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Factors Affecting the Conjugated Linoleic Acid Content of Cheddar Cheese

Hui Lin,* Terri D. Boylston,† Lloyd O. Luedecke, and Terry D. Shultz

Department of Food Science and Human Nutrition, Washington State University, Pullman, Washington 99164-6376

The effects of package type (cans and nylon vacuum pouches), milling pH (5.5, 5.7, and 5.9), additives [butylated hydroxyanisole (BHA), tyrosine, and lysine], and aging (0, 1, 3, and 6 months) on the conjugated linoleic acid (CLA) content of Cheddar cheese was determined. Fatty acid distributions and CLA, moisture, protein, lipid, and titratable acidity contents in raw milk and aged cheeses were determined. The CLA content in canned cheese (3.03 mg/g lipid) was significantly higher than in a vacuum pouch packed cheese (2.70 mg/g lipid) after 6 months of aging. Milling pHs of 5.5 and 5.9 and the addition of BHA, tyrosine, and lysine resulted in significantly lower CLA contents in cheeses aged for 6 months than the control. Multiple linear regression showed that lipid and protein contents contributed to CLA content positively. The content of oleic acid contributed significantly to the relationship between CLA and fatty acids.

Keywords: *Conjugated linoleic acid; Cheddar cheese; anticarcinogen*

INTRODUCTION

Conjugated linoleic acids (CLA) have been identified as potent antioxidants, anticarcinogens (Parodi, 1994), modulators in the immune system (Cook et al., 1993), anti-atherosclerosis agents (Lee et al., 1994), and body weight protectors (Chin et al., 1994a; Miller et al., 1994). Among the CLA isomers, *cis*-9,*trans*-11-; *trans*-9,*cis*-11-; *trans*-9,*trans*-11-; *trans*-10,*trans*-12-; and *trans*-10,*cis*-12-octadecadienoic acids are the major isomers; while *cis*-9,*cis*-11-; *cis*-10,*cis*-12-; *cis*-10,*trans*-12-; and *cis*-11,*cis*-13-octadecadienoic acids are minor isomers. The *cis*-9,*trans*-11 isomer is possibly the isomer responsible for the biological functions of CLA because it can be incorporated into biomembranes (Ha et al., 1990; Ip et al., 1991).

Dairy products, particularly cheeses, are important dietary sources of CLA (Ha et al., 1989; Chin et al., 1992; Werner et al., 1992; Lin et al., 1995). Two mechanisms have been proposed for the formation of CLA in dairy products: (1) isomerization of linoleic and linolenic acids through a biohydrogenation pathway in the rumen and (2) free radical oxidation of linoleic or linolenic acids during processing (Ha et al., 1989). Only free fatty acids can be utilized in the biohydrogenation pathway (Chin et al., 1994b) because linoleic isomerase is specific for free fatty acids with a *cis*-9,*cis*-12 diene configuration (Kepler and Tove, 1969; Kepler et al., 1970).

Several factors have been identified that affect the formation of CLA in dairy products. The addition of whey proteins or dry milk powder increased the CLA content of processed cheese and nonfat yogurt. The added proteins function as hydrogen donors to enhance isomerization reactions at the initial stage of the biohydrogenation pathway and the conversion of linoleic acid oxidation radicals to CLA (Shantha et al., 1992, 1995). Cheddar cheese heated to 80 and 90 °C in the preparation of processed cheese significantly increased CLA content as compared with the cheese heated to

* Author to whom correspondence should be addressed.
† Present address: Agri-West Laboratory, 1403 E. Lookout Rd., Richardson, TX 75082.

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70 °C (Shantha et al., 1992). Garcia-Lopez and coworkers (1994) found that heating was the only step in processing that increased CLA content in a natural cheese. Shantha et al. (1992) and Garcia-Lopez et al. (1994) suggested that elevated temperatures enhanced the formation of linoleic acid radicals, resulting in increased CLA content. Air is important in the formation of CLA through the free radical mechanism (Ha et al., 1989). The CLA content of cheese processed under atmospheric conditions was significantly higher than that of cheese processed under nitrogen (Shantha et al., 1992). Incorporation of air into butter also resulted in an increased CLA content (Shantha et al., 1995). On the other hand, the effect of aging cheese on CLA formation varies depending on cheese type and length of aging (Ha et al., 1989; Chin et al., 1992; Werner et al., 1992).

