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Conjugated linoleic acid concentration as affected by lactic cultures and added linoleic acid

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Abstract

Six lactic cultures: *Lactobacillus acidophilus* (CCRC14079), *L. delbrueckii* subsp. *bulgaricus* (CCRC14009), *L. delbrueckii* subsp. *lactis* (CCRC14078), *Lactococcus lactis* subsp. *cremoris* (CCRC12586), *Lc. lactis* subsp. *lactis* (CCRC10791), and *Streptococcus salivarius* subsp. *thermophlius* (CCRC12257) were tested for the effects of addition of 1000 and 5000 μg/ml linoleic acid, and incubation time from 0 to 48 h. The levels of conjugated linoleic acid (CLA) formation were determined by gas chromatography. A sharp increase in CLA level was observed in linoleic acid added cultures. Incubation of *L. acidophilus* in 1000 μg/ml linoleic acid added-skim milk medium for 24 h was most effective in promoting CLA formation. Increasing linoleic acid addition from 1000 to 5000 μg/ml and prolonging incubation from 24 to 48 h did not appear to enhance CLA formation.

Keywords: Conjugated linoleic acid; Starter culture; Lactic acid bacteria

1. Introduction

Conjugated linoleic acids (CLA), the isomerization products of linoleic acid, have been recognized as antioxidants, cancer inhibitors, cholesterol depressing agents, and growth promoting factors (Ha et al., 1987, 1990; Ip et al., 1991; Shultz et al., 1992).

Among all food products, ruminant products have been found to contain relatively large amounts of CLA. Dairy products, one of the major ruminant products, are rich in CLA. However, the CLA content varied. Raw milk contained 0.83–5.5 mg CLA/g fat, Brick cheese contained as high as 7.1 mg CLA/g fat, and nonfat yogurt contained 1.7–5.3 mg CLA/g fat (Chin et al., 1992; Ha et al., 1989; Shantha et al., 1995). The CLA levels of various dairy products, determined by GC methods, were 0.55–9.12 mg CLA/g fat, as summarized by Lin and Lee (1997).

The formation of CLA in dairy products has been proposed to occur through the isomerization of linoleic acids and linolenic acids in the rumen and through the oxidation of linoleic acids to form resonance-stabilized allyl radicals, followed by reprotonation of the radicals by proteins during processing (Ha et al., 1989). Biohydrogenations of linoleic acids and linolenic acids to stearic acid in the rumen through microbial enzymatic reaction also contributed to CLA formation (Chin et al., 1992, 1993; Christie, 1983; Ha et al., 1987).

Some fermented dairy products contained higher levels of CLA than nonfermented milk. Shantha et al. (1995) observed an increase in CLA content from 4.4 mg CLA/g fat of unprocessed milk to 5.3 mg CLA/g fat of a yogurt product with 0.05% fat. Ha et al. (1989) reported a higher level of CLA in Cheese Whiz of 8.81 mg CLA/g fat than in unprocessed milk of 0.83 mg CLA/g fat. Dahi, an Indian equivalent of yogurt, also contained more CLA (26.5 mg CLA/g fat) than the raw material (5.5 mg CLA/g fat) (Aneja and Murthi, 1990). Colbert and Decker (1991) evaluated the CLA content of cheese before and after ripening and found an increase in the levels of CLA at 4 to 8 weeks of ripening due to the formation of CLA from linoleic acid. More recently, Jiang et al. (1998) reported that enhanced levels of CLA were detected in certain fermented dairy products. While those reports indicated that CLA increased during fermentation, the data of Werner et al. (1992) suggested that neither different starter cultures nor aging caused changes in the CLA concentration of Cheddar-type cheeses. No changes in CLA content were observed in fermented dairy products, such as lowfat

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and regular yogurts, sour cream, and cheeses, as reported by Shantha et al. (1995).

Linoleic acid isomerase-containing microorganisms, such as *Butyrivibrio fibrisolvens* in rumen (Kepler & Tove, 1967) and the intestinal flora in rats (Chin et al., 1992, 1994) are capable of isomerizing linoleic acid to CLA. In a recent study of screening of 19 dairy starter cultures on CLA production in 25 μg/ml free linoleic acid-treated Man–Rogosa–Sharpe (MRS) broth containing 0.1% Tween-80, Jiang et al. (1998) found that only three strains of *Propionibacterium freudenreichii* were capable of producing CLA. None of the tested lactic acid bacteria cultured in MRS broth was able to produce CLA. However, they did not examine CLA-producing capacity of those lactic cultures using skim milk as the medium. Neither was the effect of higher linoleic acid addition on CLA-producing capacity of lactic cultures determined.

