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Effect of Feeding Ca-salts of Fatty Acids from Soybean Oil and Linseed Oil on c9,t11-CLA Production in Ruminal Fluid and Milk of Holstein Dairy Cows

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ABSTRACT : The objective of this study was to investigate the effect of dietary supplementation with calcium salts of soybean oil fatty acids (CaSO) and linseed oil fatty acids (CaLO) on c9,t11-CLA production in ruminal fluid and milk fat from Holstein dairy cows. Rumen fermentation, lactational performances and fatty acid profiles in ruminal fluid and milk fat were also investigated. Twenty multiparous Holstein dairy cows were allotted randomly into two groups consisting of ten cows in each group according to calving date and average milk yield. The first group of cows was fed a control (without calcium salts) diet and a treatment as 1.0% of CaSO (on DM basis) for 30 days in each period. In the second group, cows were fed the same control diet and 1.0% of CaLO as a treatment in the same manner. The forage: concentrate ratio was 52:48, and diets were formulated to contain 17% crude protein (DM basis) for both groups. Ruminal pH, protozoal numbers and the concentration of total volatile fatty acids were unchanged, however, the ruminal ammonia-N decreased by feeding CaSO or CaLO treatment compared to the control diet. The vaccenic acid (*trans*-11 C18:1; VA) in rumen fluid increased ($p<0.01$) by 169% and 153%, and the c9,t11-CLA content of rumen fluid increased ($p<0.01$) by 214% and 210% in the CaSO and CaLO treatments, respectively, compared to the control diet. In milk fatty acids, the VA content increased by 130% and 132% in the evening and morning milking times, respectively, and the c9,t11-CLA content increased by 125% in both milking times for the CaSO supplementation than that of control diet. In the case of CaLO supplementation, the VA increased by 117% and 114%, and the c9,t11-CLA increased by 96% and 94% in the evening and morning milking times, respectively, compared to the control diet. The contents of VA and c9,t11-CLA of milk fatty acids were numerically higher in the evening milking time compared to the morning milking time for control and both treatments. Finally, these results indicated that the supplementation of CaSO or CaLO treatment increased the VA and the c9,t11-CLA in both ruminal fluid and milk fat of Holstein dairy cows. (**Key Words** : Ca-salts of Fatty Acids, Conjugated Linoleic Acid, Milk Production, Rumen Fermentation, Vaccenic Acid)

INTRODUCTION

Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of octadecadienoic acid (C18:2) with conjugated double bonds, and the principal dietary sources of CLA are dairy products and other foods derived from ruminant animals (Chin et al., 1992). In recent years, CLA has been the focus of considerable research efforts due to its potential health-promoting properties associated with its anticarcinogenic properties (Ha et al., 1987; Ha et al., 1990; Ip et al., 1991), antiatherogenic in rabbits (Lee et al., 1994) and hamsters (Nicolosi et al., 1997), an antidiabetic (Houseknecht et al., 1998), affecting bone mineralization (Li and Watkins, 1998), and causing nutrient partitioning (Cook et al., 1993; Miller et al., 1994). The primary isomer of CLA is cis-9, trans-11 octadecadienoic acid, which possesses the ability to inhibit cancer, and it represents approximately 80 to 90% of the total CLA isomers in dairy products (Chin et al., 1992; Parodi, 1999). CLA derives from two sources, the rumen origin and the endogenous origin (Griinari and Bauman 1999; Griinari et al., 2000). The sequence of ruminal biohydrogenation of linoleic acid involves isomerization to form cis-9, trans-11 CLA followed by successive reduction to transvaccenic acid (*trans*-11 C18:1; VA) and stearic acid (Harfoot and Hazlewood, 1988). The VA is then converted to cis-9, trans-11 CLA by delta-9-stearoyl-CoA desaturase in ruminant tissues, and it is the major source of cis-9, trans-11 CLA secreted in milk fat (Griinari et al., 2000).

In view of the potential benefits to human health, there

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Table 1. Ingredients and nutrient content of control, Ca-salt of soybean oil fatty acid (CaSO), and Ca-salt of linseed oil fatty acid (CaLO) diets

Item	Diet		
	Control	CaSO	CaLO
	% of DM		
Corn silage	16.9	16.9	16.9
Italian rye grass silage	19.3	19.3	19.3
Alfalfa hay	15.4	15.4	15.4
Concentrate[1]	39.2	39.2	39.2
Supplement	7.7[a]	7.7[b]	7.7[b]
Mineral-vitamin mix[3]	1.5	1.5	1.5
Chemical composition			
DM %	59.5	59.5	59.5
CP	16.9	17.2	17.2
Ether extract	5.0	5.1	5.1
ADF	24.0	24.1	24.1
NDF	33.1	33.4	33.4
Ash	6.9	6.9	6.9
Ca	1.0	1.0	1.0
P	0.5	0.5	0.5
ME (Mcal/kg DM)[4]	2.77	2.80	2.80

[1] Contained 52.0% grain (flaked corn, ground corn, barley, wheat), 14.5% corn gluten feed, 5.0% brans (wheat bran, rice bran), 19.7% oil seed meals (soybean meal, rapeseed meal) 1.0% corn gluten meal, 3.6% lucern pellet and 4.2% others (molasses, $CaCO_3$, salt).

[3] Contained 2,640,000 IU/kg Vitamin A, 1,056,000 IU/kg cholecalciferol and 8,800 IU/kg vitamin E (Premix 1031; The Pillsbury Co., Minneapolis, MN).

[4] Estimated from Agriculture, Forestry and Fisheries Research Council Secretariat (1999).

[a] Contained 29.0% ground corn, 29.0% wheat, 23.0% soybean meal, 14.0% heated soybean meal and 5.0% others (molasses, $CaCO_3$).

