Effect of Sources and Levels of Carbohydrates on Fermentation Characteristics and Hydrogenation of Linoleic Acid by Rumen Bacteria *In Vitro*

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ABSTRACT : An *in vitro* study was conducted to examine the effect of sources and the addition levels of carbohydrates on fermentation characteristics, bacterial growth, and hydrogenation of linoleic acid ($C_{18:2}$) by mixed ruminal bacteria. Starch and cellobiose were added to the 200 ml non-selective basal media at the levels of 0.20 and 0.35% (w/v), respectively. Linoleic acid (66.8~79.6 mg) in the absorbed form into the pieces of nylon cloth was also added to the media of 5 treatments including control which was not added with carbohydrate. Three mls of rumen fluid strained through 12 layers of cheese cloth were added to each medium, and were incubated anaerobically in the shaking incubator of 39℃ for 24 hours. During 24 h incubation the pH in incubation media of all treatments was maintained above 6.6 by the addition of sodium bicarbonate. The pH and ammonia concentration of incubation media were not clearly influenced by the sources and addition levels of carbohydrates while additions of carbohydrates increased ($p<0.0001$) VFA concentration at the 24 h incubation. Molar proportions of acetate were reduced ($p<0.0004$) while those of propionate were increased ($p<0.0006$) by the addition of carbohydrates. But the differences in concentration and molar proportions of the VFA were small between the sources or the addition levels. Bacterial growth was faster ($p<0.0004$) in the starch added treatments than in the cellobiose added ones and control, but no differences were found between addition levels. Increased ($p<0.0487$) hydrogenation was observed from the starch added treatments compared to the cellobiose added ones, but there was no difference between addition levels. *(Asian-Aust. J. Anim. Sci. 2001. Vol. 14, No. 1 : 48-53)*

Key Words : Starch, Cellobiose, Linoleic Acid, Hydrogenation, *In Vitro*, Rumen Bacteria

INTRODUCTION

Fatty acid composition of fat in beef and milk markedly differ from those in dietary lipids. The difference is mainly due to the fact that unsaturated fatty acids in the dietary lipids are subjected to hydrogenation by rumen bacteria prior to passing into the intestinal tract (Mattos and palmquist, 1977; Wu et al., 1991; Fotouhi and Jenkins, 1992; Huang et al., 1999). *In vitro* studies with mixed cultures or pure strains of ruminal bacteria have shown that most bacteria are capable of hydrogenating linoleic acid ($C_{18:2}$) to *trans*-$C_{18:1}$ and related isomers, but only a few have the ability to hydrogenate $C_{18:2}$ completely to stearic acid (Miles et al., 1970; Harfoot et al., 1973; Kemp et al., 1975; Hazlewood et al., 1976; Fujimoto et al., 1993). Oleic acid ($C_{18:1}$) was also extensively hydrogenated to stearic acid ($C_{18:0}$) by the ruminal bacteria *in vitro* (Song and Choi, 1998; Wang et al., 1999).

Palmquist and Jenkins (1980) suggested that end product from microbial hydrogenation of C_{18}-unsaturated fatty acids was $C_{18:0}$, and this was proved by Jenkins (1993). Wu and Palmquist (1991) and Fellner et al. (1995) indicated that the extent of hydrogenation was similar to those measured *in vivo*, thus *in vitro* measurements are reliable to predict the fatty acid metabolism *in vivo*.

Lennarz (1966) suggested that the main function of bio-hydrogenation was the disposal of reducing power which is essential to bacteria living in a reduced environment. An alternative suggestion is to detoxify the unsaturated fatty acids (Kemp and Lander, 1984; Kemp et al., 1984b) since unsaturated fatty acids are toxic to many microbes in the rumen.

The extent of hydrogenation by ruminal bacteria has been affected by the levels of dietary starch and fiber (Latham et al., 1972; Leat, 1977; Gerson et al., 1985), the presence of feed particles (Kemp et al., 1984a), concentrate to roughage ratio (Leat, 1977), dietary N level (Gerson et al., 1983) and probably ruminal pH (Wang and Song, 1999). The effect of source or addition level of carbohydrate on the ruminal hydrogenation, however, was not examined since they could affect the rate of fermentation. And although we observed effect of pH at the relatively lower ranges (4.5 to 5.9) on the hydrogenation slightly as influenced by rate of fermentation of carbohydrate sources (Wang and Song, 1999), it still needs to examine the effect of carbohydrates at the higher pH.

