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Variations in Conjugated Linoleic Acid (CLA) Content of Processed Cheese by Lactation Time, Feeding Regimen, and Ripening

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Dairy products are major sources of conjugated linoleic acid (CLA); thus, an increase in CLA content can improve the quality value of dairy products. The objective of this work was to determine the effects of lactation time, feeding regimen, and ripening period on the level of CLA in processed cheese. CLA content in milk varied with the period of lactation; high in spring (April and May, about 6.8 mg CLA/g fat) and relatively low in mid summer and winter (about 4.3 mg CLA/g fat). The effects of dietary regimen and ripening period were determined in milk, which was obtained from March to May. After aging for 4 months, the cheese made from milk obtained from cows fed on pasture contained relatively higher levels of CLA compared to cheese made from milk obtained from cows fed indoors (8.12 mg CLA/g fat vs 6.76 mg CLA/g fat), but there was no difference in 7 month-aged cheeses. In both pasture and indoor feeding, 7 month-aged cheeses showed higher CLA content than 4 month-aged cheeses. The contents of stearic acid (C18:0) and linolenic acid (C18:3) were significantly higher in cheese from pasture fed cows compared to those in cows fed indoors. These findings should be helpful for the efficient production of functional dairy products with high CLA contents.

KEYWORDS: Conjugated linoleic acid (CLA); cheese; indoors; pasture; ripening; lactation time; cows

INTRODUCTION

Conjugated linoleic acid (CLA) refers to a mixture of positional and geometric isomers of linoleic acid (octadecadienoic acid), each with a conjugated double bond arrangement at 7 and 9, linoleic acid (octadecadienoic acid; LA), 8 and 10, 9 and 11, 9 and 12, 10 and 12, or 11 and 13. CLA has been shown to have a variety of preventive roles against degenerative diseases including cancer (*1−3*), atherosclerosis (*4*), reduced catabolic effect of immune stimulation (*5*), and improved the protein to fat ratio (*6*) in a variety of animal studies. Some isomers of CLA have also been linked to the enhancement of growth of lean body mass (*7*). CLA is found in many foods, but principal dietary sources are meats and the milk of ruminants (*8*). The CLA isomer profile in dairy foods varies, but the *cis*-9, *trans*-11-isomer is known to be the predominant form (as much as 90% of the total CLA).

Fatty acid compositions of animal tissue can be influenced by many factors, including nutritional status, depot, species, and time of specific feeding (*9, 10*). CLA in dairy products is derived from the isomerization of LA and linolenic acid (LNA) during the biohydrogenation by some microorganisms in the rumen, which is influenced by various dietary factors such as the source and level of lipid substrates (*11, 12*), forage-to-grain ratio, and the presence of feed particles (*13*). Attempts to increase the CLA level in cow's milk were made in various ways by controlling feeding regimens in combinations with feeding high fat substrates including n-6 and/or n-3 long chain fatty acids (*14*). This would be partly linked to the rumen bacterial population influenced by bovine diets and related environmental factors. Indeed, the population of active CLA

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producing rumen bacterium, *Butyrivibrio fibrisolvens*, decreased when cows were fed low-roughage diets (*15*).

Moreover, many quantitative data have shown the CLA content in various food items is influenced by production conditions (*16*). Parameters in cheese processing, which determine chemical alterations during fermentation such as aeration (*17*), temperature (*18*), milling pH, additives, and ripening (*19*), have been found to affect fatty acids content including CLA. In addition, the fatty acid profiles of milk have been shown to be altered by lactating season and some dietary factors (*20*−*22*). Some lactic acid bacteria are known to have CLA producing activity (*16*); thus, it was thought that fermentation during cheese ripening might also affect CLA concentration. Many studies have shown the dietary effects on CLA content in milk, but direct comparison of CLA content in cheese from milk produced under different environmental conditions was not studied. The objective of this study was to determine the effects of lactation time, bovine feeding regimen, and ripening period on the CLA content in processed cheeses.

MATERIALS AND METHODS

Choice of Farm and Plant Samples. Study of lactation time was performed in Jeju, Korea throughout the year. The farm involved had all of the typical characteristics of a cheese producing farm, native pasture, total mixed ration facility, and a sufficient number of cows to select a similar stage of lactation group. The pasture obtained for the feeding study was mainly high quality perennial grass sward harvested in June through August, and cows were fed indoor with hay and concentrate in the ratio of 7:3 in a moisture-controlled barn. Single species and bulk samples of native pastures were collected from the experimental field of the governmental experimental station in Jeju, Korea.