[b] Contained 29.0% ground corn,12.0% wheat, 23.0% soybean meal, 6.0% rapeseed meal, 14.0% heated soybean meal, 2.0% wheat bran, 5.0% molasses and 9.0% Ca salts of oil seed fatty acids (soybean or linseed oil).

are some considerable interests in developing nutritional strategies for ruminant animals to increase the c9,t11-CLA in the milk fat. The biohydrogenation in the rumen that can alter the CLA production in milk fat is affected by the type and the amount of fatty acid (FA) substrate (Noble et al., 1974), nitrogen content (Gerson et al., 1983), and forage to grain ratio (Gerson et al., 1985) in the diet. A variety of fat sources as calcium salts were fed to increase the milk concentration of CLA as a method to protect dietary lipids from ruminal biohydrogenation (Chouinard et al., 2001; Giesy et al., 2002). Dietary supplements of Ca salts of FA from soybean oil (CaSO) and linseed oil (CaLO) increased the CLA content of milk fat by three to five fold over the control diet (Chouinard et al., 2001). A few reports are available on the biohydrogenation of Ca salts of FA from palm oil (Klusmeyer et al., 1991; Wu et al., 1991) and rapeseed oil (Ferlay et al., 1993) in the rumen. As far as we are aware, little information is available regarding rumen fermentation and FA composition by the feeding of CaSO or CaLO in dairy cows.

The objectives of this study were 1) to determine the effects of CaSO or CaLO in the rumen fermentation pattern and FA composition in ruminal fluid with special references for the VA and c9,t11-CLA in dairy cows, and 2) to investigate the VA and c9,t11-CLA content in milk fatty acids by feeding of those two Ca salt supplements with the comparison of different milking periods; i.e., the evening and the morning milking periods.

MATERIALS AND METHODS

Cows and experimental design

Twenty multiparous Holstein dairy cows (mean body weight, 670 kg) of different lactation stages were fed a basal total mixed ration (TMR). Following a 2-wk feeding period on the basal TMR, the twenty cows were divided into two groups of ten cows each according to mean milk yield (31 kg/d), mean number of days postpartum (153 days) and mean lactation number (2.0). Both of the two groups were composed of cows of early, mid and late lactation stages based on the calving date. Then, two trials were conducted for two treatments; the calcium salt of soybean oil fatty acid (CaSO) and the calcium salt of linseed oil fatty acid (CaLO) using two groups of cows. The first group of 10 cows was fed the basal TMR without the Ca-salts of FA as the control and the basal TMR supplemented with 1.0% of CaSO (DM basis) as treatment. Each period of the trial lasted for 30 days including a sampling period in the last two days for both the control and CaSO treatment. The other group was fed the control diet and the basal TMR supplemented with CaLO (1.0% of DM) as treatment in the same manner.

Dietary treatments

Holstein dairy cows were fed a basal TMR during the pretreatment and treatment periods. The TMR were formulated to meet CP recommendations, and the ingredients and chemical composition of the TMR are shown in Table 1.

Dietary treatments were 1) the control (basal diet; no addition of Ca salts of fatty acids), 2) the diet supplemented with Ca salt of soybean oil fatty acids (CaSO), and 3) the diet supplemented with Ca salt of linseed oil fatty acids (CaLO). The addition of Ca salts of fatty acids from plant oils represented 1.0% of the DM intake of the diet which was added by supplementation (9.0% of supplement) in the basal diet.

Sampling of ruminal fluid and milk

Three diets were sampled to determine the DM in a forced-air oven at 65°C for 3 d. The feed intake was registered each day. The total mixed rations were kept at -20°C for chemical analysis. Samples of rumen fluid were

obtained by a suction pump using a flexible stainless stomach tube (Fujihira Industries Ltd. Tokyo, Japan), on the last two days of each period at 2 h after the feeding time. Approximately 400 ml of rumen fluid was separated from the particulate matter by straining the digesta through four layers of gauze. The ruminal fluid pH was immediately measured. The rumen fluid (1 ml) was diluted with methyl green-formalin-saline (4 ml) to count the ciliate protozoa (Mohammed et al., 2004). Approximately 100 ml of the fluid samples was stored at -20°C until subsequent analysis. Cows were milked at 0600 and 1700 h, and milk yield was recorded for four consecutive milking of the last two days of each period. Milk was sampled proportionally to the milk yield and the milk samples were split into two portions for analysis. One portion was refrigerated at 4°C for the analysis of milk composition. The other portion of each sample was stored at -30°C for the analysis of fatty acids by gas chromatography (GC).

Chemical analysis

Samples of TMR were freeze-dried, ground in a Wiley mill, and analyzed for CP, EE, Ash, ADF and NDF by AOAC (1990) and the Van Soest method (1991). The rumen fluid samples were acidified with 3 N-H_2SO_4 containing 12% metaphosphoric acid, and the concentration of the volatile fatty acid (VFA) was analyzed by gas chromatography (Model GC-8A; Shimadzu Co. Ltd, Kyoto, Japan) using a Thermon-3000 5% Shincarbon column (Shinwa Kako Co. Ltd, Kyoto, Japan) (Mohammed et al., 2004). The concentration of ammonia-N was determined using the method described by Itabashi et al. (1984). The fatty acids of the rumen fluid were determined by the method of Kamegai et al. (2001).

