Effect of Monensin and Fish Oil Supplementation on Biohydrogenation and CLA Production by Rumen Bacteria *In vitro* When Incubated with Safflower Oil*

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ABSTRACT : An *in vitro* study was conducted to examine the effect of monensin or fish oil addition on bio-hydrogenation of C_{18}-unsaturated fatty acids and CLA production by mixed ruminal bacteria when incubated with safflower oil. Commercially manufactured concentrate (1%, w/v) with safflower oil (0.2%, w/v) were added to mixed solution (600 ml) of strained rumen fluid and McDougalls artificial saliva (control). Monensin (Rumensin®, 10 ppm, w/v, MO), mixed fish oil (0.02%, w/v, absorbed to 0.2 g alfalfa hay, FO) or similar amounts of monensin and fish oil (MO+FO) to MO and FO was also added into the control solution. All the culture solutions prepared were incubated in the culture jar anaerobically at 39°C up to 12 h. Higher pH ($p<0.047$) and ammonia concentration ($p<0.042$) were observed from the culture solution containing MO at 12 h incubation than those from the culture solutions of control or FO. The MO supplementation increased ($p<0.0001$-0.007) propionate proportion of culture solution but reduced butyrate proportion at 6 h ($p<0.018$) and 12 h ($p<0.001$) of incubations. Supplementation of MO or MO+FO increased ($p<0.001$) the proportions of $C_{18:2}$. The MO alone reduced ($p<0.022$-0.025) the proportion of c9,t11-CLA compared to FO in all incubation times. The FO supplementation increased the proportion of c9,t11-CLA. An additive effect of MO to FO in the production of c9,t11-CLA was observed at 6 h incubation. *In vitro* supplementation of monensin reduced hydrogenation of C_{18}-UFAs while fish oil supplementation increased the production of CLA. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 2 : 221-225)***

Key Words : Safflower Oil, CLA, Monensin, Fish Oil, *In vitro*, Rumen Bacteria

INTRODUCTION

Attempts to increase the CLA levels in ruminant products have been made on a dietary manipulation by forage intake level or forage to concentrate ratio (Chouinard et al., 1998b; Wang et al., 2003) due to the beneficial health effects from CLA in cancer (Ha et al., 1987), atherosclerosis (Lee et al., 1994) and immunity (Michal et al., 1992). Selection of oil source (Wang et al., 2002a), supplementation type of oil (Wang et al., 2002b) and pH effect (Wang and Song, 2003) also have been important in the CLA production.

Meanwhile, supplementation of monensin which is the ionophoric antibiotics has increased the ratio of propionate to acetate (Newbold et al., 1993; Yang and Russell, 1993) while reduced CH_4 production, thus improved energetic efficiency in beef cattle (Spears and Harvey, 1984). Dietary ionophores has also been known to alter lipid metabolism in the rumen. Fellner et al. (1997) found an increased CLA proportion from the infusion of monensin (2 μg/ml) in the *in vitro* study compared to that without it. Dhiman et al. (1999) also reported that monensin supplementation (250 mg/cow/day) to the diets enhanced CLA contents in milk. Thus, the use of monensin in association with lipid supplementation may be one of the ways to increase the CLA proportion.

In addition, fish oil which is generally high in ω-3 fatty acids may also contribute to the ruminal production of CLA. The CLA concentration in milk fat increased when lactating dairy cows were fed diets supplemented with fish oil (Chouinard et al., 1998a; Dhiman et al., 1999). The effect was even greater when the diet was supplemented with fish meal and monensin together (Dhiman et al., 1999).

However, since much of the CLA found in milk is actually synthesized within the mammary gland from $C_{18:1}$ *trans*-11 through the action of stearoyl-CoA desaturase (Griinari and Bauman, 1999), effects of monensin and fish oil in the CLA production by the rumen microbes are still required to determine. The present study, therefore, was conducted to examine the effect of monensin and/or fish oil on the hydrogenating characteristics of C_{18}-unsaturated fatty acids (UFAs) and the formation of CLA by rumen bacteria.

MATERIALS AND METHODS

Preparation of rumen fluid

Rumen contents were collected at 3 h after morning feeding (0700) from two ruminally cannulated Holstein cows fed 5 kg of corn silage (60%) and concentrate (40%) for the middle lactating period on a DM basis twice daily in an equal portion and were mixed in equal portions. Analyzed contents of crude protein, ether extracts and neutral detergent fiber in concentrate were 15.8, 3.57 and 38.2%, respectively. The rumen contents were brought to

* This work was supported by Korea Research Foundation Grant (KRF-2002-041-F00040).