Therefore, the present study was conducted to examine the effect of source or addition level of carbohydrate on the ruminal hydrogenation of unsaturated fatty acids *in vitro* at the higher pH.

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MATERIALS AND METHODS

Preparation of medium and addition of carbohydrates

The of Holdeman's Sweet E broth (1977) was modified in carbohydrate levels for the experiment. Two levels (0.2% and 0.35%, w/v) starch or cellobiose each were added to 200 ml media (pH 7.0, table 1) in the flasks since those addition levels were considered to be appropriate based on the total amounts of carbohydrates in broth medium and the fermentation characteristics of previous *in vitro* experiments (Song and Choi, 1998; Wang and Song, 1999; Wang et al., 1999). Gaseous CO_2 was flushed into the autoclaved (20 min. at 121℃) medium for 2 min. after the carbohydrates were dissolved.

Preparation of rumen fluid

Rumen contents were collected at 3 h after morning feeding (0600) from the ruminally cannulated Holstein cow fed 4 kg of corn silage (60%) and commercially produced concentrates (40%) on a dry matter (DM) basis twice daily. Contents of CP, ether extracts, NDF, TDN, Ca and P in the concentrates were 14.8, 3.8, 30.2, 71, 0.62 and 0.41%, respectively. The rumen contents were brought to the laboratory, and were strained through 12 layers of cheesecloth to remove the solid particles and large protozoa. Gaseous CO_2 was flushed into the strained rumen fluid.

Preparation of linoleic acid

Total 635.5 mg $C_{18:2}$ (99% purity, Sigma Co.) was placed in a beaker and 50 ml chloroform was added. After $C_{18:2}$ was dissolved, 20 pieces of pre-weighed nylon cloth (2×3 cm) were immersed into the solution for 1 hour. Then, the chloroform was evaporated on the drying bath (Dri-Bath Type 16500, Thermolyne) at 40℃ under the N_2 gas in the hume hood. Pieces of nylon cloth containing $C_{18:2}$ were placed in a bottle and N_2 gas was flushed, and stored in a refrigerator at -4℃ until used.

Inoculation of rumen bacteria and incubation

Each piece of nylon cloth containing $C_{18:2}$ was weighed and 2 pieces of nylon cloth were placed in 200 ml of medium containing carbohydrates in a 300 ml flask. Two grams of $NaHCO_3$ was added into the flask to maintain the pH above 6.5, and then 3 mls of strained rumen fluid were inoculated into the medium of each treatment. After mixed slowly, CO_2 gas was flushed into the flask for 30 sec. And the flask was covered tightly with rubber stopper and incubated for 24 h at 39℃ in a shaking-incubator (Won Pung Scientific Co.). The shaking speed was adjusted to 80 times/min. Inoculation of rumen fluid without addition of extra carbohydrate (control) was made same as above. Therefore, total 5 treatments with duplicates were designed in each *in vitro* study and the study was repeated three times under the similar condition.

Enumeration of viable bacteria

The number of viable bacteria in medium was determined at incubation time 0 and after incubation (24 h) by the anaerobic culture techniques of Hungate (1966). First, 1 ml of medium solution from every treatment was collected and diluted to $10^5 \sim 10^7$ using the Bryant's diluting solution (1961). Then, 1 ml diluted solution was inoculated into the roll tube containing non-selective artificial medium (Scott and Dehority, 1965). The number of colony was counted after the roll tubes were incubated at 39℃ for 5 days in an incubator.

Sampling and analysis

After incubating for 6, 12 and 24 h the pH of medium solution was measured, and 5 ml of medium solution was collected for ammonia and volatile fatty acid (VFA) analysis. All samples collected were kept frozen at -20℃ until analyzed.

Ammonia concentration was determined by the method of Fawcett and Scott (1960) using the spectrophotometer. Four ml medium solution was mixed with 1 ml 25% phosphoric acid and 0.5 ml pivalic acid solution (2%, w/v) as a internal standard.

Table 1. Composition of basal medium[1]

Components	In 1000 ml
Salt solution[2]	500 ml
CysteineHCl · H_2O	0.5 g
Rumen fluid[3]	300 ml
Dextrose	1.0 g
Arabinose	1.0 g
Fructose	0.5 g
Pyruvate	1.0 g
Peptone	0.5 g
$(NH_4)_2SO_4$	0.5 g
Yeast extract	0.5 g
Hemin solution[4]	10 ml
Distilled water to	1000 ml

[1] pH of medium was adjusted to 7.0.