Milk samples refrigerated at 4°C were analyzed for fat, protein, lactose and SNF by semi-automatic infrared spectroscopy (Foss 133B, Milko-Scan; Foss Electric, Hillerod, Denmark). Milk yield and milk fat percentage were used to calculate the 4% FCM. The portions of milk stored at -30°C were analyzed for individual fatty acids. The extraction of milk fat and the fatty acid methyl esterification (FAME) of extracted fat were made by the method of Kamegai et al. (2001). FAME samples were analyzed using a gas chromatograph (Model GC-14B, Shimadzu Co., Kyoto, Japan) fitted with a flame ionization detector and integrator with a HR-SS-10 fused-silica capillary column (50 m×0.25 mm i.d.; Shinwa Kako Co. Ltd, Kyoto, Japan). Unlike the rumen samples (splitless), the split ratio in the injector port was 50:1 with a column flow of 1.0 ml/min of N_2 for the milk samples. The gas chromatography conditions were a multistage temperature program and the column oven temperature program was as follows: initial 50°C, then increased from 50°C to 180°C at 5°C/min and held for 15 min, and then raised to 220°C at 5°C/min, and finally held at 220°C for 30 min. The injector and detector temperatures were 250°C and 260°C, respectively. Peaks for individual fatty acid were identified by the retention time and comparison to known commercially prepared standards (GL Science Co. Ltd., Tokyo, Japan). The CLA standard was obtained from Rinoru Oil Mills Co. Ltd. (Tokyo, Japan). The CLA isomer reported was c-9, t-11-octadecadienoic acid.

Statistical analysis

Data were analyzed using the GLM procedure of SAS (SAS Inst., Inc. Cary, NC). The statistical model of all data, except milk fatty acids was as follows:

$$y_{ij} = u+F_i+C_j+e_{ij}$$

where y_{ij} = dependent variable for cow on treatment i; u = overall mean; F_i = the effect of the treatment (i = 1, 2); C_j = the effect of the cow (1 to 10); and e_{ij} = the error term.

The data of milk fatty acids were tested using split-plot ANOVA with the general linear models of SAS. The model was as follows:

$$Y_{ijk} = u+F_i+C_j+(FC)_{ij}+T_k+(FT)_{ik}+e_{ijk}$$

where u = the overall mean; F_i = the effect of the treatment (i = 1, 2) ; C_j = the effect of the cow (1 to 10); $(FC)_{ij}$ = the effect of the interaction between feed and cow (as error); T_k = the effect of the milking time (k = 1, 2); $(FT)_{ik}$ = the effect of the interaction between feed and milking time; e_{ijk} = the error term. Considering the split-plot linear model, F and C were tested with FC as an error term, while T and FT were tested against e. Least square means were compared using protected least significant differences. Results were expressed as least squares means. Significance was declared at $p<0.05$ and $p<0.01$ unless otherwise noted, and trends were declared at $0.05<p<0.10$.

RESULTS AND DISCUSSION

Production performances

Dry matter intake (DMI) did not differ significantly between cows fed the control diet and supplemented with the CaSO or CaLO diet (Table 3). Previous studies (Wu et al., 1991; Chouinard et al., 1998) also found the lack of an effect on DMI fed Ca salts of FA, which possessed the acceptability of the diets. The amount of Ca salts required to reach the limitation of intake depended on the experimental conditions and is probably a function of the energy requirement of the cows and the amount of energy provided by the basal ration (Chouinard et al., 1998).

The milk production increased a little amount (approximately 8.5%) for the CaSO treatment; however, a significant ($p<0.05$) increase was observed for the CaLO

Table 2. Fatty acid composition of Ca-salts of soybean oil fatty acid (CaSO) and linseed oil fatty acid (CaLO)

Fatty acid	CaSO	CaLO
	g/100 g of FAME[1]	
C12:0	0.1	0.1
C14:0	0.1	0.1
C16:0	13.2	6.3
C16:1	0.3	0.2
C18:0	4.1	3.5
C18:1	37.2	20.1
C18:2	40.2	16.2
C18:3	4.7	53.4
C20:0	0.1	0.1

[1] FAME = Fatty acid methyl ester.

treatment (approximately 11.3%) compared with the control diet (Table 3). The present study was consistent with the finding of Chouinard et al. (1998), in that milk yield increased linearly as the unsaturation of the dominant FA in the Ca salts increased. Linseed oil contains more linolenic acid than that of soybean oil (Table 2). Therefore, the FA composition of Ca salts might influence milk yield. In addition, a little higher ME contents in the treatments of TMR than the control (Table 1) might be the reason for the increase of milk production by the treatments as well. Treatments also responded for 4% FCM production in the same manner as the milk yield in the present study (Table 3).

Milk fat, protein and lactose percentages did not change for either of the treatments (Table 3). Chouinard et al. (1998) found a drop in the milk fat percentage of 26.4 and 12.1% for CaSO and CaLO, respectively, using 4.0% Ca salts of FA (on DM basis) in the diet. Our study did not show a significant decrease in the level of milk fat by feeding Ca salts of soybean or linseed oil fatty acids. It might be a reason for the lower doses of Ca salts of FA (1.0% of the DM) in the diet.

Rumen fermentation and fatty acid composition in the rumen

Table 4 shows the effects of CaSO and CaLO treatments on the ruminal conditions of dairy cows. The pH values of the rumen fluid tended to decrease ($p<0.1$) due to the Ca salts of FA supplementation. However, the protozoal count was unchanged for both treatments compared with the control. The ruminal ammonia-N concentrations fluctuated with supplementation and decreased significantly ($p<0.01$) for both treatments. On average, the rumen ammonia-N decreased by 31.7% for the supplementation of CaSO, and by 29.5% for the CaLO compared to the control diet. We are not aware of previous investigations of rumen fermentation with diets containing CaSO or CaLO supplementation. However, some previous studies of

Table 3. Production performances of dairy cows fed control, Ca salts of soybean oil fatty acid (CaSO) and linseed oil fatty acid (CaLO) diets

Variable	Treatments							
	Control (n = 10)	CaSO (n = 10)	SEM	p	Control (n = 10)	CaLO (n = 10)	SEM	p
DM intake (kg/d)	21.6	23.8	0.15	0.33	22.0	24.0	0.14	0.31
Milk yield (kg/d)	30.9	33.5	0.24	0.28	32.0	35.6	0.18	0.04*
4% FCM (kg/d)	29.9	32.6	0.21	0.19	31.4	34.3	0.14	0.02*
Fat (%)	3.85	3.82	0.03	0.79	3.88	3.76	0.02	0.52
Protein (%)	3.30	3.32	0.05	0.85	3.19	3.21	0.03	0.68
Lactose (%)	4.59	4.61	0.07	0.77	4.58	4.56	0.05	0.59

Significant levels,* $p\leq0.05$, ** $p\leq0.01$.