Since several of the previously mentioned reports indicated that cheese and yogurt products contained higher levels of CLA than nonfermented raw materials, lactic acid bacteria could produce CLA in a medium comprising dairy constituents. The objectives of this investigation were to examine six most commonly used lactic cultures, effects of linoleic acid addition to the cultures, and different incubation times, on CLA production in sterilized skim milk in vitro.

2. Materials and methods

Pure lactic acid bacteria: *Lactobacillus acidophilus* (CCRC14079), *L. delbrueckii* subsp. *bulgaricus* (CCRC 14009), *L. delbrueckii* subsp. *lactis* (CCRC14078), *Lactococcus lactis* subsp. *cremoris* (CCRC12586), *Lc. lactis* subsp. *lactis* (CCRC10791), and *Streptococcus salivarius* subsp. *thermophlius* (CCRC12257) were purchased from Culture Collection and Research Center (CCRC), Food Industrial Research Institute, Shin Chu, Taiwan, and were activated in MRS broth (Difco Lab., MI, USA) for 12 h at 37°C for all the cultures except *Lc. lactis* subsp. *cremoris* (CCRC12586), which was activated at 26°C. The activated cultures were transferred to the culture medium containing 12% skim milk powder (w/v) and incubated for 24 h at the activation temperatures. One percent of these transferred cultures was then inoculated in 20 ml culture medium (v/v). After addition of 20 or 100 mg linoleic acid (Sigma Chemical Co., St. Louis, MO, USA), the culture medium were incubated for 24 and 48 h at temperatures as previously described.

2.1. Lipid extraction

Following incubation, the medium was mixed with 200 ml chloroform: methanol (2:1, v/v), and 20 mg heptadecanoic acid (Sigma Chemical Co.) was added as the internal standard for GC analysis. After homogenizing in a Nihon Seiki universal homogenizer (Tokyo Nihon Seiki Seisakusho Co.) for 5 min at #4 setting, the mixture was centrifuged at 500×***g*** for 15 min at 4°C in a refrigerated centrifuge (Himac CR20B2, Hitachi). The lower layer was then dried with anhydrous sodium sulfate, evaporated with a rotary evaporator at 30°C, and flushed with nitrogen until dry (Ha et al., 1989; Ha & Lindsay, 1990). The residue was saponified with 1 ml 1.0 N sodium hydroxide in methanol in a 100°C water bath for 15 min. As described in the methylation procedures of Chin et al. (1992) and Station et al. (1997) using acid-catalyzed methanolysis, 6 ml of 4% HCl in methanol was added after cooling and methylated at 60°C for 20 min. The mixture was concentrated under a stream of nitrogen at room temperature and redissolved in 1 ml hexane. Finally, CLA in hexane extract was quantified by capillary GC.

2.2. GC analysis

Instrumentation used for the analyses was as follows: A DANI GC (DANI Educational, Italy) equipped with Supelcowax-10 fused silica capillary column (60 m×0.32 mm i.d., 0.25 μm film thickness, Supelco Inc., Bellefonte, PA, USA); a flame ionization detector; and a Hewlett Packard 3395 integrator (Palo Alto, CA, USA). The injection volume was 2.0 μl. The temperature of the GC oven was programmed at 150°C for 7 min and from 150°C to 220°C at the rate of 5°C/min and held for 9 min. The injector and detector temperatures were 250°C (Shantha et al., 1995). Nitrogen was used as the carrier and make-up gas. The flow rate of make-up gas was 30 ml/min. The pressure was maintained at 10 psig on the column in order to obtain the flow rate of the carrier gas at 2 ml/min. The split ratio was set at 1:50. The c-9,t-11 CLA methyl ester eluted at 48.0 min was identified by comparing the retention time with that of methylated CLA standard (Sigma Chemical Co., St. Louis, MO, USA). The area of CLA peak was calculated as μg c-9,t-11 CLA/ml medium using heptadecanoic acid as internal standard.

All data were subjected to analysis of variance for a one-factor completely randomized design and Duncan's Multiple Range Test using SAS (SAS Institute, Inc., 1986) and a significance level of 0.05 was used. Each treatment was performed in three replications.

