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Increase of Conjugated Linoleic Acid Level in Milk Fat by Bovine Feeding Regimen and Urea Fractionation

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Abstract Increasing conjugated linoleic acid (CLA) content in dairy products has been a research interest due to the potential health benefits resulted from consuming CLA. Attempts were made to obtain high level natural CLA containing fatty acid fractions from milk fat through bovine feeding of sunflower oil (SO) and urea fractionation. SO feeding changed the fatty acid profile of milk fat, increasing the CLA content five-fold at eight weeks of trial. Milk fat obtained from SO-fed cows was hydrolyzed to free fatty acids, which were then fractionated with urea at various ratios. The profiles of fatty acids were also greatly influenced by urea fractionation. Long-chain unsaturated fatty acids, including CLA, were concentrated in milk fat after the fractionation, whereas saturated long-chain counterparts were eliminated. The highest level of CLA was achieved by the fractionation at 2:1 urea/fatty acid ratio (UFR2). CLA level was elevated 2.5-fold, and the C18:1/C18:0 fatty acid ratio was increased 120 times after the fractionation. The level of CLA in high CLA-milk fat (24 mg/g fat) obtained from the feeding study was further increased through urea fractionation up to 52 mg/g fat, 10 folds as high as CLA in the control milk fat (5 mg/g fat).

Key words: Conjugated linoleic acid, sunflower oil, urea, fractionation, milk fat

Milk fat usually contains a relatively high concentration of saturated fat, which has been implicated as a risk factor for heart diseases [36]. Therefore, many attempts have been made to alter the fatty acid composition of milk fat to improve the nutritional value of dairy products [6, 17, 21]. However, some animal-derived fats are recognized as health-promoting agents, among which conjugated linoleic acid (CLA) has long been recognized as a potential inhibitor of epidermal carcinogenesis [8, 10, 13, 26]. The ability of CLA to modify plasma lipids and lipoprotein cholesterol levels has also been observed in rabbit [25, 32]. Moreover, CLA was shown to modulatethe the lipid composition of animal tissue [3]. In Zucker diabetic rats, dietary CLA (1.5%) normalized impaired glucose intolerance and lowered plasma insulin levels [12].

CLA occurs predominantly in milk and meat fats of ruminants [9, 27]; unsaturated fatty acids derived from plant material are biohydrogenated by the enzymatic reactions of rumen bacteria, and CLA emerges as a byproduct [18]. Some microorganisms accumulate bioactive lipids very rapidly in their membrane and further metabolize in a series of enzymatic reactions [16, 22]. Since CLA produced in the isomerization step is usually taken up by a saturation process [19], CLA cannot always be accumulated in a large amount in the rumen. Although the evolutionary purpose of this phenomenon is still controversial, this microbial pathway undoubtedly serves some roles in protecting rumen microbes from toxic effects of unsaturated fatty acids [15, 22]. CLA production in this pathway has been well characterized with *Butyrivibrio fibrisolvens* A38 in previous studies [19, 22].

CLA is a general term for a set of positional and geometric isomers of linoleic acid (LA) that possesses a conjugated double-bond system between the *cis*- or *trans*-configuration at positions of 8,10-, 9,11-, 10,12-, and 11,13 [8]. Among the various isomers, the *cis*-9, *trans*-11 isomer is believed to be an active form, at least for the anticarcinogenic properties. About 90% of the CLA isomers in dairy products are in the *cis*-9, *trans*-11 form, whereas only 40% of synthetically prepared CLA are in the same configuration. However, due to its low concentration in common foods, determination of the effects of naturally-produced CLA has been difficult.

