Effect of Unsaturated Fatty Acids on Cellulose Degradation and Fermentation Characteristics by Mixed Ruminal Microbes**

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ABSTRACT : This experiment was conducted to evaluate the effects of supplemental unsaturated fatty acids (UFA) on fermentation characteristics, especially on gas production, cellulose degradation and volatile fatty acid (VFA) concentration by mixed ruminal microorganisms. In order to attain this objective, unsaturated fatty acids including oleic acid (C18:1), linoleic acid (C18:2) and arachidonic acid (C22:4) were added at varying level. Mixed ruminal microbes used in this experiment were obtained from the rumen of a cannulated Holstein cow. Medium pH values after 7 d incubation were significantly affected by type and level of unsaturated fatty acids ($p<0.01$). All of UFA inhibited total gas production, and especially treatment of arachidonic acid at the levels of 0.01% gave the lowest gas production after 7 d incubation ($p<0.01$). Comparison of the population of protozoa revealed that UFA did not have any significant effect on the total protozoa number. The addition of UFA did not effect dry matter degradation. Volatile fatty acid (VFA) composition of the culture was influenced little by UFA, although the considerable amount of iso-type VFA were detected in UFA supplemented incubations. The ratio of acetic acids to propionic acids, however, was lower than control in all the treatments after 7 d incubation ($p<0.01$). (*Asian-Aust. J. Anim. Sci. 2001. Vol. 14, No. 4 : 501-506*)

Key Words : Unsaturated Fatty Acids (UFA), Cellulose Degradation, Gas Production, VFA Composition

INTRODUCTION

It has been well documented that long-chain fatty acids inhibited methane production in the rumen, while simultaneously molar proportions of propionate were increased (Czerkawski et al., 1966; Demeyer et al., 1969). Besides, Several reports exist to explain the negative effect of lipids on crude fiber digestibility (Devendra and Lewis, 1974; Pantoja et al., 1994). The speculation that inhibition is due to physical coating of fibre with lipids is no longer valid, as Øskov et al. (1978) found no effect on degradability *in sacco* of dried grass coated with tallow. Van Nevel and Demeyer (1988) have suggested that fatty acids are toxic for certain microbes, and cause shifts in the microbial composition which are responsible for the effect on crude-fibre digestion. Negative effects of the fatty acids on rumen fermentation can, at least partially, be removed through the use of protected lipids (Sutton et al., 1983) or insoluble Ca-soaps. Thus, the latter compounds do not affect fiber digestion (Olubobokun et al., 1985).

Methane production was inhibited by tallow, unsaturated fatty acids and linseed oil hydrolysate in experiments *in vitro* and *in vivo* with cows and sheep (Van Nevel and Demeyer, 1981). It was clear that the effect of unsaturated fatty acids was not entirely related to the microbial hydrogenation process competing for metabolic hydrogen (Czerkawski, 1966). It was also shown in incubations with mixed rumen bacteria *in vitro* that methane inhibition by unsaturated fatty acids was due to a direct toxic effect on methanogenesis (Jenkins and Palmquist, 1982). However, those were not still enough to explain how unsaturated fattty acids interfere with rumen fermentation.

Although relatively extensive research efforts have been made in the past to elucidate effects of fatty acids, studies reporting the effects on fermentation by isolated mixed rumen microbes are not easily found in the literature. Therefore, the objective of this experiment was to examine the effect of unsaturated fatty acids on fermentation characteristics, especially on gas production, cellulose degradation and VFA concentration by mixed rumen microbes.

MATERIALS AND METHODS

Oleic acid (C18:1), linoleic acid (C18:2), and arachidonic acid (C20:4) were added at the levels of 0%, 0.001%, 0.005%, and 0.01% to media in order to study the effect of these unsaturated fatty acids (UFA) on the fermentation and cellulose degradability by mixed ruminal microbes. All the UFA were purchased from Sigma Co. (St. Louis., MO) and were dissolved in distilled water and then sonicated by a Vibra cell sonicator (Materials Inc., Danbury, USA). This experiment was performed in 3×4 factorial design.

Mixed ruminal microbes were isolated from the rumen of a cannulated Holstein cow of 400kg body weight, which was fed rice straw and a commercial concentrate (16% crude protein) in the ratio of 70 to

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30 at 1.8% of body weight and they were divided into two equal meals.