Little research has been done to determine the effects of additives, other than proteins, on the formation of CLA in cheese products. Antioxidants may influence the formation of CLA through the free radical oxidation pathway. Although irradiation of linoleic acid with ultraviolet light in the presence of tyrosine or lysine did not produce CLA (Cawood et al., 1983), the addition of tyrosine and lysine to milk prior to cheesemaking may affect CLA formation in the cheese. Tyrosine and lysine may donate active protons to the intermediates of oxidation or biohydrogenation of linoleic or linolenic acids to enhance CLA formation.

The objectives of this study were to systematically examine the effects of several factors (including packaging, milling pH, additives, and aging) on the CLA content of Cheddar cheese. These factors may alter oxidation reactions, enzyme activities, growth and metabolism of microorganisms, and cheese maturation. Subsequently, these changes in the balance of metabolic processes in the ripening cheese may also influence CLA formation through either free radical oxidation or biohydrogenation pathways. Enhancement of the CLA content of cheeses through alterations in processing parameters would contribute to increased nutritional value and consumer acceptability of these dairy products.

MATERIALS AND METHODS

Materials and Preparation of Cheese. Raw milk was obtained from the Washington State University Dairy Farm. Holsteins at the dairy farm were confined to dry lot conditions, and ration did not include access to green chop. Standard calf rennet and Redi-Set Culture containing *Lactococcus lactis* ssp. *lactis* and L. *lactis* ssp. *cremoris* were purchased from Chr. Hansen's Laboratory, Inc. (Milwaukee, WI). Lysine, tyrosine, and BHA were purchased from Sigma Chemical Company (St. Louis, MO). The packaging materials, nylon vacuum pouches (20 × 25 cm, 3 mm standard barrier, light permeable, oxygen impermeable), were purchased from Koch Supplies (Kansas City, MO), while cans (211 × 300, with pull top) were purchased from Continental Can Co., Food Package Division (San Leandro, CA).

Cheddar cheese was made in a FT40 multi-purpose processing vessel (Armfield Technical Education Co. Ltd., Ringwood, Hampshire, England), using standard Cheddar cheesemaking procedures (Kosikowski, 1982) with treatments described later (Figure 1). Sixteen kilograms of raw milk was used for each processing batch. Lactic starter cultures grown in Phase 4 Medium, an internal buffered commercial culture medium (Waterford Food Products, Inc., Millville, UT), were used in a concentration range of 0.31−0.47% (10^{10} cfu/mL) to prepared cheese samples. Experimental cheeses (225 g) were vacuum sealed and stored in light-proof plastic bags at 7 °C. Cheeses were sampled after 0, 1, 3, and 6 months of aging. Individual cans and packages were prepared for each sampling period. Prior to sampling for the chemical analyses, the entire block of cheese was shredded and well-blended.

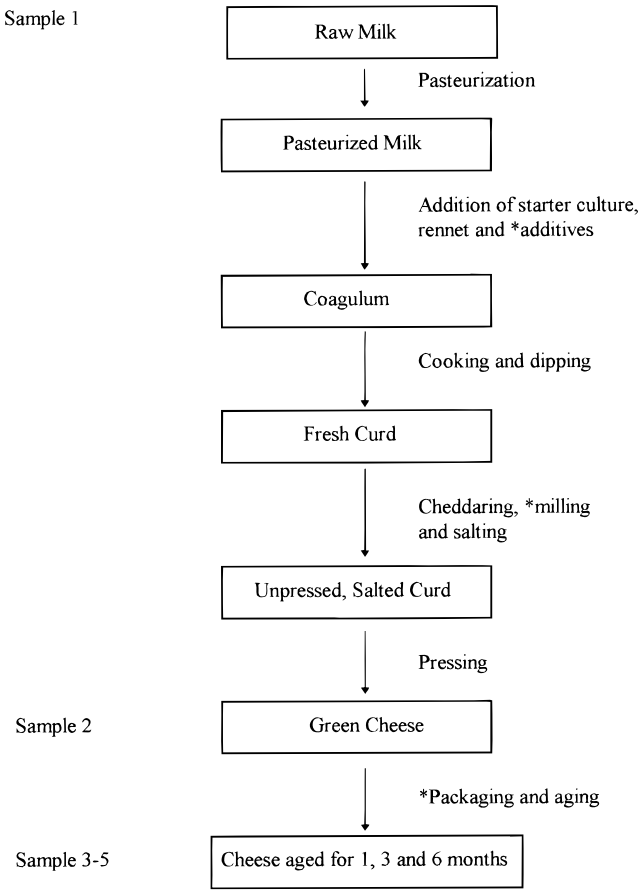

Figure 1. Processing procedure of Cheddar cheese and sampling plan. Asterisks indicate the treatment sites.