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Received September 22, 2004; Accepted November 1, 2004

Table 1. Fatty acid composition (% of total) of oils used in the current study

Fatty acids	Oil sources (%)	
	Safflower oil	Mixed fish oil
Myristic acid ($C_{14:0}$)	5.9	5.5
Pentadecanoic acid ($C_{15:0}$)	-	1.2
Palmitic acid ($C_{16:0}$)	9.4	23.6
Palmitoleic acid ($C_{16:1}$)	-	9.7
Margaric acid ($C_{17:0}$)	-	2.0
Srearic acid ($C_{18:0}$)	3.3	6.3
Oleic acid ($C_{18:1}$)	17.8	28.2
Linoleic acid ($C_{18:2}$)	62.5	3.2
Linolenic acid ($C_{18:3}$)	0.4	1.0
Arachidic acid ($C_{20:0}$)	0.3	0.5
Eicosenoic acid ($C_{20:1}$)	-	6.3
Eicosapentaenoic acid ($C_{20:5}$)	0.3	0.7
Behenic acid ($C_{22:0}$)	-	2.0
Erucic acid ($C_{22:1}$)	-	1.7
Docosahexaenoic acid ($C_{22:6}$)	-	8.0

the laboratory in a thermos in 20 min after it was taken 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. The strained rumen fluid was flushed with CO_2.

Preparation of culture solution and its incubation

Strained rumen fluid was mixed with McDougalls artificial saliva (1948) at the ratio of 1:1 under flushing of CO_2. Six grams of commercially manufactured concentrate (1% of culture solution. w/v. as-fed basis) for the growing dairy cattle with 1.2 g of safflower oil (0.2%. w/v) absorbed to 2 g ground (1 mm) alfalfa hay (2.45% EE, DM basis) were added to the 600 ml mixed solution of rumen fluid and McDougalls artificial saliva in the glass culture jar (control). and CO_2 was flushed into the culture solution for 1 minutes. Monensin (Rumensin®, 10 ppm, w/v. MO), mixed fish oil (0.02%. w/v. absorbed to 0.2 g alfalfa hay. FO). and similar amounts of monensin and fish oil (MO+FO) to MO and FO were added into the control solution. respectively. The culture jar was covered with a glass lid equipped with stirrer and was placed into a water-bath (39°C). Culture solution was again flushed with CO_2 through glass tube connected to the jars for 1 min. and was incubated up to 12 h. Stirring speed during incubation was adjusted to 120 times/min. The incubation of culture solution was done three times under the similar condition.

Sampling and analysis

pH of culture solution was measured at the incubation times of 3. 6 and 12 h by inserting the prove of pH meter into the culture solution in the jar. and 5 ml culture solution was collected for ammonia and VFA analysis. All samples collected were kept frozen at -20°C until analyzed.

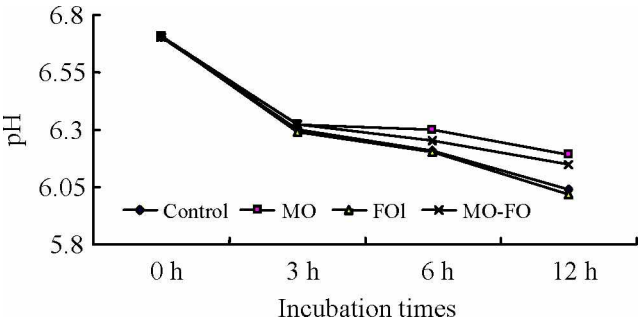

Figure 1. pH of culture solution.

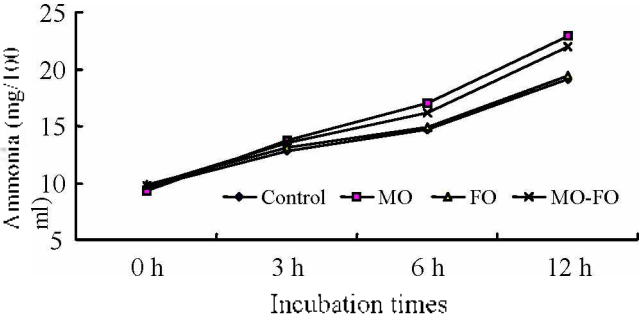

Figure 2. Ammonia concentration in culture solution.

Ammonia concentration was determined by the method of Fawcett and Scott (1960) using the spectrophotometer (DU-650). Four mls culture solution were mixed with 1 ml 25% phosphoric acid and 0.5ml 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.). In addition, fifty ml incubation solution was also collected at the incubation times of 3. 6 and 12 h. freeze dried and lipids were extracted using Folch's solution (Folch et al.. 1957). Methylation of the lipids extracted followed the method of Lepage and Roy (1986) prior to injecting into the GC using a fused silica capillary column (100 m×0.25 mm. i.d.×0.20 μm thickness. Supelco. SPTM-2.560. USA). Fatty acid composition of safflower oil and fish oil was also analyzed by the same method as for the culture solution and shown in Table 1.