Incubations were carried out for 1, 3, 5 and 7 days in 30 ml serum bottles with Butyl rubber septa (Belco Biotechnology, USA) under CO_2 at 39℃ without shaking. Serum bottles contained 9 ml modified GSM (glucose sloppy medium:mineral I solution, 165ml; mineral II solution, 165 ml; 0.1% resazurin, 1.0 ml; yeast extract, 1.0 g; peptone, 1.0 g; glucose, 1.5 g; $NaHCO_3$, 1.5 g; cystein-HCl · H_2O, 1.0 g/l L medium) by Ho and Bauchop (1991), and 50 mg Whatman No. 3 filter paper as an energy source. Mixed ruminal microbes (1 ml) were inoculated to the media with various levels of UFA under anaerobic gassing system by Hungate (1966) and Holdman et al. (1977).

Gas production was immediately measured by a gas production analyzing system modified with liquid displacement system (Beuvink et al., 1992) and pressure transducer system (Theodorou et al., 1994) at the end of the incubation. The pH of cultures was determined with Mettler Delta 340 pH meter. A portion of cultures was taken for analysis of VFA concentration and for total protozoa counting, and then, the cultures were centrifuged at 1,600×g for 15min. The residual pellets were harvested for analysis of dry matter disappearance, and the supernatant fractions were used for analysis of cellulolytic activity. After stirring the cultures, 1 ml of the cultures were added to 4 ml of MFS solution (35% formaldehyde solution, 100 ml; distilled water, 900 ml; methyl green, 0.6 g; NaCl, 8.0 g/l L MFS solution) by the method of Minato et al. (1992). Numbers of protozoa were counted with the hematocrit. Dry matter degradation rates were calculated from the differences between filter paper weight before and after incubation.

VFA concentration was analyzed by the method of Erwin et al. (1961). Supernatant in an amount of 1.0 ml, prepared as above procedure, were added to the Eppendordf tube and were mixed for 30min with 0.2 ml of 25% (vol/vol) metaphosphoric acid. After centrifuging at 1,600×g for 10 min, 0.2 ml of the supernatant were collected and used for the assay of VFA concentration using Hewlett Packard 6890 GC system.

Methane (CH_4) and CO_2 gas production (mM/l) were estimated with theoretical regression assay by Wolin (1960) and Van Soest (1982). The equations were;

(1) CO_2=acetate/2+propionate/4+3butyrate/2
(2) CH_4=CO_2-(propionate/2+butyrate)

The analyses of variance of all data from this experiment were accomplished with ANOVA procedure of SAS program. The linear and quadratic effects of variables according to incubation time were analyzed with regression procedure of SAS program (1985).

RESULTS AND DISCUSSION

Medium pH, gas production and protozoa popultion

Table 1 shows the effects of UFA on medium pH values during 7 d incubation. The addition of UFA in general resulted in linear decreases in culture pH up

Table 1. pH value as affected by unsaturated fatty acids in the supernatant of medium incubated with mixed ruminal microbes

Unsaturated fatty acids	Levels (%)	Incubation time (d)				Day effect[2]	
		1	3	5	7	L	Q
Control	-	6.56	6.17	5.93[c]	5.76[c]	**	**
Oleic acid	0.001	6.63	6.09	5.92[c]	5.76[c]	**	**
	0.005	6.59	6.09	5.89[c]	5.75[c]	**	**
	0.01	6.60	6.28	5.93[c]	5.78[c]	*	ns
Linolei cacid	0.001	6.57	6.07	5.89[c]	5.76[c]	**	**
	0.005	6.67	6.07	5.91[c]	5.80[c]	**	**
	0.01	6.61	6.11	6.09[ab]	5.86[b]	**	**
Arachidonic acid	0.001	6.64	6.08	5.94[c]	5.80[c]	**	**
	0.005	6.53	6.12	6.03[b]	5.87[b]	**	*
	0.01	6.58	6.16	6.11[a]	5.96[a]	*	ns
Effect[1]	F	ns	ns	**	**	-	-
	C	ns	*	**	**	-	-
	F*C	*	*	**	**	-	-

[1] F, among the unsaturated fatty acids; C, among the concentrations; F*C, among the unsaturated fatty acid×concentrations.
[2] Day effect: linear (L) and quadratic (Q) effects of incubation time.
* p<0.05; ** p<0.01; ns, not significant.
[a,b,c,d] Means in the same column with different letters are significantly different (p<0.05).

to 7 d incubation. The effects of UFA on pH values were more noticable in order of archidonic acid (C22:4), linoleic acid (C18:2), oleic acids (C18:1) and effects of UFA on pH were more distinct at higher inclusion levels.