Feeding Trial and Milk Sampling. Single species and bulk plant samples were collected at intervals of 15 to 20 days from March to May coinciding with the growing cycle of the pasture forage. DM intakes of supplements were measured daily for each cow, and samples of that offered were dried at 100 °C for 24 h to determine DM content. The amount of pasture eaten by each group of cows was assessed every day using a sward-sampling technique similar to that described by Stockdale and King (*23*). Bulk milk samples were obtained from 10 Holstein cows (650 kg body weight), choosing what the cows were selecting in the field and hand-plucking the samples. Whole raw milk samples from the five cows fed native pasture were collected during the morning and afternoon at the end of every month throughout the year. Milking times were at 0600 and 1600 h. One set of samples was preserved with 2-bromo-2-nitropropane-1,3-diol. The second set of samples was stored at −20 °C until analysis for fatty acid composition. Milk fatty acid analyses from morning and afternoon milkings within each month were averaged before statistical analyses. Milk that was obtained from March to May (relatively high CLA level) was promptly transported to the pilot plant for cheese production.

Cheese Manufacture. Cheeses were manufactured from the milk obtained from the cows under two different treatments. Two different vats of milk were used according to the standard method to make four different blocks of cheddar type cheese every 15 days. Briefly, combinations of starter cultures including *L. lactis* IO-1, M23, and *L. lactis ssp. cremoris* CCRC12586 (Cornell University, USA), which were typical cheese starter cultures, were added to milk (5% of milk fat) at 30 °C. Calf rennet powder (Ha-La rennet, Chr. Hansen Laboratory, Denmark) was used to coagulate the milk (0.2 g/kg milk), and the coagulated milk was cut into 0.95 cm cubes, which were then stirred and cooked at 37 °C for 30 min and held for an additional 45 min. Following cooking, curd was separated from whey, and loaves (about 15 cm wide) were formed from the curd. The 16 blocks of cheese were aged in an aging facility. Generally, a block of cheese stayed in brine for an average of 2 days for every kg of weight. Once the brine salting stage was completed, the cheese was aged in ventilated rooms at a temperature of 14−16 °C. The 16 blocks of cheeses were sampled at 4 and 7 months of ripening.

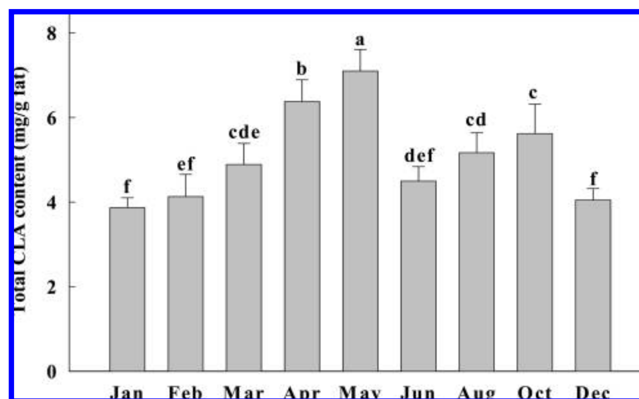

Figure 1. Seasonal variations in CLA content of milk obtained from pasture fed cows. Error bars represent standard deviations from each data point ($n = 5$). a−f: means with different superscripts are significantly different ($P < 0.05$).

Fatty Acid Analyses. All chemicals used for GC analysis were of analytical grade and purchased from Sigma (St. Louis, USA). Lipids from milk and cheese were extracted with hexane/isopropanol (3:2 v/v). Fatty acids were methylated as previously described by Kim and Liu (*24*) with some modification. Briefly, 0.5 mL of toluene and 2 mL of 5% KOH−MeOH were added to the lipids, and the samples were vortex-mixed and heated at 70 °C for 8 min, and then cooled in cold water. Two milliliters of 14% BF_3−MeOH was then added to the samples, and heated at 70 °C for an additional 8 min. The samples were cooled, and then 3 mL of 5% NaCl was added to the samples and mixed. Five milliliters of distilled water and 0.5 mL of hexane were added to extract the fatty acid methyl esters. The mixture was vortexed and centrifuged at 5,000*g* for 10 min, and then the upper phase was collected and dried with sodium sulfate. Samples were analyzed for total fatty acids including CLA isomers using an HP7890 gas chromatograph with a flame ionization detector (Hewlett-Packard 7890 Series). Fatty acid methyl esters were separated using a Supelcowax-10 fused silica capillary column (100 m × 0.32 mm i.d., 0.25 μm film thickness; Supelco, Inc., Bellefonte, PA, USA) with 1.2 mL/min of helium flow. The GC was operated at a temperature of 140 °C for 5 min, followed by heating at 2 °C/min to 240 °C, and holding for 30 min. Both the injector and detector were maintained at 260 °C. One microliter of sample was injected into the column in the split mode (50:1). The peak of each CLA isomer (*cis*-9 *trans*-11, *trans*-10 *cis*-12, *cis cis*, and *trans trans* isomers) and other fatty acids were identified and quantified by comparison with the retention time and peak area of each fatty acid standard (Sigma), respectively. Heptadecanoic acid (C17:0) was included as an internal reference before the extraction of lipids to determine the recovery of the fatty acids in each sample. The recovery of methylated fatty acids calculated in a comparison to the internal standard was higher than 80%.