The effects of three processing factors (packaging, milling pH, and additives on CLA content) were determined in separate experiments. The two types of packages used were can and nylon vacuum pouch. Milling pHs tested were pH 5.5, 5.7, and 5.9. Three additives (BHA, lysine, and tyrosine) were added at 3.5 and 7.0, 3.0 and 6.0, and 3.8 and 7.6 mg/1000 g of milk, respectively, prior to cheesemaking. The levels of BHA chosen are within the range permitted by the Federal Food, Drug and Cosmetics Act of the United States. Added levels of both lysine and tyrosine were selected to have an equivalent molar content of hydrogen donors as the two levels of BHA. The experiment was replicated twice. As shown in Figure 1, five samples were collected for each replication. Samples prepared without any additives, milled at pH 5.7, and packed in nylon vacuum pouches served as controls for all treatments. All collected samples were stored at −20 °C prior to analysis.

Reagents and Chemicals. All solvents were purchased from J. T. Baker Inc. (Phillipsburg, NJ). Hexane used for gas chromatography (GC) was HPLC grade. Methanol and chloroform used for lipid extraction were HPLC and reagent grades, respectively. Chloroform was re-distilled in glass prior to use. All other reagents were analytical grade. Fourteen percent boron trifluoride (BF_3) in methanol and fatty acid standards (i.e., linoleic acid methyl ester, heneicosanoic acid methyl ester, and heneicosanoic acid) were purchased from Sigma Chemical Company (St. Louis, MO). The CLA reference standard was obtained from Dr. Michael Pariza (Food Research Institute, University of Wisconsin).

Moisture Content. Duplicate representative portions (approximately 5 g) from each collected sample were dried in an oven at 105 °C to a constant weight (AOAC, 1984).

Protein Content. Collected milk samples were analyzed for protein content directly, while cheese samples were prepared by blending in a 0.1 N NaOH solution prior to determination as described in the UDY Protein Analyzer Manual (UDY, 1992). Duplicate protein contents were determined by the dye-binding method with Acid Orange 12, using the UDY digital colorimeter (Model 305, Fort Collins, CO).

Titratable Acidity. Titratable acidity (TA) was determined following the methods described in AOAC (1984). A total of 10 g of milk was diluted with 20 mL of CO_2-free distilled water, and cheese samples (10 g) were diluted with CO_2-free distilled water to a volume of 105 mL. Duplicate sample solutions were prepared and titrated with 0.1 N NaOH to pH 8.2, using a Schott autotitrator (Hofheim, Germany). Titratable acidity was expressed as percent of lactic acid based on the sample weight.

CLA Content and Fatty Acid Profile. Conjugated linoleic acid contents and fatty acid profiles were determined as described by Lin et al. (1995). Briefly, lipids from milk or cheese samples were extracted with chloroform and methanol as described by Bligh and Dyer (1959). The chloroform layer containing lipids was separated from the aqueous phase through centrifugation and aspiration of the methanol layer, with the choloroform layer passed through anhydrous sodium sulfate (Na_2SO_4). The chloroform−lipid extract was then concentrated through evaporation to a final volume of 10 mL. The total lipid content was determined following the nitrogen evaporation (Lin et al., 1995). Lipid extracts were stored at −75 °C until further analysis.

The lipid extracts were hydrolyzed with NaOH at 100 °C and methylated with 14% boron trifluoride in methanol at room temperature (Werner et al., 1992). Heneicosanoic acid was added to the lipid extract as an internal standard prior to hydrolysis (Lin et al., 1995). Conjugated linoleic acid and fatty acid methyl esters were analyzed on a Varian gas chromatograph (Model 3400; Varian Assoc., Inc., Sunnyvale, CA). The CLA methyl esters were separated using a Supelcowax 10 (60 m × 0.75 mm id.; phase thickness, 1.0 μm; Supelco, Bellefonte, PA) capillary column with an on-column injection port and flame ionization detector. The CLA peaks were identified by comparison with the retention time of the reference standard. Total fatty acid methyl esters were separated using an Omegawax 320 capillary column (30 m × 0.32 mm i.d.; phase thickness, 0.25 μm; Supelco, Inc.) with a split injection (20:1 split ratio). The CLA and fatty acid contents were expressed as milligrams of fatty acid per gram of lipid based on standard curves for heneicosanoic acid and linoleic acid methyl esters (Lin et al., 1995).