Total gas production, as shown in figure 1, was reduced by the addition of unsaturated fatty acids at overall incubation time ($p<0.01$). Among the unsaturated fatty acids, arachidonic acid (C22:4) gave the lowest gas production, followed by linoleic acid (C18:2), and oleic acid (C18:1). In general, gas production by mixed rumen microbes was lower at higher concentrations regardless of the types of UFAs. Methane and CO_2 production were not significantly influenced by the addition of unsaturated fatty acids (Data are not shown). Unsaturated fatty acids gave increasing methane production with longer incubation time ($p<0.01\%$), except 0.01% concentration. However, there was not apparent trend as affected by incubation time in methane production. All the unsaturated fatty acids resulted in higher CO_2 production than control after 1 d incubation ($p<0.05$), but the day effects on CO_2 production were not significant.

The fact that higher concentration of unsaturation gave the higher pH values in this experiment may indicate that the microbial hydrogenation process competed for metabolic hydrogen (Van Nevel and Demeyer, 1988), elevating pH values with the addition of UFA compared to control. The toxicity on rumen microbes by the addition of UFA, resulting in the low

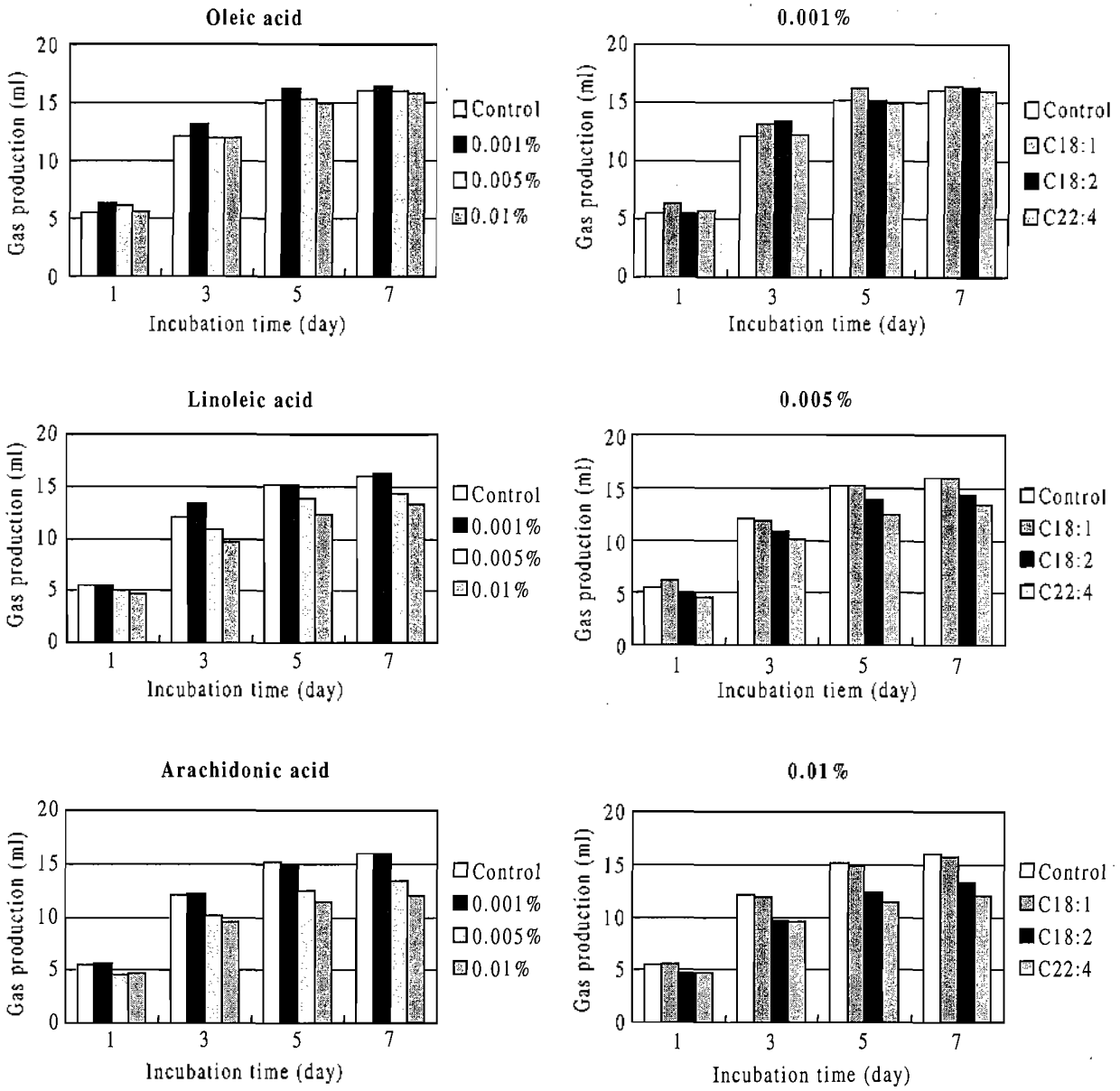

Figure 1. Total gas production (ml/0.5 g DM substrate) by mixed ruminal microbes as affected by (a) type (b) concentration of unsaturated fatty acid.

