Addition Effect of Seed-associated or Free Linseed Oil on the Formation of *cis*-9, *trans*-11 Conjugated Linoleic Acid and Octadecenoic Acid by Ruminal Bacteria *In Vitro***

J. H. Wang and M. K. Song*, Y. S. Son[1] and M. B. Chang[2]
Department of Animal Science, Chungbuk National University, Chungbuk 361-763, Korea

ABSTRACT : The effects of seed-associated or free linseed oil on fermentation characteristics and long-chain unsaturated fatty acids composition, especially the formation of conjugated linoleic acid (CLA) and octadecenoic acid (*trans*-11 $C_{18:1}$, t-$C_{18:1}$) by mixed ruminal bacteria were examined *in vitro*. Concentrate (1% of culture solution, w/v, as-fed basis) with ground linseed (0.6% of culture solution, w/v, DM basis) or linseed oil as absorbed onto ground alfalfa hay was added to 600 ml mixed solution consisting of strained rumen fluid and artificial saliva at the ratio of 1:1 in a glass culture jar. The culture jar was covered with a glass lid with stirrer, and placed into a water-bath (39°C) and incubated anaerobically up to 24 h. Seed-associated or free linseed oil did not significantly affect the pH and ammonia concentration in the culture solution. Molar percent of acetate tended to increase while that of propionate decreased with the addition of free oil treatment throughout the incubation. Differences in bacterial number were relatively small, regardless of the form of supplements. Decreasing trends in the compositions of linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) but increasing trends of stearic acid ($C_{18:0}$), t-$C_{18:1}$ and CLA compositions were found from culture contents up to 12h incubation when incubated with both ground linseed and linseed oil. The compositions of $C_{18:0}$, $C_{18:2}$ and $C_{18:3}$ were greater but those of oleic acid ($C_{18:1}$), t-$C_{18:1}$ and CLA were smaller in a culture solution containing ground linseed than those containing linseed oil. The ratio of t-$C_{18:1}$ to CLA was lower in the culture solutions containing linseed oil up to 12h incubations as compared to those containing ground linseed. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 8 : 1115-1120)***

Key Words : Oil Addition Type, Bio-hydrogenation, CLA, Trans-$C_{18:1}$, *In Vitro*, Ruminal Bacteria

INTRODUCTION

Free unsaturated fatty acids released from the degradation of dietary lipids in the rumen are subjected to bio-hydrogenation by the rumen microorganisms (Harfoot and Hazlewood, 1988). *Cis*-9, *trans*-11 conjugated linoleic acid (CLA) and octadecenoic acid (*trans*-11 $C_{18:1}$, t-$C_{18:1}$ are formed as a result of incomplete bio-hydrogenation of unsaturated fatty acids. It was known that the contents of CLA tended to be positively correlated with t-$C_{18:1}$ in the rumen (Bessa et al., 2000). However, while the CLA has received growing attention due to its beneficial health effects such as the suppressions of carcinogenesis (Belury, 1995), control of obesity (Park et al., 1997), and reductions in atherogenesis (Nicolosi et al., 1997) and diabetes (Houseknecht et al., 1998), the t-$C_{18:1}$ has been shown to negatively affect on milk production (kennelly, 1996; Kalscheur et al., 1997) and contribute to coronary heart disease (Van de Vijver et al., 1996).

Previous studies demonstrated that the milk fat concentration of CLA was dependent on the amount of dietary unsaturated fatty acids (Griinari et al., 1996), and that CLA concentrations increased as dietary level of oil or oilseed increased (McGuire et al., 1996; Kennelly, 1996). This indicates that the formations of both CLA and t-$C_{18:1}$ in the rumen are mostly dependent upon the fatty acid composition and the quantity of lipids in the diets. In addition, it can also be postulated that the form of oil supplementation affects the degree of the biosynthesis of CLA and t-$C_{18:1}$ since the degradation rate of lipids in ground or whole oil seed is in general slower than in free oil. Thus, more CLA and t-$C_{18:1}$ may be synthesized from free oil than from ground oilseed.