[2] Salts solution consists of 2.0 g NaCl, 1.0 g K_2HPO_4, 1.0 g KH_2PO_4, 0.265 g $CaCl_2 \cdot 2H_2O$, 0.409 g $MgSO_4 \cdot 7H_2O$, 10.0 g $NaHCO_3$ in 1,000 ml distilled water.

[3] Rumen fluid was obtained from cattle fed 8 kg of hay (60%) and concentrate (40%) daily, and after being strained through eight layers of cheese cloth the rumen fluid was stored frozen -20℃. It was melted and centrifuged at 3,000 rpm for 10 min to remove the large particles. The supernatant was centrifuged again at 18,000 rpm for 30 min after being autoclaved.

[4] 50 mg hemin was dissolved in 1 ml 1 N NaOH and made to 100 ml with distilled water.

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 5890II, Hewlett Packard Co.).

After incubation for 24 h nylon clothes were retracted and washed gently with distilled water for 10 sec., and were freeze dried. Incubation solution collected after 24 h incubation was also freeze dried and lipids were extracted using Folch's solution (Folch et al., 1957). 0.1 ml internal standard (1% $C_{12:0}$ in chloroform solution, w/v) was added to the extracted lipids. Methylation of the lipids was followed the method of Lepage and Roy (1986) prior to injecting into the GC. A fused silica capillary column (30 m× 0.25 mm, i.d.×0.20 μm thickness, Supelco, SPTM-2330; USA) was used. Gas chromatograph was programmed to operate at 180℃ for 1 min, then gradually increased 2 ℃/min up to 240℃ and held at 240℃ for another 2 min. Hydrogen (H_2) was used as the carrier gas. The same method as the incubation solution was applied to the GC analysis of lipids in nylon cloth.

Measurement of hydrogenation

Percent hydrogenation was confined to the $C_{18:2}$ exposed to bacteria in the medium solution from the nylon cloth since it was considered that hydrogenation of unsaturated fatty acids occurs after hydrolysis of dietary lipids in the rumen and it was not even found any trace of hydrogenation from the $C_{18:2}$ left in the nylon cloth. Thus, hydrogenation of $C_{18:2}$ was calculated by subtracting the amount of $C_{18:2}$ remained in the medium solution from the amount of $C_{18:2}$ released from the nylon cloth after 24 h incubation.

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 Duncan's Multiple Range Test (Steel and Torrie, 1980).

RESULTS

Addition of sodium bicarbonate maintained the pH of incubation media above 6.6. Despite the differences ($p<0.036$) in pH after 6 h incubation the sources and addition levels of carbohydrates did not affect the pH in the incubation media thereafter although higher incubation levels (0.35%) had the trends of lower pH than lower levels of carbohydrates (figure 1). Ammonia concentration was not influenced by the sources and addition levels of carbohydrates except for control that was highest ($p<0.048$) at 24 h incubation.

There were not significant differences in VFA production among treatments up to 12 h incubations, but addition of carbohydrates increased ($p<0.0001$) VFA production compared to the control at the 24 h incubation and the highest production of VFA was observed from 0.35% cellobiose addition (table 2). Carbohydrate addition, irrespective of the levels, did not influence on the molar proportions of acetate (C_2) and butyrate (C_4) but 0.35% cellobiose addition increased ($p<0.048$) propionate (C_3) proportion in the incubation media up to 12 h incubations. Reduced ($p<0.0004$) C_2 but increased ($p<0.0006$) C_3 proportions were observed from the carbohydrate added treatments compared to that from the control at 24 h incubation. Additions at higher levels decreased C_2 proportion while increased C_3 proportion for both starch and cellobiose. Increased ($p<0.0004$) number of viable bacteria, however, was observed only from the both levels of starch added treatments (table 2).