Statistical Analysis. Analysis of variance with mean separations using least squares means techniques was conducted to determine the influence of each processing parameter (aging, packaging, milling pH, and addition of additives) on the moisture, protein, TA, lipids, and CLA contents of the cheese (SAS, 1995).

Multiple linear regression analysis was conducted to determine the overall contribution of (1) moisture, protein, TA, and lipid contents to CLA content (sample weight basis) and (2) fatty acid contents to CLA content (lipid weight basis) of the prepared cheeses. The models were determined using stepwise regression techniques with the criterion that variables met the 0.15 significance level for entry into the model (SAS, 1995).

RESULTS AND DISCUSSION

The *cis*-9,*trans*-11 isomer was the only isomer detected and reported as CLA content in this study. More than 80% of the total CLA in foods has been reported to be the *cis*-9,*trans*-11 isomer (Ha et al., 1989; Werner et al., 1992; Chin et al., 1993), which is the biologically active form (Ha et al., 1990; Ip et al., 1991). The CLA content of all samples is presented on a sample weight basis for consumer interest. However, the discussion of the effects of processing parameters on CLA content focuses on the CLA content, as expressed on a lipid weight basis. The CLA content of the control and treatment cheeses is comparable to CLA contents of cheeses produced at Washington State University (Werner et al., 1992; Lin et al., 1995).

Table 1. Composition of Treatment and Control Cheeses Aged 6 Months[a]

treatment	moisture	protein	lipid	titratable acidity
packaging				
control (vacuum-pouch)	33.34	23.70	35.30	2.82
can	34.34*	23.65	34.20*	3.28*
milling pH				
control (5.7)	33.34	23.70	35.30	2.82
5.5	33.54	23.93	35.59	2.94
5.9	36.30*	23.40*	35.65	2.87
additives (mg/1000 g of milk)				
control (no additive)	33.34	23.70	35.30	2.82
BHA (3.5)	35.96*	23.95	34.54*	3.09*
BHA (7.0)	35.02*	24.38*	34.50*	3.15*
tyrosine (3.8)	34.40*	23.95	33.51*	2.77
tyrosine (7.6)	34.40*	24.65*	33.31*	3.02*
lysine (3.0)	35.09*	24.23*	33.82*	3.32*
lysine (6.0)	36.00*	23.75	34.01*	2.99*

[a] Means are duplicate analyses of two replications. Means with an asterisk (*) are significantly different ($P < 0.05$) from the control cheese.

Table 2. Effect of Packaging Treatment on CLA Content of Cheddar Cheese Aged up to 6 Months[a]

	CLA (mg/g of lipid)		CLA (mg/100 g of sample)	
stage[b]	pouch	can	pouch	can
milk	2.59^{bx}	2.59^{bx}	9.59^{cx}	9.59^{cx}
0-M	2.72^{abx}	2.71^{bx}	96.00^{abx}	94.93^{bx}
1-M	2.59^{bx}	2.70^{bx}	91.58^{bx}	92.66^{bx}
3-M	2.78^{ax}	2.68^{bx}	98.06^{ax}	92.50^{by}
6-M	2.70^{aby}	3.03^{ax}	95.28^{aby}	103.50^{ax}
SE[c]	0.06	0.06	1.60	1.95

[a] Means are duplicate analyses of two replications. Means with the same superscript (a−c) in a column are not significantly different ($P > 0.05$). Means with the same superscript (x, y) in a row are not significantly different ($P > 0.05$) for each CLA content category. [b] Milk, 0-M, 1-M, 3-M, and 6-M denote raw milk and the cheese aged for 0, 1, 3, and 6 months, respectively. [c] SE denotes standard error.

The composition of the control and treatment cheeses after 6 months of aging is presented in Table 1. Although the treatments did result in statistically significant ($P < 0.05$) compositional differences, in comparison to the control cheese, the composition varies less than 3% with respect to moisture, protein, and fat contents and 0.5% with respect to titratable acidity. These values are within acceptable ranges for Cheddar cheeses (Lin et al., 1995).

