Effect of C18-polyunsaturated Fatty Acids on Their Direct Incorporation into the Rumen Bacterial Lipids and CLA Production *In vitro**

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ABSTRACT : An *in vitro* study was conducted to determine the effect of C18-polyunsaturated fatty acid on direct incorporation into the rumen bacteria, bio-hydrogenation and production of CLA *in vitro*. Sixty milligrams of linoleic acid ($C_{18:2}$) or linolenic acid ($C_{18:3}$) were absorbed into the 0.5 g cellulose powder was added to the 150 ml culture solution consisting of 120 ml McDougall's buffer and 30 ml strained rumen fluid. Four *u*Ci of 1-$^{14}C_{18:2}$ or 1-$^{14}C_{18:3}$ (1 *u*Ci/15 mg each fatty acid) were also added to the corresponding fatty acids to estimate the direct incorporation into the bacterial lipids. The culture solution was then incubated anaerobically in a culture jar with stirrer at 39°C for 12 h. Ammonia concentration and pH of the culture solution were slightly influenced by the fatty acids. Amount of fatty acid incorporated into the bacteria was 1.20 mg and 0.43 mg/30 ml rumen fluid for $C_{18:2}$ and $C_{18:3}$, respectively during 12 h incubation. Slightly increased CLA (sum of *cis*-9, *trans*-11 and *cis*-10, *trans*-12 $C_{18:2}$) was obtained from the $C_{18:3}$ addition compared to that from $C_{18:2}$ after 12 h incubation *in vitro*. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 4 : 512-515)***

Key Words : Linoleic Acid, Linolenic Acid, Bio-hydrogenation, CLA, Rumen Bacteria

INTRODUCTION

Cellular lipids of rumen microorganisms are known to be generated by *de novo* synthesis and by the direct incorporation of performed precursor molecules which are of dietary origin. Knight et al. (1979) reported that acetic acid (C_2) was utilized for the synthesis of palmitic acid. However, since polyunsaturated fatty acids are not commonly synthesized by bacteria, rumen microbes are likely to incorporate the exogenous preformed fatty acids (Harfoot and Hazlewood, 1988). But the rate and the amount of incorporation of those fatty acids into the rumen bacteria have not been reported.

Meanwhile, conjugated linoleic acid (CLA) is one of the major intermediate products of bio-hydrogenation of C_{18}-polyunsaturated acids by the rumen bacteria (Harfoot and Hazlewood, 1988; Wang et al., 2003; Wang et al., 2005). The CLA has been mostly derived from the dietary linoleic acid ($C_{18:2}$, Kelly et al., 1998). Bessa et al. (2000), however, revealed the possibility of alternative pathway that may be existed in the production of CLA from linolenic acid ($C_{18:3}$) due to the extreme microbial diversity in the reticulo-rumen. Wang et al. (2002a, b) also found the possibility of CLA production from $C_{18:3}$.

The current *in vitro* study, therefore, was conducted to determine the effect of C18-polyunsaturated fatty acids on fermentation characteristics, direct incorporation into the rumen bacteria, bio-hydrogenation and production of CLA isomers (*cis*-9, *trans*-11 and *cis*-10, *trans*-12 $C_{18:2}$).

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MATERIALS AND METHODS

Preparation of rumen fluid

Approx. 4 kg rumen contents in total were collected at 2 h after morning feeding (0800) from the two non-lactating ruminally cannulated Holstein cows fed 6 kg of rice straw (50%) and concentrate (50%) on a dry matter (DM) basis twice daily in an equal portion. The rumen contents were brought to the laboratory and were blended in a Waring blender (Fisher 14-509-1) 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 protozoa. CO_2 was flushed into the strained rumen fluid.