The objective of the present *in vitro* study was to compare the effect of supplementation of seed-associated or free linseed oil on fermentation characteristics, and the formation of CLA and t-$C_{18:1}$ by ruminal bacteria.

MATERIALS AND METHODS

Preparation of rumen fluid

Rumen contents were collected 3h after the morning feeding (06:00) from ruminally cannulated Holstein cows fed 5kg of corn silage (60%) and concentrate (40%) on a DM basis twice daily. The cows were fed the concentrate supplemented with linseed oil (1%, DM basis) for 5 days

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* Address reprint request to M. K. Song. Tel: +82-43-261-2545, E-mail: mksong@cbucc.chungbuk.ac.kr
[1] Department of Animal Science, Korea University.
[2] Department of Animal Science and Technology, College of Industrial Sciences, Chung-ang University.
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prior to sampling of rumen contents for the rumen microbial adaptation to the oil. 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 strained through 12 layers of cheesecloth to remove feed particles and large protozoa. CO_2 was flushed into the rumen fluid during preparation of inocula.

Preparation of culture solution and its incubation

Strained rumen fluid was mixed 1:1 with McDougall's artificial saliva (1948) under flushing of 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 (0.6% of culture solution, w/v, DM basis) or 1.225 g of linseed oil which was absorbed to 2.25 g ground alfalfa hay (2.45% ether extracts, 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 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 the infusion purpose for 3 min, and incubated up to 24 h. Stirring speed during incubation was adjusted to 120 times/min. The incubation of culture solution was done three times with duplicate for each treatment under similar condition.

Enumeration of total viable bacteria

The number of viable bacteria in the culture solution was determined at 12 h and 24 h incubations by the anaerobic culture techniques of Hungate (1966). One ml of culture solution from each treatment was taken and diluted to 10^5-10^7 using Bryant's solution (Bryant and Robinson, 1961). Diluted solution (1 ml) was inoculated into a roll tube containing non-selective artificial medium (Scott and Dehority, 1965). The number of colonies was counted after the roll tubes were incubated at 39°C for 5 days.

Sampling and analysis

pH of culture solution was measured at the incubation times of 3, 6, 12 and 24h, 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). Culture solution (4ml) was 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 a gas chromatograph (GC, HP 5890Ⅱ, Hewlett Packard Co.). Incubation solution (50 ml) was also collected at the incubation times of 0, 3, 6, 12 and 24 h, and freeze dried, and lipids were extracted using Folch′s solution (Folch et al., 1957). Methylation of the extracted lipids followed the method of Lepage and Roy (1986) prior to injecting into the GC equipped with a fused silica capillary column (100 m×0.25 mm, i.d.×0.20 μm thickness, Supelco, SPTM-2560; USA). Octadecenoic acid (*Trans*-11 $C_{18:1}$) and *cis*-9, *trans*-11 CLA only were detected as major isomers of $C_{18:1}$ and $C_{18:2}$ (Parodi, 1997; Sehat et al., 1998). Other isomers detected were ignored because they are too low in concentrations.

Statistical analysis

The results obtained from the three 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

Compositions of C_{18}-fatty acids in linseed and concentration are presented in table 1. The amount lipid in concentrates added to culture solution was relatively small (0.346 g) compared to that from ground linseed or linseed oil (1.225 g in both) as shown in table 1. The total lipid content of 2.25 g ground alfalfa hay (DM basis) was also very small (0.05 g) and is unlikely to affect overall lipid metabolism. The possible effect of lipid content of strained rumen fluid at beginning point of the present study was ignored since both treatments had the same amount of rumen fluid and lipid content of rumen contents was small.