Statistical Analysis. Statistical differences were determined by ANOVA, with mean separations performed by the Duncan multiple range test using the general linear model procedure of the SAS statistical software package (*25*). Results were expressed as mean ± SD.

RESULTS AND DISCUSSION

Seasonal Variations in Milk CLA. The CLA content of milk fat from lactating cows, which were pasture fed was determined each month throughout the year (**Figure 1**). When cows were fed indoors, it was hard to expect significant variation in CLA content of milk; we monitored the seasonal variation of CLA in the milk from pasture feeding, which could be affected by the change of flora available to the cows. When cows were pasture fed, milk obtained in early summer season (April and May) was usually higher in CLA level than milk lactated in the other seasons. CLA content was highest in May (7.1 mg/g fat) and lowest in January (3.9 mg/g fat). The increase in CLA level of milk by pasture feeding is in agreement with previous

Table 1. Feed Intake of Cows during Lactation Periods (kg/Day)

	Mar 26−27		Apr 07−08		Apr 20−21		May 04−05		average (mean ± SD, n = 5 each)	
day	pasture	indoor	pasture	indoor	pasture	indoor	pasture	indoor	pasture	indoor
fresh	52.2	39.4	46.4	38.9	48.0	39.9	35.8	40.9	45.6 ± 6.0	39.8 ± 0.8
DM[a]	22.1	22.1	21.8	21.8	21.9	21.9	19.5	19.5	21.3 ± 1.1	21.3 ± 1.1

[a] DM = dry matter.

reports (*21, 22, 26*). The composition of milk fat appears to be subject to strong variations due to diet, stage of lactation, and environmental factors (*12, 21*). The influence of these factors on the yield and composition of milk have been extensively studied in various animal trials, and the lactation time was thought to have a strong impact on cheese quality. Seasonal variations in fatty acid content including CLA in milk fat have been reported in previous studies. Booth et al. (*27*) first reported seasonal variation for butter fat measured at 230 nm, where the wavelength for the conjugated double bonds shows its maximum absorption. McDowell and McDowell (*28*) showed that seasonal variations of CLA were in a trend similar to that of oleic acid content of butter fat, high in spring and fall, and low in winter. This may be due to the seasonal difference in dietary factors. Rego et al. (*29*) also noted that summer milk fat had much more conjugated dienoic fatty acids than winter milk fat.

The composition of microflora in the rumen varies depending on the cow's diet. Summer milk fat is usually obtained from cows fed on pasture and usually had more CLA. Pasture feeding has been known to be a good strategy to increase CLA concentration in milk fat, and the CLA level has been linear to the level of *trans*-C18:1, which is an intermediate in the biohydrogenation pathway of unsaturated fatty acids (*22, 30*). This may be attributed to the high level of dietary LA in pasture, which is a major precursor of CLA in the ruminal biohydrogenation pathway. Moreover, LA in pasture may be easily available than from dried grass for ruminal biohydrogenation. LA is toxic to rumen microorganisms, and biohydrogenation is thought to be a detoxification mechanism, but optimal LA concentration could activate ruminal biohydrogenation (*31*). Thus, supplying cows with plants high in LA would be desirable to increase CLA without adverse effects on the rumen population because triglycerides with unsaturated fatty acids such as LA and LNA in the bovine diet would be slowly released along ruminal digestion.

Butyrivibrio fibrisolvens, a well characterized bacterium for its ability in biohydrogenation and CLA formation, is a cellulolytic bacterium, which has better growth when cows are fed a high-fiber diet. When cows were fed low-roughage diets, ruminal biohydrogenation of dietary unsaturated acids may have been depressed due to a reduced number of active hydrogenating bacteria (*15*). It has been shown that lipolytic activity of the protozoal fractions also increased with low-roughage diets (*15*). Unsaturated fatty acids in a free form are more easily oxidized than in an esterified form; thus, fewer precursors for isomerization would be available for CLA production when lipolytic activity is high.