Preparation and incubation of culture

Thirty ml strained rumen fluid was mixed with 120 ml McDougall's artificial saliva (1948) under flushing of CO_2. For the measurement of direct incorporation of single fatty acid ($C_{18:2}$ or $C_{18:3}$, Sigma Co.) into the bacterial lipid, 1.5 g (1% of culture solution) lipid extracted ground corn (0.5 mm screen) and 60 mg of each fatty acid absorbed into the 0.5 g cellulose powder were added to the each 150 ml culture solution in the glass culture jar. Four *u*Ci of 1-$^{14}C_{18:2}$ or 1-$^{14}C_{18:3}$ (1 *u*Ci/15 mg each fatty acid) were also added to the corresponding fatty acids. Gaseous CO_2 was flushed into the culture solution for 1 minute. 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 1 min., and was incubated up to 12 h. Stirring speed during incubation was adjusted to 120 times/min. The *in vitro*

Table 1. Composition of C18-fatty acids in the rumen bacterial lipids

Fatty acids	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Others
Composition (%)	18.17	29.33	2.85	0.58	49.08

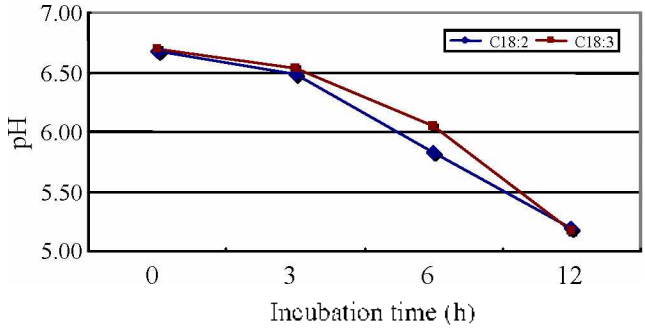

Figure 1. Changed in pH of culture solution during the incubation times as influenced by different C18 fatty acids.

study was conducted three times with three replicates per treatments per each time under the similar conditions.

Sampling and analysis

pH of culture solution was measured at the incubation times of 3, 6 and 12 h, and 5 ml culture solution was collected for ammonia and volatile fatty acid (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 the spectrophotometer (DU-650). Four ml culture solution was mixed with 1 ml 25% phosphoric acid and 0.5 ml pivalic acid solution (2%, w/v) 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 5,890 II, Hewlett Packard Co.). Two hundred ml culture 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 fatty acids 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, Supelco, SPTM-2,560; USA) was used.

For the determination of fatty acid composition of bacterial lipids, 300 ml strained rumen fluid was centrifuged at low speed (2,000×g, 4°C) for 10 min. to remove protozoa and feed particles. The supernatant was collected and was again centrifuged at high speed (22,000× g, 4°C) for 10 min. to separate bacteria. The separated bacterial pellet was then homogenized in Bryant's diluting solution (Bryant and Robinson, 1961) and centrifuged same as for separation of bacteria. Extraction of bacterial lipids, methylation and fatty acid analyses were same for the culture solution. The composition of C18-fatty acids and the total of other fatty acids in the mixed rumen bacteria was shown in Table 1.

For the measurement of incorporation of added fatty acid into the rumen bacteria, 200 ml culture solution after 12h incubation was centrifuged at low speed (2,000×g, 4°C) for 10 min. to remove feed particles. The supernatant was collected and was again centrifuged at high speed (22,000× g, 4°C) for 10min. to separate bacteria. The separated bacterial pellet was then homogenized in Bryant's diluting solution (Bryant and Robinson, 1961) and centrifuged same as for separation of bacteria. Two more times of homogenization and washing were made to remove the free radioisotopes completely and the bacterial pellet was freeze dried. Extraction of bacterial lipids was done same as for culture solution. Five ml chloroform containing bacterial lipids was transferred to the 10 ml scintillation vial and the chloroform was evaporated in the dry-bath (60°C) under the N gas. Eight ml multipurpose scintillation cocktail (Insta-gel XF, Packard Co.) was then added to the vial, and specific radio-activities (cpm) of 1-$^{14}C_{18:2}$ and 1-$^{14}C_{18:3}$ in bacteria were measured by the β-counter (Beckman LS 5801). Background cpm was also measured. Specific radio-activities of 0.1 μCi 1-$^{14}C_{18:2}$ and 0.1 μCi 1-$^{14}C_{18:3}$ were 569,681 cpm and 484,741 cpm, respectively, and background cpm was 36. Incorporated amount of each fatty acid into the bacteria was calculated by following equation: Amount of each fatty acid added to the culture solution x ((specific radioactivity (cpm) of bacterial lipid)/(total specific radioactivity (cpm) of the culture solution)).