Using the urea complexation technique, it was possible to obtain complex crystals and to selectively concentrate unsaturated fatty acids including CLA by eliminating

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saturated fatty acids in animal oil [7, 30, 37]. During the past two decades, worldwide dairy food consumption has been decreasing, whereas the low-fat product consumption has been on the increasing trend [29], which, in part, may be due to the numerous reports showing correlation between saturated fat consumption and adverse health effects, such as coronary heart disease. However, attempts have also been made to emphasize the positive effects of animal fat and to modify fat content in many foods [6, 35]. Indeed, dairy food consumption has been negatively correlated with cancer in an epidemiological study of Swedish people [20]. These findings provided firm supports that animal-based diets should also be able to contribute to human health by suitable modification of their composition.

Our study was focused on increasing the level of unsaturated fatty acids including CLA in milk fat by bovine dietary modification and urea fractionation technique. The overall process employed in this study was divided into two steps: 1) preparation of milk fat which contained high level of CLA by feeding sunflower oil (SO); 2) fractionation of the milk fatty acids with urea to eliminate saturated fatty acids. This study would envision the possibility to obtain a physiologically effective level of CLA and to study the protective effects of CLA in natural products.

MATERIALS AND METHODS

Cows and Diets

Eight lactating Holstein cows, averaging 380 kg, were used for the feeding studies. The cows were divided into two groups and were assigned to different dietary treatments. One group was given control diets without fat supplement and the other group was fed a diet with sunflower oil (5% of dry mass, Table 1). The diets were initiated from 4 weeks prior to the milk sampling. Cows had free access to water and balanced diets throughout the day, with fresh feed provided each morning.

Table 1. Ingredients and chemical composition of bovine diet and major fatty acid composition of the oil supplement.

Ingredients	Content (% DM)
Corn silage	31.5
Corn grain	27.5
Alfalfa hay	15.0
Soybean meal	13.5
Limestone	1.2
Calcium phosphate	1.0
Sodium bicarbonate	0.4
Calcium sulfate	0.4
Magnesium oxide	0.05
Protein supplement	4.0
Mineral-vitamin supplement[1]	0.25
Dietary oil supplement (SO)	5.0
C16:0	0.2
C18:0	0.3
C18:1	1.0
C18:2	3.5

Mineral-vitamin mix contained (mg/kg mix) Mn 3,500; Zn 3,000; Cu 750; Fe 20; I 85; Co15; and Se 35; retinyl acetate (2,100,000 IU per kg), cholecalciferol (390,000 IU per kg), α-tocopheryl acetate (7,500 IU per kg).

Reagents and Materials

All chemicals and solvents were of analytical grade purchased from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.). Milk used as a control and fresh sunflower oil was obtained from local sources. Conjugated linoleic acid containing 95% of *cis*-9, *trans*-11 octadecadienoic acid was from Matreya, Inc. (Pleasant Gap, PA, U.S.A.). Fatty acids used as standards were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Preparation of Fatty Acids

The saponification process was slightly modified from that used in our previous study [21]. Briefly, a milk fat sample (5 g) obtained from cows on different dietary regimen was dissolved in 50 mL of 1 M potassium hydroxide in 95% ethanol, and hydrolyzed at 70°C for 1 h with internal standard (heptadecanoic acid). To the saponified mixture, distilled water was added and the unsaponifiable matter was extracted into hexane (2×20 ml). The aqueous layer was acidified with 2 N HCl. The mixture was transferred to a separatory funnel and the liberated fatty acids were extracted with hexane (2×20 ml). The hexane layer, containing free fatty acids, was washed with 10 ml water and dried over anhydrous sodium sulfate. Fatty acids were then recovered using a rotary evaporator under vacuum.

Urea Fractionation

The dried fatty acid sample (5 g) was dissolved in 50 ml of alcohol mixture (ethanol:methanol=5:1). Subsequently, urea was added into the alcohol mixture with the ratio (urea/fatty acid ratio) of 1:1 (UFR1) or 2:1 (UFR2) or 3:1 (UFR3) and dissolved at 70°C. The solution was crystallized at −20°C for 24 h. Crystals were removed by gravity filtration on a Buchner funnel. To recover the CLA-containing fraction, the aqueous solution was acidified to pH 4–5 with 2 N HCl; equal volume of hexane was subsequently added and the mixture was extracted in a separatory funnel three times. The hexane extract was dried over anhydrous sodium sulfate and evaporated until completely dried using a rotary evaporator. The dried fatty acids were weighed to determine the yield of fractionation. The second fractionation was performed with the same final concentration of urea.