Table 4. Ruminal pH, protozoal count, ammonia-N, total VFA, and the molar proportion of VFA of cows fed control, Ca salts of soybean oil fatty acid (CaSO) and linseed oil fatty acid (CaLO) diets

Item	Treatments							
	Control	CaSO	SEM	p	Control	CaLO	SEM	p
pH	6.67	6.35	0.05	0.10[a]	6.56	6.31	0.05	0.07[a]
Protozoa ($\times10^4$/ml)	42.2	43.1	0.18	0.80	41.5	40.2	0.19	0.70
Ammonia-N (mg/dl)	20.8	14.2	0.52	0.01**	20.0	14.1	0.49	0.01**
Total VFA (m*M*)	148.6	143.7	0.93	0.13	146.2	144.8	0.97	0.93
VFA composition (mol/100 mol)								
Acetate	64.5	61.7	0.53	0.01**	65.6	62.3	0.49	0.01**
Propionate	18.9	19.8	0.45	0.47	17.0	18.7	0.47	0.26
Butyrate	10.5	12.5	0.28	0.01**	11.4	12.8	0.22	0.01**
Isobutyrate	1.74	2.00	0.11	0.16	1.92	1.99	0.11	0.83
Valerate	1.85	2.03	0.13	0.10[†]	1.88	2.13	0.18	0.20
Isovalerate	1.81	1.96	0.15	0.34	1.71	1.98	0.12	0.13
Aetate:propionate	3.4	3.1	0.40	0.23	3.9	3.3	0.35	0.13

Significant levels, * $p\leq0.05$, ** $p\leq0.01$. Trends, [a]$p\leq0.10$.

Table 5. Effects of Ca salts of soybean oil fatty acid (CaSO) and linseed oil fatty acid (CaLO) on ruminal fatty acid composition of dairy cows

Fatty acid	Treatments							
	Control	CaSO	SEM	p	Control	CaLO	SEM	p
	g/100 g of FAME[1]							
C12:0	0.46	0.37	0.02	0.07[a]	0.52	0.47	0.03	0.10[a]
C12:1	0.17	0.16	0.03	0.57	0.19	0.21	0.01	0.24
C13:0	4.54	4.03	0.15	0.20	4.95	4.03	0.05	0.10[a]
C13:1	0.19	0.23	0.04	0.02*	0.18	0.23	0.01	0.01**
C14:0	1.76	0.74	0.03	0.01**	1.70	0.84	0.02	0.01**
C14:1	0.74	0.75	0.02	0.89	0.57	0.69	0.01	0.08[a]
C15:0	1.58	0.78	0.02	0.01**	2.20	0.72	0.06	0.01**
C15:1	0.55	0.58	0.02	0.36	0.55	0.59	0.04	0.46
C16:0	27.13	17.44	0.18	0.01**	24.11	16.10	0.21	0.01**
C16:1	0.66	0.55	0.01	0.09[1]	0.79	0.73	0.05	0.11
C17:0	1.45	0.78	0.01	0.01**	1.28	0.84	0.03	0.01**
C17:1	0.38	0.46	0.01	0.01**	0.41	0.49	0.02	0.07[a]
C18:0	29.51	33.33	0.23	0.05*	29.88	35.00	0.11	0.01**
C18:1	19.65	21.08	0.04	0.20	20.77	22.52	0.25	0.34
C18:2	4.20	4.02	0.07	0.53	3.95	3.97	0.05	0.95
C18:3	0.42	0.41	0.02	0.94	0.47	0.58	0.02	0.01**
VA[2]	2.48	6.67	0.03	0.01**	2.38	6.02	0.03	0.01**
c9,t11-CLA[3]	1.46	4.59	0.05	0.01**	1.26	3.90	0.03	0.01**

[1] FAME = Fatty acid methyl ester. [2] VA = *trans*-vaccenic acid. [3] c9,t11-CLA = *cis*-9, *trans*-11conjugated linoleic acid.
Significant levels, * p≤ 0.05, ** p≤0.01. Trends, [a] p≤0.10.

saturated fat sources observed that the addition of Ca salts of palm oil FA in the low forage diet increased the ruminal pH without changing the ruminal concentration of ammonia -N and VFA (Klusmeyer et al., 1991). Moreover, the supplementation of animal fat (tallow and grease) to the animal diets decreased the ruminal ammonia -N associated with the reduced number of protozoa (Onetti et al., 2001). In our study, ruminal ammonia-N was decreased by the supplementation of CaSO or CaLO. It might be occurred due to the decrease of deamination in the rumen or might be the decrease of the number of rumen protozoa in the rumen. The reason of the first one might be acceptable, since the number of protozoa was unchanged by CaSO or CaLO supplementation in the present study. Thus, the CaSO or CaLO supplementation might therefore reduce the rate of deamination as well as decrease the ammonia-N concentration in the rumen. However, further study is necessary to understand properly the reason for the decrease of ruminal ammonia-N by feeding Ca-salts supplementation in dairy cows.