Form of oil supplementation did not affect the pH of the culture solution over the incubation period (figure 1). No significant differences were also observed in ammonia concentration between oil types although levels of ammonia were numerically higher with seed-associated oil than with

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

Feed components	Ether extract (%, DM)	Composition of C_{18}-fatty acids (%)			
		Stearic acid ($C_{18:0}$)	Oleic acid ($C_{18:1}$)	Linoleic acid ($C_{18:2}$)	Linolenic acid ($C_{18:3}$)
Linseed	34.04	2.75	16.9	13.9	59.2
Concentrate	6.42	4.77	23.5	43.1	4.5

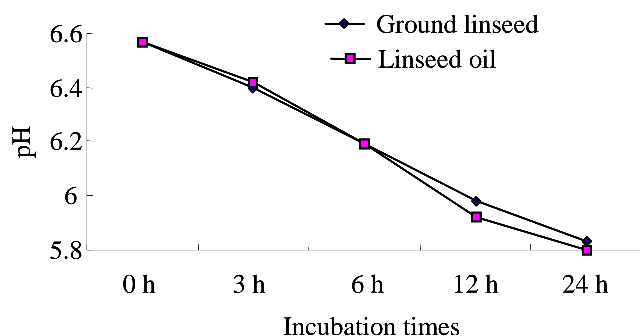

Figure 1. pH of culture solution.

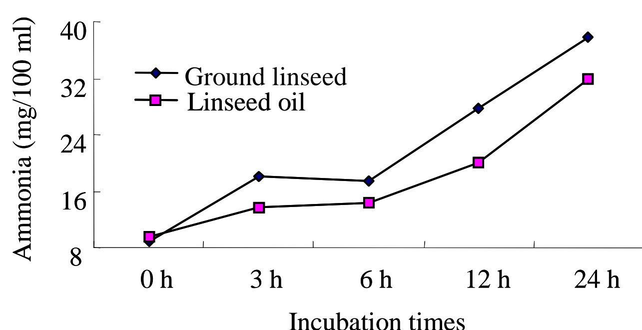

Figure 2. Ammonia concentration in culture solution.

free oil (figure 2).

Addition of oil tended to decrease the total VFA concentrations in culture solution compared to the addition of ground linseed (table 2). There were no significant differences on molar percent of major VFAs, but molar percent of acetate (C_2) tended to increase while that of propionate (C_3) decreased slightly in the free oil added treatment throughout the incubation times (table 2). Differences in bacterial number were relatively small between the two treatments (table 2).

Decreasing trends in the compositions of linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) but increasing trends of stearic acid ($C_{18:0}$), t-$C_{18:1}$ and CLA compositions were clearly found from culture contents at least up to 12 h incubation when incubated with both ground linseed and linseed oil (table 3). The effects of oil addition type on fatty acid composition in culture solution were clear from 6 h incubation. The compositions of $C_{18:0}$, $C_{18:2}$ and $C_{18:3}$ were greater but those of oleic acid ($C_{18:1}$), t-$C_{18:1}$ and CLA were lower in a culture solution containing ground linseed than in those containing linseed oil. Up to 12 h, ratios of t-$C_{18:1}$ to CLA were lower in culture solutions containing linseed oil as compared to those containing ground linseed.

DISCUSSION

In the present study, the degradations of ground linseed and free oil, and the bio-hydrogenation of C_{18}-unsaturated fatty acids mostly resulted from bacterial activity as protozoa were seldom observed in the strained rumen fluid. Although pH of culture solution was not influenced by the addition form of oil (figure 1) addition of seed-associated oil increased slightly concentrations of ammonia (figure 2) and total VFA (table 2). The increases in ammonia and total VFA might be due to the degradation of ground linseed supplemented. But slightly increases in C_2 and decreases in C_3 proportions from the addition of oil as compared to ground linseed are likely due to the ground alfalfa which was used for the absorption of linseed oil.

