was incubated. While the CLA proportion increased with pH in culture solution containing rapeseed, CLA was almost not detected from the incubation of linseed.

Table 4. Composition (%) of C_{18}-fatty acids in culture solution[1] when incubated with ground linseed

Fatty acids	pH of culture solution[1), 2)]				SEM[3)]	Pr>F[4)]
	4.5	5.3	6.1	6.9		
	----------3 h----------					
$C_{18:0}$	25.05	21.93	24.32	23.33	1.529	0.563
$C_{18:1}$	14.25^{b}	15.53^{a}	15.32^{a}	16.08^{a}	0.213	0.016
T11-$C_{18:1}$ [5)]	2.66^{c}	3.74^{c}	6.67^{b}	8.69^{a}	0.360	0.000
CLA[6)]	ND	0.03	0.08	0.06	0.052	0.743
$C_{18:2}$	13.05^{a}	13.04^{a}	11.03^{ab}	10.08^{b}	0.486	0.027
$C_{18:3}$	27.95^{ab}	29.78^{a}	26.65^{ab}	25.20^{b}	0.701	0.038
	----------6 h----------					
$C_{18:0}$	25.02	22.82	30.66	28.23	1.793	0.118
$C_{18:1}$	14.44	15.53	15.43	16.87	0.745	0.288
T11-$C_{18:1}$	2.77^{b}	4.46^{b}	8.18^{a}	10.57^{a}	0.785	0.007
CLA	ND	ND	ND	ND		
$C_{18:2}$	12.81^{a}	12.49^{a}	8.75^{b}	7.89^{b}	0.376	0.001
$C_{18:3}$	27.55^{a}	28.20^{a}	21.53^{b}	18.72^{b}	0.752	0.002
	----------12 h----------					
$C_{18:0}$	25.92^{b}	23.23^{b}	39.95^{a}	28.98^{b}	1.870	0.011
$C_{18:1}$	13.93	15.09	15.52	17.44	0.632	0.070
T11-$C_{18:1}$	2.73^{b}	5.33^{b}	15.19^{a}	16.30^{a}	1.164	0.002
CLA	ND	ND	0.05	ND	0.027	0.478
$C_{18:2}$	12.71^{a}	11.54^{a}	4.25^{b}	5.42^{b}	0.412	0.0001
$C_{18:3}$	27.21^{a}	27.69^{a}	8.22^{b}	11.77^{b}	1.075	0.0004

[1)] pH of ruminal culture was adjusted with 30% of H_2SO_4 or NaOH solution. [2)] Means in the same row with different superscripts differ. ND, not detected. [3)] Standard error of the mean. [4)] Probability levels. [5)] *Trans*-11 octadecenoic acid. [6)] *Cis*-9, *trans*-11 isomer of linoleic acid.

Table 5. Composition (%) of C_{18}-fatty acids in culture solution[1] when incubated with ground rapeseed

Fatty acids	pH of culture solution[1) 2)]				SEM[3)]	Pr>F[4)]
	4.5	5.3	6.1	6.9		
	----------3 h----------					
$C_{18:0}$	40.08	40.91	36.38	26.44	3.807	0.155
$C_{18:1}$	13.25	12.88	15.07	18.36	1.757	0.252
T11-$C_{18:1}$ [5)]	3.95	5.85	5.53	7.47	0.614	0.066
CLA[6)]	0.80	0.79	1.12	1.48	0.206	0.200
$C_{18:2}$	12.00	10.39	11.63	13.02	1.675	0.749
$C_{18:3}$	8.19	7.59	10.68	13.59	1.906	0.249
	----------6 h----------					
$C_{18:0}$	41.37^{a}	40.57^{a}	39.95^{a}	24.45^{b}	2.596	0.025
$C_{18:1}$	12.62^{b}	12.24^{b}	14.10^{b}	19.87^{a}	1.158	0.028
T11-$C_{18:1}$	4.08^{c}	6.12^{b}	6.93^{b}	11.12^{a}	0.442	0.001
CLA	0.70^{c}	0.78^{c}	1.18^{b}	1.70^{a}	0.093	0.005
$C_{18:2}$	11.37	10.40	8.70	9.64	1.222	0.471
$C_{18:3}$	7.41	7.73	9.62	13.08	1.226	0.090
	----------12 h----------					
$C_{18:0}$	40.55	40.29	43.05	32.64	2.194	0.099
$C_{18:1}$	12.88	12.80	13.29	15.13	0.837	0.303
T11-$C_{18:1}$	4.04^{d}	7.59^{c}	9.98^{b}	15.18^{a}	0.354	0.0001
CLA	0.71^{c}	0.70^{c}	1.42^{b}	2.01^{a}	0.069	0.0002
$C_{18:2}$	11.87^{a}	8.77^{a}	4.22^{b}	3.32^{b}	1.002	0.012
$C_{18:3}$	7.54	7.34	8.54	10.98	1.019	0.181