Packaging. The effect of packaging treatment on the CLA content of Cheddar cheese is shown in Table 2. No significant difference ($P < 0.05$) in CLA content, as expressed on lipid or sample weight basis, was found between the cheese vacuum packed in nylon pouches or cans through 3 months of aging. The significantly higher ($P < 0.05$) CLA content in the canned cheese as compared to the pouch packed cheese at 6 months of aging may be attributed to an enhancement of the free radical oxidation mechanism for CLA formation. The interior of a canned cheese has more free space than the interior of a pouch packed cheese. Therefore, a canned cheese likely contains more residual air than a pouch packed cheese at the initial aging stage. Since air enhances lipid oxidation, a greater content of allyl radicals could be formed in a canned cheese (Ha et al., 1989), which can be converted into CLA through pro-

Table 3. Effect of Milling pH Treatment on CLA Content in Cheddar Cheese Aged up to 6 Months[a]

	CLA (mg/g of lipid)			CLA (mg/100 g of sample)		
stage[b]	control	pH 5.5	pH 5.9	control	pH 5.5	pH 5.9
milk	2.59^{bx}	2.73^{ax}	2.73^{ax}	9.59^{cx}	10.31^{dx}	10.31^{dx}
0-M	2.72^{abx}	2.82^{ax}	2.62^{abx}	96.00^{abxy}	100.61^{ax}	90.50^{bcy}
1-M	2.59^{by}	2.64^{ay}	3.03^{ax}	91.58^{by}	94.18^{aby}	104.91^{ax}
3-M	2.78^{ax}	2.43^{aby}	2.72^{ax}	98.06^{ax}	86.58b^{cy}	95.84^{bx}
6-M	2.70^{abx}	2.24^{by}	2.22^{by}	95.28^{abx}	79.62^{cy}	76.90^{cy}
SE[c]	0.06	0.06	0.13	1.60	1.38	4.40

[a] The milling pH for control group was 5.7. Means are duplicate analyses of two replications. Means with the same superscript (a−d) in a column are not significantly different ($P > 0.05$). Means with the same superscript (x, y) in a row are not significantly different ($P > 0.05$) for each CLA content category. [b] Milk, 0-M, 1-M, 3-M, and 6-M denote raw milk and the cheese aged for 0, 1, 3, and 6 months, respectively. [c] SE denotes standard error.

tonation of the radicals. The CLA content in the canned samples increased in the second 3 months of aging, suggesting that the conversion of allyl radicals into CLA became pronounced during this time period. The highest CLA content of the canned cheese was attained after 6 months of aging, while the vacuum pouch packed cheese reached its maximum CLA content following 3 months of aging. An extended aging period may allow conversion of the *cis*-9,*trans*-11 CLA isomer to the *trans*-9,*trans*-11 CLA isomer (Nichols et al., 1951) and further hydrogenation of CLA to monoenoic and stearic acids (Hughes et al., 1982; Harfoot and Hazlewood, 1988). The formation of CLA isomers continued to increase throughout the 6 months of aging in the canned cheese. However, the CLA contents of the vacuum pouch packed cheese aged for 3 and 6 months were not significantly different, indicating a stabilization of CLA content.

Milling pH. The change of milling pH from 5.7 to 5.5 and 5.9 resulted in a decreased CLA content in cheese aged 6 months (Table 3). Conjugated linoleic acid contents of all three pH treatments were similar on a lipid weight basis at the initial aging stage. After 1 month of aging, CLA content in cheese milled at pH 5.9 was higher than the other two pH treatments. Optimal activity of lipolytic enzymes occurs at approximately pH 6.0 (Walstra and Jenness, 1984; Kitchen, 1985). The milling pH of 5.9 could contribute to greater enzyme activity than treatments at lower pHs. Thus, at the higher pH, greater lipolysis may have resulted in a higher content of free fatty acids and precursors for formation of CLA through the biohydrogenation pathway. However, with aging periods of more than 1 month, the CLA contents of cheeses milled at pH 5.9 decreased, indicating decomposition of the CLA isomers. For the cheeses milled at pH 5.7, the standard milling pH, the CLA content increased steadily to a maximum value following 3 months of aging.