As shown in Table 4, the average ruminal concentration of total VFA, the molar proportions of propionate, isobutyrate, valerate and isovalerate, as well as the acetate:propionate ratio, were not affected significantly when the cows were fed diets containing CaSO or CaLO. However, the molar proportion of acetate was decreased, and butyrate was significantly increased ($p<0.01$) for both treatments (Table 4). The shifted rumen fermentation towards butyrate at the expense of acetate has been shown for the first time in the case of CaSO or CaLO supplementation, and it can be attributed to the associated significant ($p<0.01$) increase in the butyrate proportion in milk fat in the present study (Tables 6 and 7). This result is inconsistent with previous studies (Klusmeyer et al., 1991; Ohajuruka et al., 1991) in which acetate and butyrate did not differ significantly for Ca salts of long chain FA from palm oil. This might be a reason for varying the unsaturation of FA in Ca salts. Saturated FA is more stable than the unsaturated FA in the rumen. Ca salts of unsaturated FA were not completely inert to the rumen, and it dissociated to some extent in the rumen (Sukhija and Palmquist, 1990).

The rumen is the site of microbial lipid metabolism. Lipolysis of dietary glycolipids, phospholipids and triglycerides leads to free fatty acids that are hydrogenated by microbes to more saturated end products. The fatty acid composition in the rumen was altered with CaSO or CaLO treatment compared with the control diet (Table 5). As shown in Table 5, the total medium chain fatty acids (≤C:15) decreased by 35.5 or 30.1%, and the total long chain FA (≥C:16) increased by 20 or 22% for CaSO or CaLO, respectively, than the control. The stearic acid (C18:0) increased by 13% for the CaSO ($p<0.05$) or 17% for the CaLO ($p<0.01$) treatments compared with the control diet. The changes in the proportions of saturated and unsaturated fatty acids were primarily results of decreasing the concentrations of C12:0, C14:0 and C16:0, and increasing the proportions of C18:1, C18:2, VA and CLA in

Table 6. Effect of Ca salts of soybean oil fatty acid (CaSO) and milking time on the fatty acid composition of milk fat from dairy cows

Fatty acid	Control		CaSO		SEM	p	
	Evening	Morning	Evening	Morning		Effect of CaSO	Effect of milking time
	---------- g/100 g of FAME[1] ----------						
C4:0	1.43	1.34	1.86	1.80	0.05	0.01**	0.17
C6:0	2.03	1.92	1.93	1.97	0.10	0.72	0.77
C8:0	1.91	2.19	1.57	2.01	0.12	0.44	0.01**
C10:0	3.17	3.37	3.10	3.23	0.06	0.61	0.01**
C12:0	3.73	3.91	3.44	3.62	0.05	0.13	0.01**
C12:1	0.08	0.09	0.08	0.09	0.01	0.97	0.02*
C13:0	0.10	0.10	0.11	0.11	0.01	0.53	0.12
C13:1	0.14	0.14	0.12	0.12	0.01	0.14	0.89
C14:0	12.18	12.45	11.11	11.47	0.11	0.02*	0.01**
C14:1	1.13	1.16	1.08	1.07	0.01	0.67	0.48
C15:0	1.10	1.10	1.02	1.01	0.01	0.17	0.56
C15:1	0.32	0.31	0.28	0.28	0.01	0.07[a]	0.18
C16:0	31.13	32.01	25.68	25.83	0.21	0.01**	0.02*
C16:1	1.54	1.61	1.45	1.58	0.05	0.45	0.6
C17:0	0.57	0.56	0.58	0.59	0.03	0.67	0.95
C17:1	0.28	0.28	0.40	0.38	0.02	0.01**	0.70
C18:0	9.54	9.07	10.98	10.07	0.11	0.01**	0.07[a]
C18:1	23.36	22.39	27.29	26.64	0.25	0.01**	0.01**
C18:2	1.84	1.88	2.10	2.15	0.05	0.04*	0.38
C18:3	0.39	0.39	0.36	0.36	0.02	0.20	0.33
VA[2]	0.67	0.62	1.54	1.44	0.03	0.01**	0.13
C9,t11-CLA[3]	0.64	0.61	1.44	1.37	0.03	0.01**	0.10[a]

[1] FAME = Fatty acid methyl ester. [2] VA = *trans*-vaccenic acid. [3] c9,t11-CLA = *cis*-9, *trans*-11conjugated linoleic acid.
Significant levels,* p≤0.05, ** p≤0.01. Trends, [a]p≤0.10.

the rumen fluid with the CaSO or CaLO diet compared with the control (Table 5). The total unsaturated fatty acids (USFA) were significantly ($p<0.05$) high in the rumen fluid of supplemental CaSO treatment (36.8% of the total FA) than that of the control diet (28.2% of the total FA). In case of the CaLO treatment, the total USFA was also high ($p<0.01$) (37% of the total FA) than the control diet (28.8% of the total FA). The reason for the alteration of the FA profile of the rumen fluid is not clear in this study; however, it might be a cause of added Ca salts of unsaturated FA and its dissociation in the rumen.

The concentration of VA in the ruminal digesta increased ($p<0.01$) by 169% and by 153% for the CaSO and CaLO treatments, respectively, than the control diets, which were accumulated in the rumen (Table 5). The increase in the VA concentration, and the VA to C18:0 ratio (Table 5) are an indication of the incomplete biohydrogenation of unsaturated FA with CaSO or CaLO supplements. The mechanisms of incomplete biohydrogenation of unsaturated fatty acid are not clear in this experiment for both treatments; however, it might involve inhibition of the enzyme that catalyzes the final biohydrogenation step in the rumen (AbuGhazaleh et al., 2002).