Gas Chromatograph Analysis

Derivatization of milk fat was carried out as previously described [21]. Briefly, milk fat samples (20 mg) were

methylated with 2 ml of 1% H_2SO_4 in methanol. The mixture was heated for 1 h in a 20-ml glass tube at 70°C, and 5 ml of 5% NaCl was then added. The fatty acid esters were extracted twice with 2 ml hexane. The hexane layer was washed with 4 ml of 4% potassium bicarbonate solution and dried over anhydrous sodium sulfate. The solution was then filtered through filter paper (Whatman No. 1) by gravity, and the solvent was removed in a rotary evaporator under vacuum.

The samples were analyzed for CLA isomers and total fatty acid profile in an HP5890 gas chromatograph with a flame ionization detector (FID). Fatty acid methyl esters were separated by using a Supelcowax-10 fused silica capillary column (60 m×0.53 mm i.d., 0.5 μm film thickness; Supelco. Inc., Bellefonte, PA, U.S.A.) at a flow rate of 2.4 ml/min helium. GC conditions were as follows: injector temp. 200°C; oven programmed, held at 40°C for 5 min, then increased to 220°C at 20°C/min, held for 40 min; detector temp. 250°C. One μl of sample was injected into the column in the splitless mode.

Quantification of Fatty Acids

Each fatty acid peak was identified and quantified by comparing with the retention time and peak area of fatty acid standards, respectively. The fatty acid content was expressed as percent (%) of total milk fat. Recoveries of fatty acids and the internal standard were higher than 80% after methylation. All the analyses were performed in triplicate and mean values are shown. For all variables, differences between treatments were determined by using a student *t*-test ($p<0.05$).

RESULTS

CLA Enrichment by Bovine Feeding

Sunflower oil (SO) was chosen as a fat substrate for feeding studies, because of its high level of linoleic acid (LA; 70%). The dry mass (DM) intake was relatively constant throughout the feeding trial (20–25 kg/day). Milk fat from cows treated with different diets were extracted and analyzed for the CLA content and other fatty acid profiles. The concentration of CLA was affected by bovine diet during the oil supplement. When cows were fed diet containing SO at 5% of DM, the average CLA content in milk fat was higher (2.4%) than the control group (0.5%) ($P<0.05$). The levels of octadecenoic acid (C18:1) and stearic acid (C18:0) were also elevated by SO treatment. However, CLA content in milk fat from oil-treated cows could not be maintained throughout the study. CLA level of milk fat obtained from the SO-treated cows proportionally increased with treatment periods and then declined after 8 weeks of feeding studies (Fig. 1).

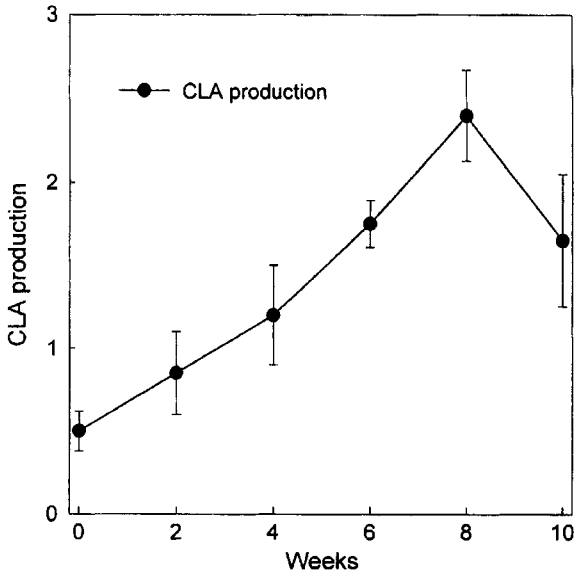

Fig. 1. Changes in CLA level during lactation. Cows were fed sunflower oil. CLA level was measured for 12 weeks (n=5).