Additives. Butylated hydroxyanisole functions as an antioxidant through the formation of a resonance stabilized radical intermediate upon the donation of hydrogen from the phenol ring structure. Tyrosine and lysine also contain active protons in their phenol and amine groups, respectively. The functional groups of these compounds are able to donate hydrogen to the intermediates of free radical oxidation and biohydrogenation reactions. The donation of hydrogen may convert linoleic acid radicals to CLA (Ha et al., 1889) and isomerize linoleic acid to CLA, thereby increasing CLA contents, but may also decrease CLA contents by enhancing the hydrogenation of CLA to monoenoic and stearic acids (Posati et al., 1975; Hughes et al., 1982).

In Table 4, the CLA content in the BHA-treated groups decreased during pre-aging processing from raw milk to green cheese. Decomposition, isomerization, or saturation reactions attributed to the antioxidant activity of BHA may contribute to the observed decrease in the CLA content during the initial stages of processing. This observation suggests that free radical oxidation was the predominant mechanism of CLA formation prior to aging. During aging, the biohydrogenation pathway may be the dominant mechanism for CLA formation because anaerobic conditions are preferred by this pathway (Kepler et al., 1966). The ability of BHA to donate hydrogen enhanced formation of CLA during the initial month of aging. However, further donation of hydrogen after 1 month of aging may have allowed the reduction of CLA to monoenoic or saturated fatty acids and thus decreased CLA content. The addition of BHA at both levels produced similar effects at most stages except the last stage, where the low-level BHA-treated cheese had a significantly lower CLA content than the high-level BHA-treated cheese.

The addition of tyrosine also reduced the CLA content,

Table 4. Effect of Additives on CLA Content in Cheddar Cheese Aged up to 6 Months[a]

		additives (mg/1000 g of milk)					
		BHA		tyrosine		lysine	
stage[b]	control	3.5	7.0	3.8	7.6	3.0	6.0
		Lipid Weight Basis (mg/g of lipid)					
milk	2.59^{by}	2.62^{axy}	2.68^{axy}	2.87^{ax}	2.72^{axy}	2.78^{axy}	2.66^{bxy}
0-M	2.72^{abx}	2.20^{bz}	2.38^{ayz}	2.60^{bxy}	2.35^{byz}	2.68^{abx}	2.46^{cy}
1-M	2.59^{bx}	2.69^{ax}	2.80^{ax}	2.02^{cxy}	1.88^{cy}	2.60^{bx}	2.70^{bx}
3-M	2.78^{ay}	2.09^{bz}	2.26^{byz}	2.47^{byz}	2.41^{byz}	2.73^{ay}	2.93^{ax}
6-M	2.70^{abw}	1.81^{cz}	2.10^{by}	2.19^{cy}	2.07^{cy}	2.40^{cx}	2.50^{cx}
SE[c]	0.06	0.12	0.12	0.06	0.06	0.05	0.05
		Sample Weight Basis (mg/100 g of sample)					
milk	9.59^{cx}	9.65^{dx}	9.91^{cx}	10.48^{cx}	10.05^{cx}	10.11^{dx}	9.88^{dx}
0-M	96.00^{abw}	76.62^{bz}	82.08^{axy}	88.42^{ax}	80.12^{ay}	91.66^{ax}	84.95^{cy}
1-M	91.58^{bx}	93.23^{ax}	96.48^{ax}	68.73^{bz}	64.14^{bz}	88.71^{by}	92.85^{bx}
3-M	98.06^{ax}	72.16^{bz}	77.75^{byz}	84.09^{ay}	82.11^{ay}	93.41^{ax}	100.58^{ax}
6-M	95.28^{abw}	62.59^{cz}	72.44^{by}	74.20^{by}	70.29^{by}	80.49^{cx}	83.28^{cx}
SE	1.60	4.65	4.65	1.81	1.81	1.35	1.35

[a] Means are duplicate analyses of two replications. Means with the same superscript (a−d) in a column are not significantly different ($P > 0.05$). Means with the same superscript (w−z) in a row are not significantly different ($P > 0.05$). [b] Milk, 0-M, 1-M, 3-M, and 6-M denote raw milk and the cheese aged for 0, 1, 3, and 6 months, respectively. [c] SE denotes standard error.