The concentration of c9,t11-CLA in the rumen also markedly increased ($p<0.01$) with Ca salt supplementation (Table 5). It increased by 214% for the CaSO (CaSO vs. control; 4.6% vs. 1.5%) and by 210% for the CaLO treatments (CaLO vs. control; 3.9% vs. 1.26%). The objective of our study was to determine the effect of treatments on the ruminal fatty acids content, especially VA and c9,t11-CLA contents in the rumen. The large amount of VA and c9,t11-CLA in the rumen of cows fed CaSO or CaLO compared with the controls indicated the incomplete biohydrogenation of polyunsaturated fatty acids in the rumen. Thus, c9,t11-CLA could escape the rumen, and also VA could be converted to c9,t11-CLA in the mammary glands to increase the milk concentration of c9,t11-CLA (Tables 6 and 7). This is the first report on the fatty acids profile in the ruminal fluid feeding CaSO or CaLO in dairy cows, as far as we are aware.

Milk fatty acid profile

Most of the short and medium-chain fatty acids in the milk fat were unchanged or tended to decrease for the supplementation of CaSO or CaLO treatments, except the butyrate (Tables 6 and 7). The decrease indicated a lower *de novo* FA synthesis within the mammary glands that has been attributed to a direct inhibition of mammary acetyle-coenzyme A carboxylase activity because of the increased mammary uptake of long chain fatty acids from plasma triacylglycerols (Storry, 1988). The addition of CaSO or CaLO to the ration caused a significant ($p<0.01$) decrease in

Table 7. Effect of Ca salts of linseed oil fatty acid (CaLO) and milking time on the fatty acid composition of milk fat from dairy cows

Fatty acid	Control		CaLO		SEM	p	
	Evening	Morning	Evening	Morning		Effect of CaLO	Effect of milking time
	g/100 g of FAME[1]						
C4:0	1.41	1.41	1.71	1.72	0.05	0.01**	0.94
C6:0	1.81	1.86	1.82	1.93	0.03	0.55	0.01**
C8:0	2.65	2.13	1.69	1.75	0.15	0.03*	0.13
C10:0	3.17	3.32	3.22	3.33	0.05	0.91	0.02*
C12:0	3.77	3.92	4.10	4.06	0.04	0.43	0.07[a]
C12:1	0.11	0.11	0.11	0.10	0.01	0.94	0.80
C13:0	0.10	0.11	0.13	0.13	0.01	0.15	0.50
C13:1	0.13	0.13	0.12	0.12	0.01	0.03*	1.0
C14:0	12.18	12.47	11.99	11.96	0.18	0.43	0.47
C14:1	1.31	1.31	1.27	1.24	0.01	0.78	0.36
C15:0	1.10	1.10	1.10	1.10	0.01	0.65	0.62
C15:1	0.31	0.29	0.26	0.25	0.01	0.01**	0.03*
C16:0	30.26	31.27	26.10	26.88	0.23	0.01**	0.01**
C16:1	1.73	1.73	1.62	1.55	0.04	0.20	0.48
C17:0	0.58	0.56	0.65	0.63	0.01	0.25	0.14
C17:1	0.28	0.26	0.40	0.38	0.01	0.09[a]	0.03*
C18:0	9.49	9.23	10.40	10.02	0.14	0.15	0.03*
C18:1	23.43	22.40	25.90	24.97	0.21	0.01**	0.01**
C18:2	1.77	1.75	1.95	2.03	0.05	0.02*	0.55
C18:3	0.41	0.41	0.36	0.35	0.02	0.09[a]	0.77
VA[2]	0.60	0.58	1.30	1.24	0.02	0.01**	0.13
c9,t11-CLA[3]	0.66	0.63	1.29	1.22	0.02	0.01**	0.14

[1] FAME = Fatty acid methyl ester. [2] VA = *trans*-vaccenic acid. [3] c9,t11-CLA = *cis*-9, *trans*-11conjugated linoleic acid.
Significant levels, * $p \leq 0.05$, ** $p \leq 0.01$. Trends, [a] $p \leq 0.10$.

the proportion of saturated FA C16:0 (Tables 6 and 7). The low concentration of C16:0 in milk fat may be considered beneficial to human health due to the association of C16:0 with hypercholesterolemic effects (McGuire et al., 1997). The proportions of long chain fatty acids, namely, C18:0, C18:1, C18:2, VA and CLA increased significantly ($p<0.01$ and $p<0.05$) for the CaSO or CaLO treatments compared with the controls (Tables 6 and 7). This result is supported by the previous study of Chouinard et al. (1998). The greater production of long-chain fatty acids in the milk is associated with the increase of mammary uptake of long chain FA from plasma triacylglycerols (Storry, 1988). As shown in the rumen FA composition (Table 5), more long chain USFAs were produced with the supplementation of Ca salts, which were in turn found in milk FA.

The fatty acid profile in milk fat has altered by the milking time too. The short and medium chain fatty acids tended to decrease ($p<0.10$) in most cases for the evening milking time compared with the morning milking time in the CaSO or CaLO treatments. The C16:0 showed a significant decrease and the C18:0 tended to increase in the evening milking time for both of the treatments group. The degree of unsaturation of long chain fatty acids specially for C18:1, VA and CLA were greater in the evening milking time than the morning milking time for both treatments of CaSO or CaLO (Tables 6 and 7). This is the first report on varying the unsaturation of long chain fatty acids in the milk of different milking times.

The VA production in milk significantly ($p<0.01$) increased for both CaSO or CaLO treatments (Tables 6 and 7). It increased by 130% and 132% for CaSO (Table 6), and by 117% and 114% for CaLO treatments (Table 7) in the evening and morning milking time, respectively, than the control. It also tended to increase ($p<0.10$) in the evening milking time. The VA concentration in the milk FA was lower than the rumen FA for both treatments. This might be a result of the delta-9-stearoyl-CoA desaturase activity to produce c9,t11-CLA from the VA in ruminant tissues (Griinari et al., 2000).