3. Results and discussion

A large increase ($p < 0.05$) in total plate counts from 6.0–7.0 to 7.5–9.1 log CFU/ml were found in the media of six lactic cultures with 0, 1000, and 5000 μg/ml linoleic acid added as incubation time increased from 0 to 24 h (Fig. 1). However, changes in total plate counts

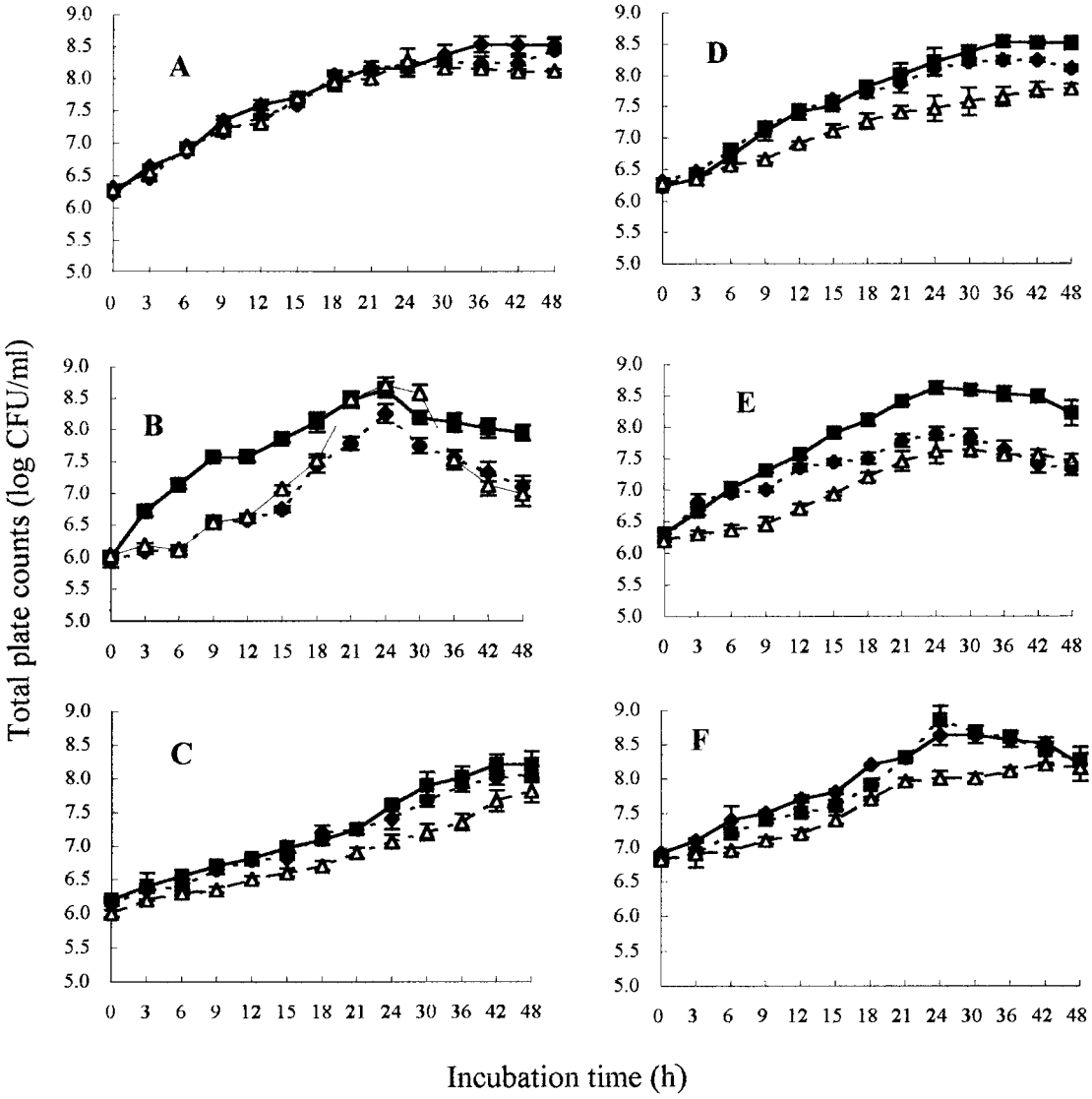

Fig. 1. Changes in total plate counts of six lactic cultures in sterilized skim milk with free linoleic acid added at different incubation times [amount of linoleic acid added (μg/ml): 0 (■), 1000 (◆), 5000 (△)]. A: *L. acidophilus* CCRC 14079, incubated at 37°C; B: *L. delbrueckii* subsp. *bulgraricus* CCRC 14009, incubated at 37°C; C: *L. delbrueckii* subsp. *lactis* CCRC 14078, incubated at 37°C; D: *Lc. lactis* subsp. *cremoris* CCRC 12586, incubated at 26°C; E: *Lc. lactis* subsp. *lactis* CCRC 10791, incubated at 37°C; F: *Str. salivarius* subsp. *thermophilus* CCRC 12257, incubated at 37°C.