Table 5. Effect of Treatments on TA in Cheddar Cheese Aged up to 6 Months[a]

	stage[b]					
treatment	milk	0-M	1-M	3-M	6-M	SE[c]
packaging						
control (vacuum pouch)	0.16^{cx}	2.22^{bx}	2.82^{ax}	2.80^{ay}	2.82^{ay}	0.09
can	0.16^{cx}	2.44^{bx}	2.68^{by}	3.54^{ax}	3.28^{ax}	0.12
milling pH						
control (5.7)	0.16^{cx}	2.22^{bx}	2.82^{ax}	2.80^{ax}	2.82^{ax}	0.09
5.5	0.16^{dx}	2.40^{cx}	2.78^{bx}	2.51^{cy}	2.94^{ax}	0.02
5.9	0.16^{dx}	1.72^{cy}	2.49^{by}	2.47^{by}	2.87^{ax}	0.07
additives (mg/1000 g of milk)						
control (no additive)	0.16^{cx}	2.22^{bxy}	2.82^{ax}	2.80^{az}	2.82^{az}	0.09
BHA (3.5)	0.15^{dx}	1.64^{cz}	1.99^{bz}	3.11^{ay}	3.09^{axy}	0.10
BHA (7.0)	0.15^{dx}	1.56^{cz}	2.10^{by}	3.24^{ax}	3.15^{ax}	0.10
tyrosine (3.8)	0.16^{dx}	1.52^{cz}	2.21^{by}	2.99^{ayz}	2.77^{abz}	0.05
tyrosine (7.6)	0.16^{dx}	1.89^{cyz}	2.67^{bxy}	2.85^{abz}	3.02^{ay}	0.05
lysine (3.0)	0.16^{cx}	2.03^{by}	2.21^{by}	3.12^{ay}	3.32^{ax}	0.05
lysine (6.0)	0.16^{dx}	2.44^{cx}	2.97^{bx}	3.36^{ax}	2.99^{by}	0.05

[a] Means are duplicate analyses of two replications. Means with the same superscript (a−d) in a row are not significantly different ($P > 0.05$). Means with the same superscript (x−z) within the same column are not significantly different ($P > 0.05$). [b] Milk, 0-M, 1-M, 3-M, and 6-M denote raw milk and the cheese aged for 0, 1, 3, and 6 months, respectively. [c] SE denotes standard error.

which was attributed to the antioxidant activity of tyrosine. The addition of two levels of tyrosine did not result in significant differences in the CLA content of cheeses.

Unlike BHA and tyrosine, lysine does not have a phenol ring structure. As shown in Table 4, the CLA content in the low-level lysine-treated group was comparable to the control group at most stages except after 6 months of aging where the lysine-treated cheese had a significantly lower CLA content than the control. The high level of added lysine decreased the CLA content at the 0 and 6 month aging stages but increased CLA content at the 3 month aging stage. Cawood and colleagues (1983) also found that irradiating the incubated fatty acids (containing linoleic acid) with lysine did not produce CLA, which agrees with observations in this study regarding the lysine-treated cheeses at the stages between milk and 1 month aging. Increased CLA content in the cheese treated with the high level of added lysine at the 3 month aging stage and decreased CLA contents in the cheeses treated with both levels of lysine during the last 3 months of aging may be attributed to the ability of lysine to donate hydrogen to the intermediates of the biohydrogenation pathway and to CLA, respectively. As described previously, the donation of hydrogen to the intermediates of the biohydrogenation pathway enhances formation of CLA, whereas the donation of hydrogen to CLA enhances the conversion of CLA to more saturated fatty acids.

Relationship between CLA Content and Composition of Cheeses. The quantitative analyses of major cheese components indicated that the treatments did not significantly affect moisture, lipid, and protein contents but did affect TA (Table 5). Titratable acidity in canned cheeses was significantly higher than in a vacuum pouch packed cheeses after 3 months of aging, indicating higher enzyme activity of cultures. Accordingly, higher isomerization and biohydrogenation activity of culture may exist in the canned cheese as compared with pouch packed cheese, which support the observations of higher CLA content in the canned cheese than in pouch packed cheese in the second 3 months of aging. Compared with the control (pH 5.7), the milling pH of 5.9 resulted in a continuous decrease in TA from the initial to the 3 month aging stage, while the milling pH of 5.5 resulted in a decrease in TA only at the 3 month aging stage. Milling pH of 5.5 and 5.9 may retard the production of lactic acid, indicating that pH 5.7 was an optimum milling pH for enzymatic activities of the starter culture. The observations of optimum enzymatic activities in the canned cheeses and the cheeses milled at pH 5.7 were related to the optimum CLA contents of these cheeses. No differences in TA were found between the control and milling pH treated samples after 6 months of aging. The addition of additives generally decreased TA at the 0 and 1 month aging stages but increased TA at the 3 and 6 month aging stages, suggesting that a retarded growth of bacteria existed in these cheeses, explaining the decreased CLA content in these additive treated cheeses at latter aging stages.