Our experiments were limited to the comparison of the two diets, however, if we compared the two control groups of both treatments, there was a little difference in the concentration of VA in the ruminal fluid and in the milk FA. When CaSO or CaLO was added to the diet as treatment, the concentration of VA in both the rumen and milk were higher for the CaSO treatment than the CaLO treatment compared to control diet. A previous study (Chouinard et al., 1998) showed that the proportion of VA was highest for the cows fed the CaSO diet than the CaLO diet. Tamminga and Doreau (1991) mentioned that C18:3 often completely hydrogenated to C18:0, however, the hydrogenation of C18:2 was not complete, and gave rise to the formation of

different isomers of C18:1, including VA. The CaSO contained the highest proportion of C18:2 (Table 2). This might be a cause for the higher production of VA in the rumen and milk fat of the CaSO treatment than that of the CaLO treatment as compared to the control diet of both treatments.

The c9,t11-CLA increased significantly ($p<0.01$) for the CaSO or CaLO treatments than the control (Tables 6 and 7). The increase in c9,t11-CLA production was observed to be 125% and 124.5% higher in the CaSO treatment than the control (Table 6) for the evening and morning milking period, respectively. In the case of CaLO treatment, the c9,t11-CLA increased by 96% for the evening milking time and by 94% for the morning milking time (Table 7). Chouinard et al. (1998) found a marked increased of CLA for CaSO (5-fold higher than the control) or CaLO (4-fold). However, we found a marked increase of c9,t11-CLA (4-fold) in the rumen FA rather than the milk FA for both treatments. Our study confirmed the enhancement of c9,t11-CLA production in the milk fat by feeding CaSO or CaLO, and also established that these treatments enhanced the c9,t11-CLA in ruminal FA without any disturbance of rumen fermentation. The c9,t11-CLA production tended to increase ($p<0.10$) in the evening milking time for the CaSO treatment and numerically increased for the CaLO treatment. We were aware that no previous data have shown the differences of the c9,t11-CLA production in different milking times. As this study was the first report on the effect of fatty acids composition including CLA in different milking times by feeding CaSO or CaLO, the reason for the differences of FA composition was not clearly understood in this study. Further study on the milking time effect might be suggested to understand the mechanisms of the variation of FA composition in milk.

Finally, it can be concluded from the present study that the VA and c9,t11-CLA content in the ruminal FA were increased remarkably by the feeding CaSO or CaLO diets in dairy cows. Results from the present research confirmed that the CLA content of cow's milk fat increased by both CaSO and CaLO diets in dairy cows. The value obtained in this trial was substantially higher than the average milk fat value of 0.3 to 0.6% c9,t11-CLA reported in the literature (Dhiman et al., 1999). The milk FA profile was also altered by both treatments and the milking time. The concentration of VA and c9,t11-CLA in the milk FA also numerically increased in the evening milking time.

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REFERENCES

Abu-Ghazaleh, A. A., D. J. Schingoethe, A. R. Hippen, K. F. Kalscheur and L. A. Whitlock. 2002. Fatty acid profiles of milk and rumen digesta from cows fed fish oil, extruded soybeans or their blend. J. Dairy Sci. 85:2266-2276.

Agriculture, Forestry and Fisheries Research Council Secretariat. 1999. Japanese feeding standard for dairy cattle. Japan Livestock Industry Association, Tokyo.

AOAC. 1990. Official Methods of Analysis. 15th edn. Association of Official Analytical Chemists, Arlington, Virginia.

Chin, S. F., W. Liu, J. M. Storkson, Y. L. Ha and M. W. Pariza. 1992. Dietary sources of conjugated dienoic isomers of linoleic acid, newly recognized class of anticarcinogens. J. Food Comp. Anal. 5:185-197.

Chouinard, P. Y., L. Corneau, W. R. Butler, Y. Chilliard, J. K. Drackley and D. E. Bauman. 2001. Effect of dietary lipid source on conjugated linoleic acid concentrations in milk fat. J. Dairy Sci. 84:680-690.

Chouinard, P. Y., V. Girard and G. J. Brisson. 1998. Fatty acid profile and physical properties of milk fat from cows fed calcium salts of fatty acids with varing unsaturation. J. Dairy Sci. 81:471-481.

Cook, M. E., C. C. Miller, Y. Park and M. W. Pariza. 1993. Immune modulation by altered nutrient metabolism: Nutritional control of immune-induced growth depression. Poult. Sci. 72:1301-1305.

Dhiman, T. R., G. R. Anand, L. D. Satter and M. W. Pariza. 1999. Conjugated linoleic acid content of milk from cows fed different diets. J. Dairy Sci. 82:2146-2156.

Ferlay, A., J. Chabort, Y. Elmeddah and M. Doreau. 1993. Ruminal lipid balance and intestinal digestion by dairy cows fed calcium salts of rapseed oil fatty acids or rapseed oil. J. Anim. Sci. 71:2237-2245.

Gerson, T., A. John and B. R. Sinclair. 1983. The effect of dietary N on *in vitro* lipolysis and fatty acid hydrogenation in rumen digesta from sheep fed diets high in starch. J. Agric. Sci. (Camb.) 101:97-101.

Gerson, T., A. John and A. S. D. King. 1985. The effects of dietary starch and fiber on the *in vitro* rates of lipolysis and hydrogenation by sheep rumen digesta. J. Agric. Sci. (Camb.) 105:27-30.

Giesy, J. G., M. A. McGuire, B. Shafii and T. W. Hanson. 2002. Effect of dose of calcium salts of conjugated linoleic acid (CLA) on percentage and fatty acid content of milk fat in mid lactation Holstein cows. J. Dairy Sci. 58:2023-2029.

Griinari, J. M. and D. E. Bauman 1999. Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk in ruminants. In: Advances in Conjugated Linoleic Acid Research I (Ed. M. P. Yurawecz, M. M. Mossoba, J. K. G. Kramer, M. W. Pariza and G. J. Nelson). AOCS Press, Champaign, Illinois. pp. 180-200.