[1)] pH of ruminal culture was adjusted with 30% of H_2SO_4 or NaOH solution. [2)] Means in the same row with different superscripts differ.
[3)] Standard error of the mean. [4)] Probability levels. [5)] *Trans*-11 octadecenoic acid. [6)] *Cis*-9, *trans*-11 isomer of linoleic acid.

DISCUSSION

Infusion of 30% H_2SO_4 or NaOH solution at 20 min interval maintained pH in culture solution within the acceptable ranges (±5% of pH for each designated level). It is also considered that lipid content in concentrate was so low that its effect on fatty acid composition in culture solution containing oilseeds could be very small. The fermentation of concentrate and biohydrogenation of C_{18}-unsaturated fatty acids were considered to be the results of

bacterial activity in the present study since protozoa were seldom found from the rumen fluid strained through 12 layers of cheese cloth as observed in previous *in vitro* study (Wang et al., 2002b).

Based on the ammonia concentration (Figure 1). VFA production (Tables 2 and 3) and composition of C_{18}-fatty acids (Tables 3 and 4). relatively lower pH (4.5 and 5.3) depressed an overall fermentation and the degree of hydrogenation in culture solution containing both linseed and rapeseed than higher pH levels (6.1 and 6.9). The fermentation characteristics as influenced by pH are agreeable with earlier studies. Ruminal pH values below 6.0 depressed cellulolytic activity and alter ruminal microflora (Stewart. 1977). Kopecny and Wallace (1982) suggested that optimized pH for proteolytic activity was 5.5-7.0 in mixed rumen bacteria. Lipolysis was also reduced at pH not exceeding 6.0 (Van Nevel and Demeyer. 1996).

Cis-9. *trans*-11 isomer is the principal dietary form of CLA as suggested by Chin et al. (1992) and Sehat et al. (1998). The CLA is generated mainly from *cis*-9. *cis*-12 linoleic acid ($C_{18:2}$) through the isomerization by ruminal bacteria and its sequential reduction step yields *trans*-11 ocdecenoic acid (Harfoot and Hazlewood. 1988). The CLA was mostly detected in the culture solution containing rapeseed but little from linseed in this study (Tables 4 and 5). This difference can be due to the difference in proportion of $C_{18:2}$ between oilseeds in which $C_{18:2}$ were much higher in rapeseed than in linseed (Table 1).

The present results showed that higher pH level increased the CLA production in culture solution containing rapeseed. Based on the fermentation characteristics (Figure 1. Tables 2 and 3) this might be related to the degradation rate of oilseeds as influenced by pH of culture solution. Wang and Song (1999. 2001) observed that lower pH derived from greater addition level of carbohydrate reduced the release of free $C_{18:2}$ from ground oilseeds *in vitro*. Van Nevel and Demeyer (1996) also found a reduced lipid hydrolysis *in vitro* at pH not exceeding 6.0. This indicates that lipolysis as the initial step is obligatory for further hydrogenation process.

The effect of pH on lipolysis. however. may not mean that the formation of CLA and *trans*-11 ocdecenoic acid from C_{18}-polyunsatutated fatty acids are not restricted by pH. Kepler et al. (1970) reported that the *cis*-12, *trans*-11 octadecenoic acid isomerase catalysing the transformation of $C_{18:2}$ into CLA needed the free COOH radical which occurs more at higher pH. Romo (1995) found that lowering the pH from 6.8 to 5.8 caused an accumulation of *trans*-11 ocdecenoic acid *in vitro*. In addition. Hughes et al. (1982) isolated and characterised *Butirivibrio fibrisolvens* *cis*-9. *trans*-11 octadecadienoic acid reductase and concluded it had a maximum activity at neutral pH ranges.

In conclusion. within the range of pH in which fermentation occurs (mainly, pH 6.1 and 6.9), the formation of *cis*-9. *trans*-11 CLA and *trans*-11 octadecenoic acid by rumen bacteria increases with the increment of pH when ground oilseeds are incubated *in vitro*.

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