from 24 to 48 h of incubation varied with strain of starter culture in the treatments with no linoleic acid added. Total plate counts after 48 h incubation with no linoleic acid added were higher ($p < 0.05$) with *Lactobacillus acidophilus*, *Lactococcus lactis* subsp. *lactis*, and *Streptococcus salivarius* subsp. *thermophlius* inoculated, lower ($p < 0.05$) with *L. delbrueckii* subsp. *lactis* inoculated, and no change ($p > 0.05$) with *L. delbrueckii* subsp. *bulgaricus* and *Lc. lactis* subsp. *cremoris* inoculated. No significant difference ($p > 0.05$) in CLA levels was found among 0, 24, and 48 h incubations in the treatments with no linoleic acid added, as shown in Table 1, due to the exhaust of available linoleic acid for CLA conversion, indicating the need of linoleic acid addition for CLA formation. Both CLA levels and total plate count rose as incubation time increased from 0 to 24 h in 1000 and 5000 μg/ml linoleic acid-added treatments. However, no significant increase ($p > 0.05$) in CLA levels was observed as incubation time increased from 24 to 48 h due to the completion of CLA conversion at 24 h incubation. This suggested that incubation time longer than 24 h was unnecessary for promoting CLA production in 1000/5000 μg/ml linoleic acid-added culture media.

The levels of CLA and linoleic acid in sterilized skim milk before fermentation were 7.2 and 23.4 (g/ml, respectively. Since the fermentation process was suggested to increase CLA concentrations of the milk products (Aneja & Murthi, 1990; Shantha et al., 1992), CLA levels rose to 14.5–19.5 μg/ml in the medium with no linoleic acid added after 0, 24, and 48 h incubations (Table 1). No notable differences ($p > 0.05$) in CLA

Table 1
Formation of CLA (μg/ml) by six lactic cultures in sterilized skim milk with free linoleic acid added at different incubation times

Linoleic acid added (μg/ml)	Lactic culture	Incubation time (h)		
		0[a,x]	24[a,x]	48[a,x]
0	*L. acidophilus*[d]	18.0[a,x]	18.5[a,x]	17.5[a,x]
	L. delbrueckii subsp. *bulgaricus*[d]	19.0[a,x]	18.0[a,x]	16.6[a,x]
	L. delbrueckii subsp. *lactis*[d]	17.0[a,x]	17.5[a,x]	19.0[a,x]
	Lc. lactis subsp. *cremoris*[e]	15.0[a,x]	15.5[a,x]	14.5[a,x]
	Lc. lactis subsp. *lactis*[d]	16.5[a,x]	18.0[a,x]	14.5[a,x]
	Str. salivarius subsp. *thermophilus*[d]	18.0[a,x]	19.5[a,x]	16.5[a,x]
1000	*L. acidophilus*[d]	23.0[a,x]	105.5[c,y]	106.5[c,y]
	L. delbrueckii subsp. *bulgaricus*[d]	21.5[a,x]	86.5[b,y]	85.5[b,y]
	L. delbrueckii subsp. *lactis*[d]	23.5[a,x]	77.5[ab,y]	77.5[ab,y]
	Lc. lactis subsp. *cremoris*[e]	21.5[a,x]	63.0[a,y]	68.5[a,y]
	Lc. lactis subsp. *lactis*[d]	20.0[a,x]	77.5[ab,y]	71.0[ab,z]
	Str. salivarius subsp. *thermophilus*[d]	25.0[a,x]	73.5[ab,y]	68.0[a,y]
5000	*L. acidophilus*[d]	25.0[ab,x]	91.5[b,y]	93.5[c,y]
	L. delbrueckii subsp. *bulgaricus*[d]	24.0[ab,x]	86.0[ab,y]	66.0[ab,z]
	L. delbrueckii subsp. *lactis*[d]	23.0[ab,x]	52.0[c,y]	51.0[a,y]
	Lc. lactis subsp. *cremoris*[e]	26.0[b,x]	70.0[a,y]	61.0[ab,y]
	Lc. lactis subsp. *lactis*[d]	24.0[ab,x]	76.5[ab,y]	72.0[b,y]
	Str. salivarius subsp. *thermophilus*[d]	18.0[a,x]	82.5[ab,y]	57.0[ab,z]