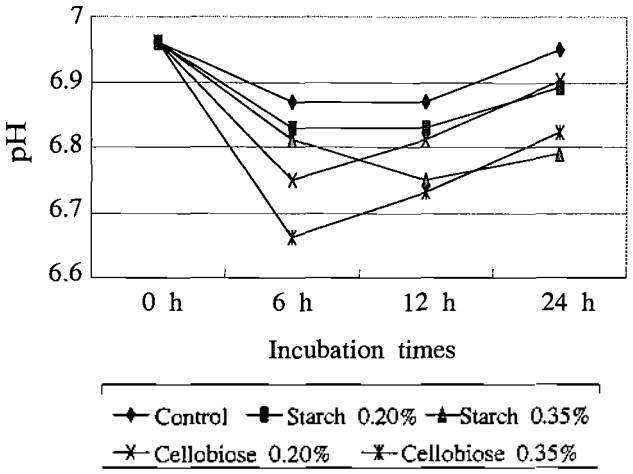

Figure 1. pH of media at various sampling times

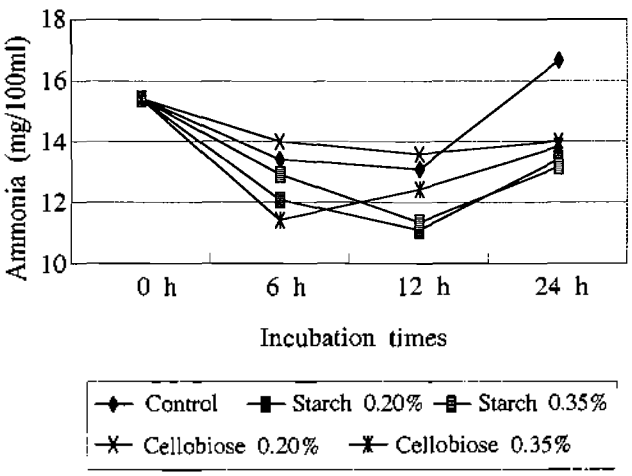

Figure 2. Ammonia concentration in incubation media at various sampling times

Table 2. Molar proportion and concentration of VFA and number of viable bacteria in incubation medium[1]

Items	Control	Starch[2]		Cellobiose[2]		SEM[3]	Pr>F[4]
		0.20 %	0.35 %	0.20 %	0.35 %		
	---------- 6 h ----------						
Total VFA (mmoles/100 ml)	22.6	26.7	27.1	26.7	25.4	0.936	0.762
Molar proportion (mmoles/100 mmoles)							
Acetate (C_2)	53.1	54.9	54.4	56.1	55.8	1.325	0.489
Propionate (C_3)	22.8	22.4	22.4	22.9	22.7	0.934	0.982
Butyrate (C_4)	19.3	20.0	19.5	18.9	18.6	0.418	0.114
C_2/C_3	2.33	2.47	2.44	2.45	2.45	0.082	0.748
	---------- 12 h ----------						
Total VFA (mmoles/100 ml)	37.1	39.7	42.4	40.2	41.4	1.839	0.223
Molar proportion (mmoles/100 mmoles)							
Acetate (C_2)	47.9	46.7	46.6	45.6	43.9	1.156	0.171
Propionate (C_3)	35.6^{b}	36.7^{b}	36.2^{b}	37.6^{ab}	40.3^{a}	1.111	0.048
Butyrate (C_4)	15.5	14.8	14.3	14.9	14.3	0.533	0.365
C_2/C_3	1.35^{a}	1.27^{a}	1.29^{a}	1.21^{ab}	1.09^{b}	0.059	0.056
	---------- 24 h ----------						
Total VFA (mmoles/100 ml)	41.2^{d}	50.9^{c}	55.7^{b}	55.3^{b}	64.2^{a}	0.845	0.0001
Molar proportion (mmoles/100 mmoles)							
Acetate (C_2)	44.6^{a}	41.7^{b}	39.9^{c}	40.2^{c}	37.1^{d}	0.396	0.0004
Propionate (C_3)	34.3^{c}	37.2^{c}	40.4^{b}	42.7^{b}	47.8^{a}	0.827	0.0006
Butyrate (C_4)	14.3	14.8	14.4	11.8	10.3	0.928	0.061
C_2/C_3	1.30^{a}	1.12^{b}	0.99^{c}	0.94^{c}	0.78^{d}	0.017	0.0001
Bacteria ($\times 10^7$)[5]	44.5^{b}	71.7^{a}	76.0^{a}	38.8^{b}	41.0^{b}	5.435	0.0004

[1] Means in the same row with different superscripts differ.
[2] Addition levels (%, w/v) of starch or cellobiose addition in 200 ml broth medium.
[3] Standard error of the mean.
[4] Probability levels.
[5] Number of total viable bacteria in incubation media after 24 h incubation. Mean bacterial number in the incubation medium at 0 h incubation was 91×105 cells per ml.