fermentation as discussed above, also might be related to higher pH values than control. Czerkawshi et al. (1966) and Van Nevel and Demeyer (1981) confirmed that gas production was inhibited by tallow, unsaturated fatty acids and linseed oil hydrolysate in experiments with cow and sheep. It was concluded that unsaturated fatty acids were related to the microbial hydrogenation process competing for metabolic hydrogen (Van Nevel and Demeyer, 1988). Jenkins and Palmquist (1982), on the other hand, reported that gas inhibition by unsaturated fatty acids was due to a direct toxic effect on ruminal methanogens. Both competing of the metabolic hydrogen and toxic effect might have reduced total gas production by the addition of unsaturated fatty acids in this experiment, which were related to reduced total protozoa numbers and DM degradation rate as seen in table 2. Toxic effect of unsaturated fatty acids on rumen microbes may be evidenced by the lower gas production and digestion of diets, besides the degree of unsaturation might also affect microbial hydrogenation process competing for metabolic hydrogen, resulted in lowered total gas production. Kurihara et al. (1997) revealed that the supplementations of oleic acid and linoleic acid reduced the methane production by 3.6 l/kg DM and 4.0 l/kg DM, respectively. Henderson (1973) and Prins et al. (1972), in the meantime, reported that the growth of *Methanobacterium ruminantium* or *Metahnobacterium* M.O.H was severely inhibited by unsaturated fatty acids.

The effects of unsaturated fatty acids were not entirely related to the microbial hydrogenation process competing for metabolic hydrogen (Czerkawski et al., 1966). Jenkins and Palmquist (1982) revealed that methane inhibition by unsaturated fatty acids was due to a direct toxic effect on methanogens. However, in the current experiment there was not significant reduction of methane production by treatments of various unsaturated fatty acids but lowered total gas production. Thus, it seems that effects on the microbial hydrogenation process of unsaturated fatty acids were stronger than toxic effect on ruminal methanogens in this experiment.

The population of protozoa as affected by unsaturated fatty acids revealed that total protozoa numbers were not significantly influenced by the addition of unsaturated fatty acids (Data are not shown). The population of protozoa was not significantly affected by the concentration of unsaturated fatty acids and incubation time, although the addition of unsaturated fatty acid tended to decrease them. Total protozoa numbers were highest after 3 d incubation, but there was no significant trend as affected by incubation time with the addition of unsaturated fatty acid. Kurihara et al. (1997) reported that the treatments of stearic acid, oleic acid and linoleic acid had not significant effects on the population of protozoa compared with control, which is similar to the result of this experiment.

Dry matter degradation rate

The influence of the type and level of UFA on dry matter degradation rate is defined in table 3.

Table 2. Dry matter degradation (%) of filter paper by mixed ruminal microbes as affected by unsaturated fatty acids

Unsaturated fatty acids	Levels (%)	Incubation time (d)				Day effect[2]	
		1	3	5	7	L	Q
Control	-	2.31	31.59^{abcd}	69.06^{a}	80.94^{a}	**	*
Oleic acid	0.001	4.30	25.10^{de}	44.68^{cd}	67.04^{bc}	**	ns
	0.005	nd[3]	35.04^{ab}	44.29^{cd}	62.92^{cd}	*	*
	0.01	4.61	33.73^{abc}	51.05^{bc}	65.68^{bc}	ns	ns
Linolei cacid	0.001	nd	39.27^{a}	64.06^{a}	77.35^{ab}	**	**
	0.005	3.48	31.80^{abcd}	65.81^{a}	68.58^{abc}	**	**
	0.01	nd	21.10^{e}	40.13^{d}	73.32^{abc}	*	*
Arachidonic acid	0.001	4.12	39.35^{a}	54.17^{b}	73.62^{abc}	**	*
	0.005	3.76	27.70^{bcde}	50.86^{bc}	62.62^{cd}	**	ns
	0.01	nd	25.80^{cde}	52.76^{b}	52.41^{d}	**	**
Effect[1]	F	ns	ns	**	**	-	-
	C	ns	*	**	**	-	-
	F*C	ns	**	**	**	-	-

[1] F, among the unsaturated fatty acids; C, among the concentrations; F*C, among the unsaturated fatty acid × concentrations.
[2] Day effect: linear(L) and quadratic(Q) effects of incubation time.
[3] nd, not detected.
* $p<0.05$; ** $p<0.01$; ns, not significant.
[a,b,c,d,e] Means in the same column with different letters are significantly different ($p<0.05$).