Enrichment of CLA by Urea Fractionation

Urea-fat complexes were formed using urea/fatty acid ratios of 1:1 (UFR1) and 2:1 (UFR2). At each concentration, fractionation was performed twice consecutively. The patterns of changes in CLA concentration were similar in both the control milk fat and milk fat from SO-fed cows, and the CLA content varied depending on the urea/fatty acid ratio. The yield of total fatty acid declined as the urea/fatty acid ratio increased (Table 2). The CLA level increased up to 2.6 times at UFR1 and 3 times at UFR 2 in control fat, and no significant difference was found with high-CLA milk fat. Both the recovery of fatty acids after fractionation and the content of CLA with higher urea/fatty acid ratio (UFR3) were lowered. However, the amount of solvent

Table 2. Mean value of CLA concentration in fractionated milk fat.

Urea/fatty acid ratio	Fractionation	CLA (% fat)	Yield of total CLA (%)
Control	Control fat	0.5	100
	Hi-CLA fat	2.4	100
UFR1	Control fat	1.3	65
	Hi-CLA fat	4.7[1]	60
UFR2	Control fat	1.5	42
	Hi-CLA fat	5.2[1]	40
UFR3	Control fat	0.9	25
	Hi-CLA fat	3.5[1]	23

*The fatty acid levels were expressed as %CLA of total milk fat for controls and %CLA of crystallized samples of fractionated milkfat (n=3); [1]High-CLA fat has higher content than control fat in each group ($P<0.05$).

Table 3. Fatty acid profile (% of total fatty acids).

Fatty acids	Control		UFR1		UFR2	
	Control	Hi-CLA	Control	Hi-CLA	Control	Hi-CLA
C6:0	1.8	1.3	2.4	2.0	1.2	1.0
C8:0	1.2	0.7	7.2	6.3	2.4	2.3
C10:0	2.5	1.3	12.5	6.7	6.0	3.5
C12:0	3.0	1.8	7.9	3.2	6.2	2.4
C14:0	10.0	7.9	2.3	1.5	10.6	0.9
C14:1	1.4	1.3	5.6	4.8	2.0	1.8
C16:0	27.5	21.4	0.8	0.5	3.1	1.6
C16:1	2.5	2.7	4.4	3.6	6.0	3.4
C18:0	11.4	12.8	0.1	0.2	0.1	0.3
C18:1	26.0	35.3	25.8	40.3	37.0	50.5
C18:2	2.7	3.0	14.8	12.4	16.4	16.4
C18:3	0.4	0.2	2.3	2.0	2.9	2.2
CLA	0.5	2.4	1.3	4.7	1.5	5.2
Others	9.1	7.9	11.6	12.2	5.6	8.5

The fatty acid levels were expressed as % milk fat; Values represent means (n=3); Short chain fatty acids (C4:0) were not detected due to the loss during the evaporation.

added during the fractionation did not affect the yields of total fat and CLA (data not shown).

Fatty Acid Profile

Total fatty acid profile was obtained by the comparison of the GC profiles of the fatty acids with those of fatty acid standards, and the composition after the fractionation was determined for the major fatty acids in milk fat (Table 3). Feeding of SO also altered the total fatty acid profile to a great extent. The levels of palmitic acid (C16:0) and stearic acid (C18:0) increased, whereas shorter chain saturated fatty acid levels (C8:0, C10:0, C12:0, C14:0) decreased. Butyric acid (C4:0) was not detected after fractionation due to its volatility and loss during evaporation.