Statistical analysis

The results obtained were subjected to least squares analysis of variance according to the general linear models procedure of SAS (1985) and the data among treatments were compared using S-N-K Test (Steel and Torrie. 1980).

RESULTS

Higher pH ($p<0.047$. Figure 1) and ammonia concentration ($p<0.042$. Figure 2) were observed from the

Table 2. Concentration and molar proportion of VFA in culture solution when incubated with safflower oil

Items	Treatments [1]				SEM[2]	Pr>F [3]
	Control	MO	FO	MO-FO		
	3 h					
Total VFA (mmoles/100 ml)	48.52	52.70	50.32	48.37	1.632	0.837
Molar proportion (mmoles/100 mmoles)						
Acetate (C_2)	47.89	46.73	47.43	47.17	0.914	0.337
Propionate (C_3)	24.74^b	26.96^a	24.74^b	26.95^a	0.289	0.007
Butyrate	21.48	20.04	21.67	20.05	0.785	0.397
C_2/C_3	1.93^a	1.73^b	1.91^a	1.74^b	0.031	0.018
	6 h					
Total VFA (mmoles/100 ml)	55.63	57.06	58.79	58.68	0.818	0.135
Molar proportion (mmoles/100 mmoles)						
Acetate	43.67	43.68	44.13	44.01	0.705	0.951
Propionate	25.16^b	29.54^a	24.48^b	29.01^a	0.525	0.005
Butyrate	25.13^a	20.37^b	25.36^a	20.59^b	0.801	0.018
C_2/C_3	1.73^a	1.47^b	1.80^a	1.51^b	0.034	0.006
	12 h					
Total VFA (mmoles/100 ml)	67.76	66.54	65.65	65.31	0.428	0.051
Molar proportion (mmoles/100 mmoles)						
Acetate	42.74	41.51	41.86	41.43	0.385	0.206
Propionate	21.90^d	29.95^a	25.33^c	28.23^b	0.250	0.0001
Butyrate	28.44^a	21.05^b	26.54^a	22.44^b	0.533	0.001
C_2/C_3	1.95^a	1.38^d	1.65^b	1.46^c	0.001	0.0001

[1] Control: without monensin and fish oil, MO: monensin, FO: fish oil, MO-FO: monensin and fish oil.
Means in the same row with different superscripts differ significantly.
[2] Standard error of the mean. [3] Probability levels.

culture solution containing MO at 12 h incubation than those from control or FO. Supplementation of MO did not affect the VFA concentration, but increased ($p<0.0001$-0.007) propionate (C_3) proportion over the all collection times of culture solution while reduced butyrate (C_4) proportion at 6 h ($p<0.018$) and 12 h ($p<0.001$) of incubations (Table 2). Concentration and proportion of VFA were not greatly affected by FO supplementation.

Supplementation of MO or MO-FO lowered ($p<0.001$-0.002) the proportion of $C_{18:0}$ throughout the incubation, while enhanced the proportions of $C_{18:1}$ at 12 h ($p<0.035$), and $C_{18:2}$ at 3 h ($p<0.001$) and 6 h ($p<0.0009$) of culture solution (Table 3). Supplementation of MO alone reduced ($p<0.022$-0.025) the proportion of c9,t11-CLA compared to FO in all incubation times but increased ($p<0.025$) the proportion of c9,t11-CLA compared to that of control at 12 h incubation. Reversed trends to the profiles of most fatty acids in culture solution containing MO, however, were obtained from the supplementation of FO (Table 3). An additive effect of MO to FO in the production of c9,t11-CLA was only observed at 12 h incubation. Production of t10,c12-CLA was relatively small compared to c9,t11-CLA and its proportional trend for all incubation times as influenced by supplementation sources followed the cases of c9,t11-CLA.

DISCUSSION

The patterns of the changes with incubation time in pH, ammonia concentration and VFA indicate the normal fermentation and they responded to the supplemented sources. The data of pH (Figure 1) and proportions of C_3 and C_4 (Table 1) in culture solution in the present study may indicate that MO (10 ppm, w/v) affected the fermentation as it probably reduce the Gram-positive bacterial growth which are sensitive to MO (Russell, 1987).