Table 3. VFA concentration (mM/l) by mixed ruminal microbes as affected by unsaturated fatty acids after 3 day incubation

Unsaturated fatty acids	Levels (%)	VFA concentration (mM)						
		Acetic acid	Propionic acid	Butryic acid	Isobutyric acid	Valeric acid	Isovaleric acid	C_2/C_3 ratio
Control	-	21.13^{b}	13.17	8.29	3.90	4.54	8.16^{a}	1.60
Oleic acid	0.001	35.72^{a}	17.57	10.74	4.57	4.95	5.73^{abcd}	2.05
	0.005	16.15^{b}	11.65	7.20	3.35	3.42	3.58^{bcd}	1.40
	0.01	16.80^{b}	12.98	9.93	4.71	5.04	6.71^{abc}	1.30
Linolei cacid	0.001	19.87^{b}	10.95	8.90	4.84	5.08	4.88^{abcd}	1.85
	0.005	18.58^{b}	12.72	9.58	4.51	6.99	5.81^{abcd}	1.47
	0.01	19.49^{b}	13.65	7.80	3.38	5.76	3.38^{cd}	1.41
Arachidonic acid	0.001	18.39^{b}	10.40	6.94	3.20	5.86	2.67^{a}	1.84
	0.005	17.98^{b}	16.20	6.58	3.37	4.72	5.09^{abcd}	1.10
	0.01	20.68^{b}	21.16	8.48	4.62	4.00	7.36^{ab}	0.98
Effect[1]	F	ns	ns	ns	ns	ns	ns	**
	C	ns	ns	ns	ns	ns	ns	**
	F*C	*	*	ns	ns	ns	*	**

[1] F, among the unsaturated fatty acids; C, among the concentrations; F*C, among the unsaturated fatty acid × concentrations.
* $p<0.05$; ** $p<0.01$; ns, not significant.
^{a,b,c,d} Means in the same column with different letters are significantly different ($p<0.05$).

Added UFA had a minimal effect on dry matter digestion on 1 d incubation, while distinct effects were obtained from day 3. All three UFA depressed dry matter degradation at all three inclusion levels. However, effects of level of UFA were varied according to the type of UFA, which is obvious from the significant interactions between the type and level of UFA.

Prins et al. (1972) and Henderson (1973) reported the supplementation of unsaturated fatty acids lowered DM degradation rate possibly due to the toxicity for ruminal microbes. Yang et al. (2000) also reported that polyunsaturated fatty acids had the possibility to show an inhibiting effect on ruminal fermentation and digestion, which agree with those obtained from present study. Sutton et al. (1983), however, reported that the addition of linseed oil and coconut oil had no significant effects on DM degradation rate. Present results do not provide any clue, however, whether effects of unsaturated fatty acids on the DM digestibilities were due to the toxicity or other factors, which needs further investigation.

VFA concentration

Overall VFA concentration and ratio of acetate to propionate tended to decrease as the level of added UFA in the medium increased (table 3). In addition, UFA with higher saturation gave lower ratio of acetate and propionate ($p<0.01$) than control. The level of UFA also influenced the ratio with a general trend of higher value at 0.001% than control. The proportion of acetate to propionate decreased with longer incubation time with oleic acid ($p<0.01$), but with linoleic and arachidonic acid incubation time did not significantly influence the ratio.

Kurihara et al. (1997) reported that the addition of linoleic acid (C18:2) significantly reduced total VFA, however, they did not determine the concentrations of acetate and propionate. On the contrary, Sutton et al. (1983) showed that total VFA concentration remained unchanged when unsaturated fatty acids were supplied, but Tamminga et al. (1983) found a 20% decrease when the cows received 12% tallow in the concentrates. There was no apparent relation between methane production and propionate production though methane inhibition with a simultaneous increase in propionate production is in general agreement.

The addition of oleic acid at the concentration of 0.001% and arachidonic acid at the concentration of 0.01% increased both acetate and propionate productions. However, at the other concentration of unsaturated fatty acids, productions of acetate and propionate tended to be lower than those of control. These results seem to be related to the low DM degradation rate. Cellulolytic microbes might have been inhibited by unsaturated fatty acids resulting in low fermentation, thus it caused lower VFAs production.

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