Noticeably, concentration of the long-chain saturated fatty acids (carbon number higher than 14) was selectively removed after the fractionation. Stearic acid, a major saturated fatty acid, decreased 100-fold after the fractionation with both UFR1 and UFR2, and palmitic acid level decreased to a lesser extend, especially in UFR2. The decrease in saturated fatty acid content was more evident in fatty acids with higher carbon numbers (Table 3).

In the control fat, mono-unsaturated fatty acids such as palmitoleic acid (C16:1) and oleic acid (C18:1) increased up to 2-fold at UFR2. CLA level was maximized with UFR2, and the increases of other long-chain unsaturated fatty acids such as LA and linolenic acid (C18:3) were more evident (Table 3). Fractionation with higher ratios (UFR3) did not further influence the fatty acid profile (data not shown).

DISCUSSION

Nutritional manipulation of the ruminants provides a strategy to alter the content and composition of milk fat. The quality and composition of milk fat are influenced by various interacting dietary factors, including the proportion of concentrate to forage ratio, and amounts of fatty acid supplements and its composition [1, 15, 31]. Dietary supplementation of LA, a major fatty acid in SO, has been shown to greatly affect the milk fat composition [17]. Fatty acid profiles of ruminal fat are also highly dependent on the feeding regimen, because unsaturated fatty acids are subject to ruminal transformation [23]. Moreover, Davis and Brown [5] showed in an earlier study that oil supplements increased *trans*-C18:1 as well as other isomers of unsaturated fatty acids produced in the rumen. The production of CLA, particularly the *cis*-9, *trans*-11 isomer, has been thought to be the first step in the biohydrogenation reaction of LA present in cow's feed [18]. Indeed, the strong correlation between *cis*-9, *trans*-11 CLA isomer and LA content of feeding material has been reported in other studies [17]. However, this phenomenon cannot be simply illustrated by the first-order reaction between the substrate and the product, because CLA emerges as an intermediate in the process of ruminal biohydrogenation [22].

The decline of CLA in cows fed SO for a long term (<8 weeks) could also be partially explained by the effects on rumen bacteria caused by abnormal dietary situation. Plant oil feeding might have induced the adaptation of the active rumen bacteria to toxic substrate levels or selected some microorganism which had high activity in CLA accumulation. The long-term exposure to the toxic fat substrates, however, could eventually affect the growth of CLA-producing bacteria itself. Thus, a large-scale LA input for the CLA accumulation was not always advantageous for the CLA accumulation in cow's tissue or milk, and the feeding trials should be performed with careful consideration concerning the optimal period of substrate exposure and the level of substrate included in the feed.

Urea fractionation can be a useful method for fatty acid separation because the inclusion of the large amount of fats into crystals could be achieved economically with simple equipments and with a low energy requirement [37]. We previously used the method to increase CLA level in a normal milk fat, and 2.5-fold CLA increase was attained [21]. In the present study, the CLA concentration was slightly enhanced by lowering the temperature for crystallization (-20°C), and by extending crystallization time (24 h), by which urea might have formed more stable crystals with unsaturated fatty acids.

CLA and other long-chain fatty acids were efficiently concentrated in the noncrystallized fraction with urea, and the fractions were easily separated by filtration. Complexation depends on the configuration of the fatty acid moieties,

because urea selectively forms a complex with high-carbon-number alkanes. Thus, fatty acids could be fractionated with urea, based on the degree of unsaturation and the chain length, and saturated and monoethylenic fatty acids could be efficiently removed by forming a crystal with urea in certain ratios. Moreover, the mild conditions applied during the fractionation may not affect the molecular structures of the unsaturated fatty acids with one or two double bonds [7]. In fact, the ratio C18:1/C18:0, an indicator of the elimination of saturated fatty acids, was about 2 in the control groups and increased up to 120 times after the urea fractionation with UFR2.