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 Test (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

pH of culture solution lowered with incubation times but was not influenced by the fatty acid except for 6 h incubation which is slightly ($p<0.102$) higher from $C_{18:3}$ than from $C_{18:2}$ addition (Figure 1). No differences were observed in ammonia concentration between fatty acids although $C_{18:2}$ slightly ($p<0.081$) decreased concentration compared to $C_{18:3}$ (Figure 2). Total VFA concentration increased with incubation time and was higher ($p<0.025$) for $C_{18:3}$ than for $C_{18:2}$ addition at 3 h incubation and slightly increased at the other incubation times in $C_{18:3}$ added treatment (Table 2). Addition effect of single C18-polyunsaturated fatty acid was not found in molar proportion of VFA, but butyrate proportion was unexpectedly high in both additions of $C_{18:2}$ and $C_{18:3}$. The

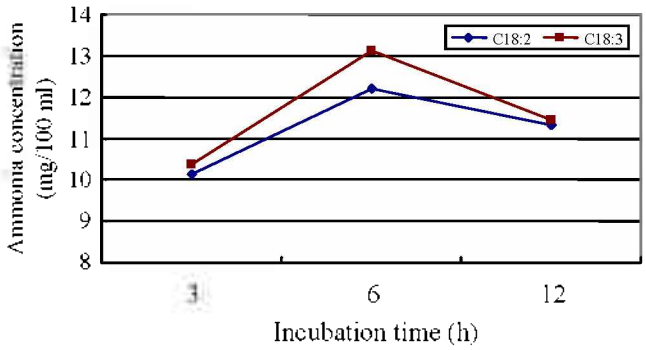

Figure 2. Changes in ammonia concentration in culture solution during the incubation times as influenced by different C18 fatty acids.

C_2:C_3 ratio tended to be higher for $C_{18:3}$ than for $C_{18:2}$.

Incorporation of added $C_{18:2}$ into the bacterial lipid during 12 h incubation was higher ($p<0.0009$) as 1.20 mg/30 ml rumen fluid than that of $C_{18:3}$ (0.43 mg) in the present study (Table 3). Percent incorporation of $C_{18:2}$ also increased compared to that of $C_{18:3}$.

Composition of C18-fatty acids in culture solution after 12 h incubation was shown in Table 4. As expected, compositions of $C_{18:2}$ and $C_{18:3}$ were high as 54.5% and 44.25%, respectively, in culture solution but the $C_{18:1}$ composition was similar between added fatty acids. Production of CLA consisting of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers was slightly increased by the $C_{18:3}$ addition compared to $C_{18:2}$ addition (Table 4).

Cellular lipids of rumen microbes have been known to be generated by *de novo* synthesis, and acetic acid (C_2) was the major precursor for the synthesis of palmitic acid (Knight et al., 1979). However, since polyunsaturated fatty acids are not commonly synthesized by bacteria, rumen microbes may incorporate the exogenous preformed fatty acids (Harfoot and Hazlewood, 1988), and their postulation is supported by the present study. The higher incorporation of $C_{18:2}$ than $C_{18:3}$ in the present study might be related to the relevant composition of rumen bacterial lipids in which composition of $C_{18:2}$ is higher as 2.85% than $C_{18:3}$ (0.58%).

Table 3. Direct incorporation of single C18-polyunsaturated fatty acid into the rumen bacteria in the culture solution after 12 h incubation

Items	$C_{18:2}$	$C_{18:3}$	SEM[1]	Pr>F[2]
Incorporated fatty acid (mg/30 ml rumen fluid)	1.20[a]	0.43[b]	0.117	0.0009
Percent incorporation	1.99[a]	0.71[b]	0.196	0.0009

[a,b] Means in the same row with different superscripts differ ($p<0.05$).
[1] Standard error of the means. [2] Probability level.