Based on the proportion of $C_{18:0}$, both MO and FO slightly reduced the rate of hydrogenation of C_{18}-unsaturated fatty acids added to the culture solution by rumen bacteria. But their actions in hydrogenation were different depending on the stage of hydrogenation related to incubation time (Table 2). Fellner et al. (1997) reported that MO inhibits the growth of *Butyrivibrio fibrisolvens*, Gram-positive bacterium which has been actively involved the in ruminal hydrogenation. They also reported that the MO increased the total CLA isomers. But the result of present study in t10,c12-CLA composition is consistent with results of Fellner et al. (1997) only at the 12 h incubation (Table 3). This result may indicate the time dependent effect of monensin. Results from studies with lactating dairy cows fed the diets supplemented with the monensin have been varied. Sauer et al. (1998) reported an increase, whereas both Dhiman et al. (1996) and Chouinard et al. (1998b) observed no effect of monensin on CLA concentration of milk fat from cows. However, the reason for the differences in CLA production between above two studies has not been defined.

Table 3. Composition (%) of C_{18}-fatty acids in culture solution when incubated with safflower oil

Fatty acids	Treatments [1]				SEM [2]	Pr>F [3]
	Control	MO	FO	MO-FO		
	3 h					
$C_{18:0}$	47.28^{a}	39.95^{c}	43.87^{b}	38.59^{c}	0.586	0.001
$C_{18:1}$	16.03	14.05	16.56	15.59	0.962	0.399
$C_{18:2}$	11.95^{b}	22.03^{a}	13.31^{b}	20.77^{a}	0.753	0.001
c9, t11-CLA	0.60^{bc}	0.54^{c}	0.75^{a}	0.69^{ab}	0.028	0.022
t10, c12-CLA	0.46	0.37	0.44	0.30	0.114	0.753
Total CLA	1.06	0.91	1.19	0.99	-	-
$C_{18:3}$	0.82	1.71	1.24	1.60	0.248	0.186
	6 h					
$C_{18:0}$	52.67^{a}	47.14^{b}	49.16^{ab}	42.93^{c}	1.011	0.010
$C_{18:1}$	16.59	13.13	16.30	16.21	0.892	0.137
$C_{18:2}$	8.27^{b}	15.13^{a}	9.01^{b}	15.00^{a}	0.479	0.001
c9, t11-CLA	0.49^{b}	0.46^{b}	0.74^{a}	0.69^{a}	0.037	0.014
t10, c12-CLA	0.28	0.13	0.43	0.33	0.095	0.314
Total CLA	0.77	0.59	1.17	1.02	-	-
$C_{18:3}$	1.11	1.40	1.25	1.42	0.266	0.827
	12 h					
$C_{18:0}$	61.68^{a}	48.49^{c}	55.08^{b}	44.16^{c}	1.309	0.002
$C_{18:1}$	10.98^{b}	17.82^{ab}	13.28^{ab}	20.13^{a}	1.464	0.035
$C_{18:2}$	6.99	9.98	9.66	11.11	0.757	0.070
c9, t11-CLA	0.39^{c}	0.63^{b}	0.68^{b}	1.14^{a}	0.099	0.025
t10, c12-CLA	0.18^{b}	0.39^{a}	0.31^{b}	0.51^{a}	0.069	0.045
Total CLA	0.57	1.02	0.99	1.65	-	-
$C_{18:3}$	1.03	1.09	0.90	1.02	0.194	0.911

[1] Control: without monensin and fish oil, MO: monensin, FO: fish oil, MO-FO: monensin and fish oil.
Means in the same row with different superscripts differ significantly.
[2] Standard error of the mean. [3] Probability levels.

An increased c9,t11-CLA proportion in culture solution in present study is well agreed with the results of Dhiman et al. (1999) in which they fed cow either a control diet, supplementations of 3% fish meal, 250 mg of monensin/cow/per day or fish meal and monensin together. Relevant contents of CLA were 5.3, 8.6, 6.8 and 8.9 mg/g of milk fatty acids, respectively. Chouinard et al. (1998a) and Chilliard et al. (1999) also reported an increased concentration of CLA in milk fat from fish oil supplementation.

In general, fish oil contains relatively long-chain poly-unsaturated fatty acids (PUFAs, Table 1). But effect of fish oil on the ruminal bio-hydrogenation of the PUFAs has not been well defined. Further study, therefore, is required to examine how the fish oil act on lipolysis, hydrogenation of UFAs and CLA production by rumen bacteria.

In conclusion, *in vitro* supplementation of monensin increased pH, ammonia concentration and propionate proportion in the culture solution, but reduced hydrogenation of C_{18}-UFAs. Meanwhile, fish oil did not affect the fermentation characteristics, but increased the production of CLA.

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