The technique of fatty acid fractionation by urea complex has been well established in the area of marine oil industry, and often applied for omega-3 fatty acid separation, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from marine oils [4, 11, 14, 30, 37]. The beneficial roles of such polyunsaturated fatty acids have been well documented for lowering serum triacylglycerols and cholesterol [24]. High concentration of omega-3 fatty acids was obtained from seal blubber oil with urea to fatty acid ratio of 4.5, and less unsaturated fatty acids were efficiently removed [37]. With milk fat in our study, however, polyunsaturated fatty acids with less degree of unsaturation were not efficiently fractionated. In fact, the fractionation at higher urea concentration (UFR3) was found unnecessary. To obtain higher unsaturated fatty acid concentration in milk fat, the study for the optimization of variables such as duration of crystallization and temperature should be followed. We are currently investigating the optimal condition for highly-purified CLA production from plant oil sources using urea fractionation (data not published).

The fractionation rate of each fatty acid may vary, even with the same concentration of urea. Major long-chain unsaturated fatty acids in milk fat, such as oleic acid (C18:1) and LA (C18:2), were not included in the urea complex and separated completely in the aqueous fraction. Thus, the degree of elimination of unsaturated fatty acid may depend on the concentration of urea used in the fractionation. The fractionation of other unsaturated fatty acids could also be possible under optimized conditions. For example, fractionation of oleic acid, the most abundant unsaturated fatty acid in milk fat, was greatly enhanced by urea. The increased dietary level of oleic acid could enhance the plasma level of CLA, because mono-unsaturated fatty acids seem to serve as substrates for desaturase in a variety of tissue which are responsible for endogenous synthesis of CLA [2]. On the other hand, the high degree of unsaturation in fractionated milk fat achieved by the fractionation technique could allow milk fat to be easily oxidized. Therefore, the stability of fat-modified dairy products, using the fractionated milk fat, should be maintained by including suitable antioxidants in the diet of lactating cows or in milk fat itself [1].

Short-chain fatty acids with carbon numbers <14 were also concentrated after the fractionation. The advantages of these fatty acids have been previously reported: Rat coloncyte proliferation was decreased by supplementing short-chain fatty acids *in vitro*, indicating that they may suppress tumor cell growth *in vivo* [28]. Moreover, short- and medium-chain fatty acids do not contribute to energy deposition in adipose tissue. Instead, they are rapidly hydrolyzed from triglyceride, passing through mucosa cells without intracellular esterification, and are directly transported to the liver [33]. Considering the anticarcinogenic effect of CLA, the crystallized milk fat could have a synergistic health promoting effect with other unsaturated fatty acids and short-chain fatty acids concentrated in the fraction. Moreover, the increase of unsaturated fatty acids in milk fat could meet with the consumer needs and probably boost the consumption of dairy products.

Besides, the CLA content in usual dairy products is lower than the effective level for the anticarcinogenic effects. Dairy products contain about 5 mg CLA/g fat, and 0.1% of CLA in a diet was shown to be enough to reduce mammary tumor occurrence. A 350-g rat fed a diet with 0.1% CLA would have about 15 mg CLA/day. This would be equivalent to a daily CLA intake of 3 g for a 70 kg human by extrapolation, which is about three times higher than the estimated usual CLA consumption in western countries [10]. CLA consumption may be much less for Asian people due to their low consumption of dairy products. Therefore, it would be desirable to concentrate CLA in the milk fat to maximize the health-promoting effect of CLA, which could help protect dairy consumers from various types of cancer.

Dairy cow is a main source of CLA, and several studies showed the potential to increase CLA level and to alter the fatty acid profile of milk fat by modification of bovine diet to change the environment for rumen bacteria. Results presented herein showed the possibility to concentrate natural CLA to a biologically effective level by the combination of an animal feeding trial and a chemical reaction. Future research should be pursued to maximize CLA level in the milk fat through optimized urea complexation in a combination with other purification procedure, by which CLA could be concentrated in an economic way. This may lead to the practical application of CLA as an ingredient in supplementary and functional foods.

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