The rate of bio-hydrogenation of C_{18}-unsaturated fatty acids in culture solution was very rapid in the present *in vitro* study (table 3) when compared to the fatty acid composition of linseed oil (table 1). Similar results were observed in the previous studies (Song and Choi, 1998; Huang et al., 1999; Wang and Song, 2001). Because dietary lipids should be hydrolyzed prior to the hydrogenation of C_{18}-polyunsaturated fatty acids, the addition oil in a seed associated or free form may affect the ruminal synthesis of CLA and t-FA. Kepler et al. (1970) reported that the *cis*-12, *trans*-11 octadecenoic acid isomerase which catalyses the trans-formation of linoleic acid into CLA needed the free COOH radical. This also implies a lipolysis of the dietary lipid fraction before isomerization takes place. In the present study, the addition of linseed oil increased the extent of hydrogenation of $C_{18:2}$ and $C_{18:3}$, and the degree of isomerization of $C_{18:2}$ compared to that of ground linseed, resulting in the increased proportions of t-FA and CLA (table 3). Thus, increased proportions of t-FA and CLA as influenced by increased hydrogenation of $C_{18:2}$ and $C_{18:3}$ from free oil supplementation might be due to the increased degradation rate of free oil as compared to oil in the ground linseed.

Accumulation of t-FA may indicate that bio-hydrogenation of C_{18}-unsaturated fatty acids does not proceed to completion as observed in the rumen by Jenkins (1993). It was also found that accumulated free $C_{18:2}$ blocked conversion of t-FA to $C_{18:0}$, resulting in increased t-FA concentration (Jenkins, 1993). Kim et al. (2000) reported that growing cultures did not produce significant amounts of CLA until the $C_{18:2}$ concentration was high enough to inhibit bio-hydrogenation. This inhibitory effect of $C_{18:2}$ on the bio-hydrogenation in the rumen may indicate a biological limitation in controlling the simultaneous production of both t-FA and CLA. Proportion of $C_{18:2}$ in

Table 2. Concentration, molar proportion of VFA and number of viable bacteria in culture solution when incubated with linseed

Items	Type of oil source		SEM[1)]	Pr>F[2)]
	Ground linseed	Linseed oil		
		3 h		
Total VFA (mmoles/100 ml)	59.62	57.48	8.453	0.874
Molar proportion (mmoles/100 mmoles) :				
Acetate (C_2)	48.87	50.67	1.786	0.549
Propionate (C_3)	25.99	24.88	1.294	0.608
Butyrate (C_4)	18.25	17.97	0.467	0.711
C_2/C_3	1.89	2.05	0.171	0.581
		6 h		
Total VFA (mmoles/100 ml)	69.05	66.43	8.423	0.846
Molar proportion (mmoles/100 mmoles) :				
Acetate (C_2)	48.91	50.19	1.767	0.659
Propionate (C_3)	25.94	24.74	1.320	0.587
Butyrate (C_4)	18.54	18.83	0.361	0.623
C_2/C_3	1.89	2.04	0.172	0.615
		12 h		
Total VFA (mmoles/100 ml)	80.17	79.22	7.458	0.931
Molar proportion (mmoles/100 mmoles) :				
Acetate (C_2)	48.15	49.07	1.650	0.733
Propionate (C_3)	25.62	24.33	1.124	0.502
Butyrate (C_4)	19.33	19.75	0.423	0.555
C_2/C_3	1.89	2.02	0.154	0.591
Bacteria ($\times 10^7$)	23.15	19.45	8.009	0.986
		24 h		
Total VFA (mmoles/100 ml)	90.66	87.36	8.197	0.803
Molar proportion (mmoles/100 mmoles) :				
Acetate (C_2)	47.95	49.08	1.695	0.684
Propionate (C_3)	24.72	23.45	1.018	0.470
Butyrate (C_4)	19.62	20.03	0.486	0.611
C_2/C_3	1.95	2.10	0.157	0.558
Bacteria ($\times 10^7$)	8.80	9.10	5.754	0.977

[1)] Standard error of the mean.
[2)] Probability levels.

linseed oil is small (13.9%) at the beginning of incubation (table 1). But rapid trans-formation of high $C_{18:3}$ proportion (59.2%) in oil (table 1) to $C_{18:2}$ might inhibit the further complete hydrogenation to $C_{18:0}$, resulting in increased accumulations of t-FA and CLA up to 12h incubation (table 3). But it is not known that this phenomenon observed from the batch culture occurs similarly in the rumen because bio-hydrogenation as well as the oil degradation is highly dependant upon the retention time of the oil source.