Table 3. Hydrogenation of linoleic acid ($C_{18:2}$) during 24 h incubation[1]

Items	Control	Starch[2]		Cellobiose[2]		SEM[3]	Pr>F[4]
		0.20 %	0.35 %	0.20 %	0.35 %		
$C_{18:2}$ input (mg)[5]	67.5	66.8	71.6	79.8	70.6	6.992	0.540
$C_{18:2}$ release (mg)[6]	56.2	55.9	49.9	49.7	65.4	7.865	0.597
$C_{18:2}$ hydrogenation[7]							
mg	54.2	54.7	48.5	45.1	61.3	7.718	0.559
%	95.6^{ab}	97.9^{a}	96.9^{a}	90.0^{b}	94.1^{ab}	2.505	0.048

[1] Means in the same row with different superscripts differ.
[2] Addition levels (%, w/v) of starch or cellobiose addition in 200 ml broth medium.
[3] Standard error of the mean.
[4] Probability levels.
[5] Amount of $C_{18:2}$ absorbed into the nylon cloth prior to incubation.
[6] Amount of $C_{18:2}$ released into the incubation media during 24 h incubation.
[7] hydrogenation of $C_{18:2}$ was calculated by subtracting the amount of $C_{18:2}$ remained in the medium solution from the amount of $C_{18:2}$ released from the nylon cloth after 24 h incubation.

Absorbed $C_{18:2}$ into the nylon cloth was in the range of 66.8~79.8 mg for all treatments including control. During 24 h incubation the amounts of $C_{18:2}$ released into the incubation media from nylon cloth were 56.2, 55.9, 49.9, 49.7 and 65.4 mg, for the treatments of control, 0.20 and 0.35% of starch, and 0.20 and 0.35% of cellobiose additions, respectively (table 3).

Addition of starch increased ($p<0.048$) hydrogenation (96.9~97.9%) of $C_{18:2}$ compared to control (95.6%) and cellobiose (90.0~94.1%) added treatments, but the addition levels of carbohydrates did not affect the hydrogenation by rumen bacteria. The overall mean hydrogenation was 95.1%.

DISCUSSION

The fermentation of media containing extra carbohydrates (starch and cellobiose) and the hydrogenation of $C_{18:2}$ considered to be the results of bacterial activity in the present study since protozoa were seldom found from the strained rumen fluid under the microscope due to the straining of rumen contents through 12 layers of cheese cloth. Based on the pH (figure 1), ammonia concentration (figure 2) and VFA production and viable number of bacteria (table 2), the fermentation by ruminal bacteria *in vitro* was in the normal proceed and the interference in fermentation by added $C_{18:2}$ should be minor as the amount of it released into the incubation media was small (table 3). Fermentation thus seemed to be influenced by the sources and addition levels of carbohydrates as well as added buffer ($NaHCO_3$).

Despite the extent of hydrogenation of USFA *in vitro* was similar to those measured *in vivo* as indicated by Wu and Palmquist (1991) and Fellner et al. (1995), hydrogenation (%) by ruminal bacteria in this *in vitro* study was very high as shown in table 3. The results obtained were similar to those of studies of Bickerstaffe et al. (1972) in which 90% of dietary $C_{18:2}$ was hydrogenated in the rumen of lactating goats. Mattos and Palmquist (1977), however, found that 68% of $C_{18:2}$ was hydrogenated in lactating cows and Wu et al. (1991) reported that 63.79% of $C_{18:2}$ was hydrogenated in the rumen. Fellner et al. (1995) observed based on *in vitro* study that hydrogenation of infused $C_{18:2}$ into the rumen averaged 77%.

The extent of hydrogenation of unsaturated fatty acids by ruminal bacteria have been affected by mainly dietary regimes such as levels of dietary starch and fiber (Latham et al., 1972; Leat, 1977; Gerson et al., 1985), the presence of feed particles (Kemp et al., 1984a), dietary N level (Gerson et al., 1983). In this study, however, most of experimental conditions were very similar among carbohydrate added treatments except for the differences in addition levels of starch and cellobiose. The effect of carbohydrate addition on pH of incubation media was relatively small due to the added sodium bicarbonate as buffer (figure 1). This result indicates that the extent of hydrogenation by mixed rumen bacteria might be affected by the carbohydrate source in this *in vitro* study which was in turn positively related with bacterial growth (table 2) where higher hydrogenation of $C_{18:2}$ was observed from the addition of starch than from that of cellobiose. Wang et al. (1999) also found the increased hydrogenation of $C_{18:1}$ *in vitro* from the starch compared to those from the dextrose and cellulose.

It is concluded that the sources and the levels of carbohydrates added to the incubation media reflected the VFA production and the bacterial growth, but hydrogenation of $C_{18:2}$ was influenced by the carbohydrate source under the higher pH of the present *in vitro* experimental condition. It remains, however, to examine the relationships between the duration of incubation and rate of degradation of carbohydrate sources, and hydrogenation of C_{18}-USFA.

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