Dietary Regimen and Cheese Ripening. From the seasonal experiment for differences in milk fat, we performed the study on the effects of dietary regimen and cheese ripening on CLA content of processed cheese from milk obtained in the early summer season (from March to May), which had relatively high CLA levels. Feed intake (fresh) of cows during lactation periods was higher in the pasture than in the indoor group, but DM intake was equal (**Table 1**). Cheese (5.8−10.3 mg CLA/g fat) produced from the milk in this study contained relatively higher levels of CLA compared with cheeses (2.9−7.1 mg CLA/g fat)

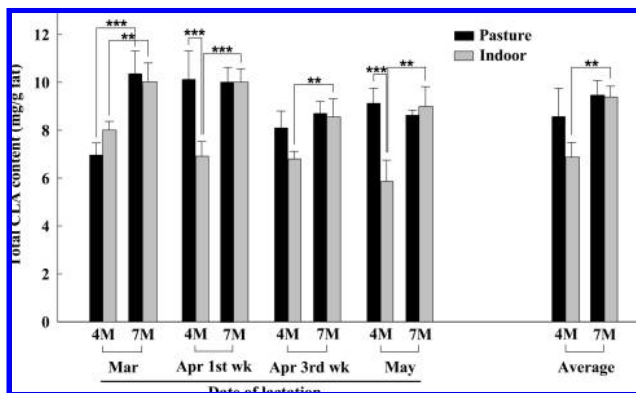

Figure 2. Effects of lactating time, feeding regimen, and ripening on CLA content in cheese. 4 M = 4 month-aged cheese; 7 M = 7 month-aged cheese. Error bars represent standard deviations from each data point (n = 5). ** $P < 0.01$; *** $P < 0.001$.

in other studies (*8*). In both pasture and indoor feeding, the extension of ripening period increased CLA content in cheese (**Figure 2**). When cows were pasture fed, the CLA level was generally higher in 4 month-aged cheese, but there was no significant increase with 7 month-aged cheese, compared to the cheese obtained from indoor feeding. The differences of CLA level between pasture and indoor feeding in 4 month-aged cheeses were the most significant in milk obtained during the first week in April and May. In both pasture and indoor feeding, the CLA content in processed cheese was increased by ripening for 4 months compared with that in raw milk samples. However, CLA level in 7 month-aged cheese did not show a significant difference between pasture and indoor feeding. This suggests that the CLA level in milk from indoor feeding can be affected strongly by the long-term ripening process of cheese.

The present study indicated that environmental factors during ripening have an impact on CLA content in cheese and that it could be influenced in a different manner depending on the cow's dietary regimen. Heating temperature during cheese processing could also be a factor for increase in CLA concentration in processed cheese. Cheddar cheese heated to 80−90 °C was shown to have significantly increased CLA content as compared with cheese heated to 70 °C (*18*). Fernandez-Garcia et al. (*32*) reported that heating was the only step, which could enhance the CLA content during cheese processing, and suggested that high temperature enhanced the formation of LA radicals resulting in a conjugated system in the fatty acid backbone. The ripening period of cheese also affected the CLA content of cheese, especially in cheese obtained from pasture-fed cows. In the present study, although it was unclear why ripening was more influential in increasing CLA content in cheese from indoor feeding, it was feasible that the microflora responsible for cheese ripening was affected by the different feeding regimen and resulting milk composition. Some lactic acid bacteria were shown to increase CLA content during cheese fermentation in our previous study (*16*). Cheese quality can be affected by milk treatment and the milk processing procedure. In addition, pH could be a key factor for CLA production since

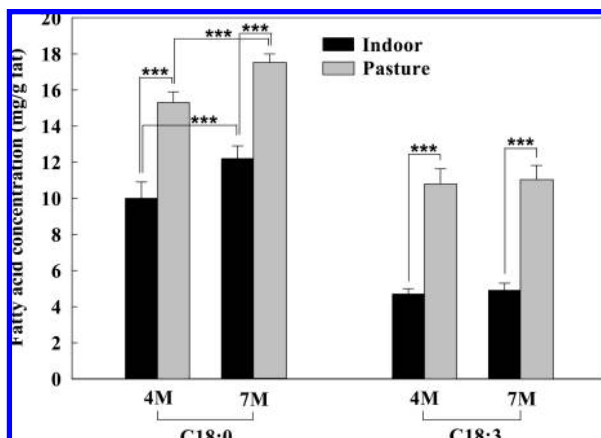

Figure 3. Effects of feeding regimen and ripening on stearic acid (C18:0) and linolenic acid (C18:3) content in cheese. 4 M = 4 month-aged cheese; 7 M = 7 month-aged cheese. Error bars represent standard deviations from each data point ($n = 5$). ** $P < 0.01$; *** $P < 0.001$.

LA isomerization is a pH dependent enzymatic reaction. Acidic environment induced by high concentrate indoor feeding in early spring and winter could be antagonistic to CLA production because the optimal condition for the responsible bacteria for CLA production was shown to be near neutral (*31*).

Most of the other fatty acids in cheese were not under significant variation except stearic acid (SA, C18:0) and linolenic acid (LNA, C18:3). As shown in **Figure 3**, SA and LNA were significantly higher in cheese obtained from pasture feeding in comparison to indoor feeding, and the ripening for 7 months significantly increased SA levels. It appears that the high content of LNA in the pasture is reflected in milk fatty acid profiles. Moreover, the higher SA concentration with 7 month-aged cheese may be due to more biohydrogenation occurring during ripening.

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