Table 6. Regression Statistics for Prediction of Conjugated Linoleic Acid (CLA) Content[a] Based on the Contents of Major Components and Selected Fatty Acids[b]

major components			selected fatty acids		
	intercept	overall r^2		intercept	overall r^2
	−154.2770	0.0997		0.7514	0.2873
	slope	partial r^2		slope	partial r^2
protein	3.6782	0.0531	18:1ω9	0.3817	0.2034
lipid	4.3718	0.0466	18:1ω7	0.7029	0.0077
			18:0	−0.9038	0.0332
			16:0	0.7549	0.0311
			14:0	−1.2076	0.0195

[a] Milligrams of CLA (*cis*-9,*trans*-11 isomer)/g of sample based on the contents of major components; milligrams of CLA (*cis*-9,*trans*-11 isomer)/g of lipid based on the contents of fatty acids. [b] Major components or fatty acids of which the content was significantly ($P < 0.15$) correlated with CLA content.

A fairly low correlation coefficient (r^2) value 0.0997 was obtained from multiple linear regression analysis between CLA content and content of major components, including moisture, lipid, protein, and TA (Table 6). Protein and lipid contents were significant contributors to the relationship between CLA and major component contents. The positive relationship between the amount of protein and CLA confirms the enhancement effect of proteins on CLA formation found by previous investigators (Cawood et al., 1983; Iversen et al., 1984; Ha et al., 1989; Shantha et al., 1992, 1995). Proteins may enhance CLA formation by acting as hydrogen donors (Cawood et al., 1983; Ha et al., 1989) or binding oxidation inductors such as iron, lipoxidase, and riboflavin, etc. (Shantha et al., 1992). Since CLA and CLA

precursors are of lipid origin, it is not surprising to find a positive relationship between the content of CLA and lipids. The positive relationship between the content of CLA and proteins or lipids was also observed in a survey study of 15 different commercial cheese types (Lin et al., 1995).

Five fatty acids were identified as significant contributors to explaining the CLA content in the cheeses (Table 5). The C18:1ω9 fatty acid was the most important contributor because of the high partial correlation coefficient (r^2). Conjugated linoleic acid and oleic acid isomers, C18:1ω9 and C18:1ω7, are the products of biohydrogenation of linoleic and linolenic acids (Kepler et al., 1966; Kemp et al., 1975; Hazlewood and Dawson, 1976). Therefore, an increase in CLA can be accompanied by an increase in the aforementioned oleic acid isomers, as indicated by positive relationships between content of CLA and C18:1ω9 or C18:1ω7. Similarly, Lin and co-workers (1995) observed a positive relationship between CLA and oleic acid contents in a survey of CLA contents in dairy products. The accumulation of final products of enzymatic reactions generally inhibits further enzyme activity and slows down the reaction process. Accordingly, stearic acid (C18:0), as the final product of biohydrogenation, can inhibit biohydrogenation reactions and thus reduce the production of CLA through the biohydrogenation pathway. The inhibitory effect of stearic acid was suggested by the negative relationship between the amount of CLA and stearic acid (Table 6). The relationship between CLA content and the content of C16:0 or C14:0 possibly represents an indirect relationship between CLA content and the content of C16:0 or C14:0. Factors that contribute to an increase in CLA content may also contribute to an increase or decrease in C16:0 or C14:0 content, respectively.

CONCLUSIONS

The processing parameters investigated in this study (type of package, milling pH, and addition of additives) had significant effects on the CLA content of cheddar cheeses aged for 6 months. Although the differences noted in this study by altering the factors selected may not represent practical significance, further research to understand the mechanisms responsible for CLA formation may make it possible to increase the CLA content of cheddar cheeses to have a positive nutritional benefit. Due to the complex nature of the cheese, several hypotheses have been proposed on the basis of these results, to explain the mechanisms of CLA formation. The free-radical oxidation mechanism is proposed to be the major mechanism for CLA formation prior to aging, while biohydrogenation is proposed to predominate during aging. These pathways have been suggested as the major pathways for not only the formation of CLA from linoleic and linolenic acids but also the further conversion of CLA to more saturated fatty acids.

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