Griinari, J. M., B. A. Corl, S. H. Lacy, P. Y. Chouinard, K. V. V.

Nurmela and D. E. Bauman. 2000. Conjugated linoleic acid is systhesized endogenously in lactating dairy cows by delta-9-desaturase. J. Nutr. 130: 2285-2291.

Ha, Y. L., N. K. Grimm and M. W. Pariza. 1987. Anticarcinogens from fried ground beef: heat-altered derivatives of linoleic acid. Carcinogenesis. 8:1881-1887.

Ha, Y. L., J. Storkson and M. W. Pariza. 1990. Inhibition of benzo(a)pyrene-induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. Cancer Res. 50:1097-1101.

Harfoot, C. G. and G. P. Hazlewood. 1988. Lipid metabolism in the rumen. In: The Rumen Microbial Ecosystem (Ed. P. N. Hobson and C. S. Stewart). Blackie Academic & Professional, London, England. pp. 285-322.

Houseknecht, K. L., J. P. Vanden Heuvel, S. Y. Moya-Camarena, C. P. Portocarrero, L. W. Peck, K. P. Nickel and M. A. Belury. 1998. Dietary conjugated linoleic acid normalizes impaired glucose tolerance in the Zucker diabetic fatty *falfa* rat. Biochem. Biophys. Res. Commun. 244:678-682.

Ip, C., S. F. Chin, J. A. Scimeca and M. W. Pariza. 1991. Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. Cancer Res. 51:6118-6124.

Itabashi, H., T. Kobayashi and M. Matsumoto. 1984. The effects of rumen ciliate protozoa on energy metabolism and some constituents in rumen fluid and blood plasma of goats. Jpn. J. Zootech. Sci. 55:248-256.

Kamegai, T., M. Kasai and I. Ikeda. 2001. Improved method for preparation of the methyl ester of conjugated linoleic acid. J. Oleo Sci. 50:237-241.

Klusmeyer, T. H., G. L. Lynch, J. H. Clark and D. R. Nelson. 1991. Effects of calcium salts of fatty acids and proportion of forage in diet on ruminal fermentation and nutrient flow to duodenum of cows. J. Dairy Sci. 74:2220-2232.

Lee, K. N., D. Kritchevsky and M. W. Pariza. 1994. Conjugated linoleic acid and atherosclerosis in rabbits. Atherosclerosis. 108:19-25.

Li, Y. and B. A. Watkins. 1998. Conjugated linoleic acids alter bone fatty acid composition and reduce *ex vivo* prostaglandin E_2 biosynthesis in rats fed n-6 or n-3 fatty acids. Lipids. 33:417-425.

McGuire, M. A., M. K. McGuire and M. S. McGuire. 1997. Bovinic acid: The natural CLA. In: Proceedings of the 1997 Cornell Nutr. Conf. Feed Manuf., Rochester, NY. Cornell University, Ithaca, New York. pp. 217-226.

Miller, C. C., Y. Park, M. W. Pariza and M. E. Cook. 1994. Feeding conjugated linoleic acid to animals partially overcomes catabolic responses due to endotoxin injection. Biochem. Biophys. Res. Commun. 198:1107-1112.

Mohammed, N., N. Ajisaka, Z. A. Lila, K. Hara, K. Mikuni, K. Hara, S. Kanda and H. Itabashi. 2004. Effect of horseradish oil on methane production and ruminal fermentation *in vitro* and in steers. J. Anim. Sci. 82:1839-1846.

Nicolosi, R. J., E. J. Rogers, D. Kritchevsky, J. A. Scimeca and P. J. Huth. 1997. Dietary conjugated linoleic acid reduces plasma lipoproteins and early aortic atherosclerosis in hypercholesterolemic hamsters. Artery. 22:266-277.

Noble, R. X., J. H. Moore and C. G. Harfoot. 1974. Observations on the pattern of biohydrogenation of esterified and unesterified linoleic acid in the rumen. Br. J. Nutr. 31: 99-108.

Ohajuruka, O. A., Z. Wu and D. L. Palmquist. 1991. Ruminal metabolism, fiber, and protein digestion by lactating cows fed calcium soap or animal-vegetable fat. J. Dairy Sci. 74:2601-2609.

Onetti, S. G., R. D. Shaver and M. A. McGuire. 2001. Effect of type and level of dietary fat on rumen fermentation and performance of dairy cows fed corn silage-based diets. J. Dairy Sci. 84:2751-2759.

Parodi, P. W. 1999. Conjugated linoleic acid and other anticarcinogenic agents of bovine milk fat. J. Dairy Sci. 82:1339-1349.

Storry, J. E. 1988. The effect of dietary fat on milk composition. In: Recent Developments in Ruminant Nutrition-2 (Ed. W. Haresign and D. J. A. Cole). Butterworths, London, England. pp. 111.

Sukhija, P. S. and D. L. Palmquist. 1990. Dessociation of calcium soaps of long-chain fatty acids in rumen fluid. J. Dairy Sci. 73: 1784-1787.

Tamminga, S. and M. Doreau. 1991. Lipids and rumen digestion. In: Rumen Microbial Metabolism and Ruminant Digestion (Ed. J. P. Jouany). Paris, France. pp. 151-163.

Van Soest, P. J., J. B. Robertson and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583-3597.

Wu, Z. and D. L. Palmquist. 1991. Synthesis biohydrogenation of fatty acids by ruminal microorganisms *in vitro*. J. Dairy Sci. 74:3035-3046.

Wu, Z., O. A. Ohajuruka and D. L. Palmquist. 1991. Ruminal synthesis, biohydrogenation, and digestibility of fatty acids by dairy cows. J. Dairy Sci. 74:3025-3034.