Table 4. Addition effects of single C18-polyunsaturated fatty acid on composition of C18-fatty acids and CLA in culture solution after 12 h incubation

Fatty acids (%)	$C_{18:2}$	$C_{18:3}$	SEM[1]	Pr>F[2]
$C_{18:0}$	28.72	28.14	2.247	0.8165
$C_{18:1}$	4.72	6.71	0.811	0.8036
$C_{18:2}$	54.50[a]	3.05[b]	3.374	0.0001
$C_{18:3}$	0.48[b]	44.25[a]	1.712	0.0001
CLA[3]	0.86	1.24	0.181	0.1231
Other fatty acids	11.72	16.61	1.290	0.1349

[a,b] Means in the same row with different superscripts differ ($p<0.05$).
[1] Standard error of the means. [2] Probability level.
[3] CLA, sum of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 $C_{18:2}$ isomers.

Table 2. Addition effects of single C18-polyunsaturated fatty acid on VFA production in the culture solution

Items	$C_{18:2}$	$C_{18:3}$	SEM[1]	Pr>F[2]
		3 h		
Total VFA (mmoles/100 ml)	11.9[b]	16.4[a]	1.314	0.031
Molar proportion (mmoles/100 mmoles)				
Acetate (C_2)	34.07	37.96	2.186	0.2702
Propionate (C_3)	23.87	23.23	0.506	0.1903
Butyrate	33.87	31.13	1.605	0.4612
C_2/C_3	1.45	1.64	0.116	0.2531
		6 h		
Total VFA (mmoles/100 ml)	18.2	18.6	1.621	0.1928
Molar proportion (mmoles/100 mmoles)				
Acetate (C_2)	38.10	39.26	0.473	0.3570
Propionate (C_3)	24.16	23.64	0.889	0.5354
Butyrate	30.16	29.85	0.922	0.3385
C_2/C_3	1.59	1.69	0.110	0.3329
		12 h		
Total VFA (mmoles/100 ml)	21.9	25.5	1.103	0.2655
Molar proportion (mmoles/100 mmoles)				
Acetate (C_2)	41.20	43.27	1.744	0.4206
Propionate (C_3)	24.98	22.99	1.653	0.4150
Butyrate	27.56	26.67	0.818	0.4611
C_2/C_3	1.72	1.92	0.174	0.4274

[a,b] Means in the same row with different superscripts differ ($p<0.05$).
[1] Standard error of the means. [2] Probability level.

indicating that the requirement of each C18-polyunsaturated fatty acid for the synthesis of bacterial lipids may differ.

Kelly et al. (1998) indicated that CLA was mostly derived from the dietary $C_{18:2}$ in the rumen. Bessa et al. (2000), however, revealed the possibility of alternative pathway in the production of CLA from $C_{18:3}$. In the present study, slightly increased CLA (*cis*-9, *trans*-11 and *cis*-10, *trans*-12 $C_{18:2}$) composition (Table 4) from $C_{18:3}$ compared to $C_{18:2}$ indicates that $C_{18:2}$ may not be the single precursor for the production of CLA in the rumen. Wang et al. (2002a) also found greater CLA production from linseed which is higher in $C_{18:3}$ composition than rapeseed from *in vitro* study. Similar results were obtained from the *in vitro* study by Wang et al. (2002b) when ground linseed and linseed oil were incubated. Further studies, however, are still required to confirm the bacterial synthesis of CLA from $C_{18:3}$ under various fermentation conditions.

In conclusion, incorporation amounts of $C_{18:2}$ and $C_{18:3}$ into the rumen bacteria in the present *in vitro* study were found to be related to the relevant composition of rumen bacterial lipids. The strong possibility that rumen bacteria can produce CLA from $C_{18:3}$ was also found under the current fermentation condition.

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