Another aspect to consider is the relative ratio of t-FA to CLA due to the impacts of both bio-hydrogenation intermediates on human health although both of them are derived from rumen bio-hydrogenation of C_{18}- poly USFA. Both CLA and t-FA were detected in fat depots and milk (Solomon et al., 2000). In the present study, proportions of both intermediates were higher and the ratios of t-FA to CLA were lower from the culture solution containing linseed oil than from that containing ground linseed.

CONCLUSION

Addition of oil in the seed associated or free form affected the *in vitro* bio-hydrogenation of C_{18}-fatty acids. Especially, linseed oil increased the proportions of both t-FA and CLA, but this effect might be influenced by the rate of oil degradation. Linseed oil lowered the ratio of t-FA to CLA as compared to the ground linseed. But further work is required to define the manipulation strategies that increase CLA without producing t-FA.

Table 3. Composition (%) of C_{18}-fatty acids in culture solution

Fatty acids	Type of oil source[1]		SEM[2]	Pr>F[3]
	Ground linseed	Linseed oil		
	0 h			
$C_{18:0}$	25.90	24.41	3.782	0.816
$C_{18:1}$	12.50	12.91	0.721	0.731
t-$C_{18:1}$	2.76	3.23	0.645	0.655
CLA	ND [4]	ND [4]	-	-
$C_{18:2}$	12.60	13.66	0.636	0.359
$C_{18:3}$	29.27	26.83	3.752	0.691
	3 h			
$C_{18:0}$	39.71	33.52	5.338	0.499
$C_{18:1}$	9.44	10.88	1.369	0.536
t-$C_{18:1}$	5.91	8.24	0.493	0.079
CLA	0.78	1.58	0.356	0.250
t-$C_{18:1}$/CLA	8.17	5.83	2.112	0.515
$C_{18:2}$	7.96	8.53	0.881	0.691
$C_{18:3}$	18.73	18.70	3.078	0.996
	6 h			
$C_{18:0}$	47.07	37.94	4.045	0.252
$C_{18:1}$	8.39	11.89	1.011	0.134
t-$C_{18:1}$	6.01^b	12.80^a	0.413	0.007
CLA	0.90	3.65	0.771	0.128
t-$C_{18:1}$/CLA	6.86	3.84	1.311	0.245
$C_{18:2}$	6.01	5.63	0.539	0.662
$C_{18:3}$	14.68	9.79	2.078	0.238
	12 h			
$C_{18:0}$	50.50^a	41.56^b	1.237	0.036
$C_{18:1}$	8.87^b	13.36^a	0.492	0.023
t-$C_{18:1}$	11.41^b	19.71^a	0.250	0.002
CLA	2.06	4.19	0.924	0.244
t-$C_{18:1}$/CLA	5.56	5.18	1.118	0.836
$C_{18:2}$	3.42	2.02	0.257	0.061
$C_{18:3}$	7.19^a	2.19^b	0.251	0.005
	24 h			
$C_{18:0}$	53.16	46.09	2.317	0.164
$C_{18:1}$	9.47	12.56	1.017	0.164
t-$C_{18:1}$	11.98	19.40	1.817	0.102
CLA[6]	2.09	3.05	0.304	0.157
t-$C_{18:1}$/CLA	5.70^b	6.35^a	0.019	0.002
$C_{18:2}$	2.28^a	1.11^b	0.150	0.031
$C_{18:3}$	4.38^a	1.16^b	0.149	0.004

[1] Means in the same row with different superscripts differ.
[2] Standard error of the mean.
[3] Probability levels.
[4] Not detected.

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