[abc] Means of each linoleic acid addition in the same column followed by the same superscripts are not significantly different ($p > 0.05$).
[xyz] Means in the same row followed by the same superscripts are not significantly different ($p > 0.05$).
[d] Incubated at 37°C.
[e] Incubated at 26°C.

concentration were observed among the treatments of six lactic cultures without addition of linoleic acid, which suggested that the strain of lactic culture did not affect CLA formation when skim milk was used as the medium. This finding was confirmed by Werner et al. (1992) who reported that starter cultures did not influence CLA concentrations of Cheddar-type cheeses.

However, the strain of starter culture and fermentation time affected CLA formation as 1000 μg/ml linoleic acid was added, and the CLA concentrations increased to 21.5–107 μg/ml after fermentation, as shown in Table 1. A sharp increase ($p < 0.05$) in CLA levels from 20.0-25.0 to 63.0–105 μg/ml in the culture media with 1000 μg/ml linoleic acid added was found when incubation time increased from 0 to 24 h. CLA level did not change ($p > 0.05$) as incubation time increased to 48 h except with *Lc. lactis* subsp. *lactis*, in which the level decreased from 77.5 to 71.0 μg/ml after 48 h incubation. Among all the 1000 μg/ml linoleic acid-added treatments, inoculation of *L. acidophilus* resulted in the largest yield of CLA at level of 105 μg/ml after 24 h incubation at 37°C. However, the level remained unchanged as incubation time increased to 48 h. Therefore, inoculation of *L. acidophilus* for 24 h was suggested for promoting CLA production in 1000 μg/ml linoleic acid-added skim milk medium. The conversion rate of linoleic acid to CLA, in 1000 μg/ml linoleic acid-added culture media incubated at 37°C for 24 h, was in the range of 6.3% (*Lc. lactis* subsp. *cremoris*) to 10.5% (*L. acidophilus*). All the lactic cultures tested in this study were capable of producing CLA in sterilized skim milk, which explained why some fermented dairy products had higher levels of CLA than nonfermented milk as previously described (Aneja & Murthi, 1990; Chin et al., 1992; Ha et al., 1989; Shantha et al. 1995). While none of the lactic cultures was able to produce CLA in MRS broth (Jiang et al., 1998), lactic cultures produced CLA in skim milk, indicating that skim milk as the medium was better for lactic cultures to produce CLA. The reasons were probably due to the neutralization of the inhibitory effect of fatty acids by milk protein (Boyaval et al. 1995) and prevention of CLA oxidation by alkyl radicals formed from the milk protein, especially sodium caseinate and low molecular weight (0.5–5 kDa) whey protein (Shantha & Decker, 1993), in the skim milk. The capacity of lactic cultures to produce CLA in linoleic acid-added skim milk after incubation was further confirmed by two recent CLA studies regarding the inhibitory effect of dairy additives (Lin et al., 1998) and linoleic isomerase from *L. delbrueckii* subsp. *bulgaricus* (Lin et al., 1999).

The levels of CLA in the treatments of 5000 μg/ml linoleic acid addition were 18.0–93.5 μg/ml, as shown in Table 1. Similar trends found in 1000 μg/ml linoleic acid-added treatments were observed in these treatments, in which a significant increase ($p < 0.05$) in CLA level from 18.0–26.0 to 52.0–91.5 μg/ml was detected as incubation time increased from 0 to 24 h. However, CLA level remained unchanged ($p > 0.05$) as incubation time increased to 48 h except with *L. delbrueckii* subsp. *bulgaricus* and *Str. salivarius* subsp. *thermophlius*. The levels went lower ($p < 0.05$) at 48 h of incubation in these two cultures. Inoculation of *L. delbrueckii* subsp. *lactis*

resulted in the smallest yield of CLA at a level of 52.0 μg/ml in 5000 μg/ml linoleic acid-added medium among all the lactic cultures after 24 h incubation. No significant difference ($p > 0.05$) in CLA level among the other 5 cultures was observed at this incubation time. The highest amount of CLA was produced in the medium inoculated with *L. acidophilus* among the treatments of 48 h incubation. However, no significant difference ($p > 0.05$) in CLA level inoculated with *L. acidophilus* was found between 24 and 48 h of incubation. Above all, inoculation of all the strains except *L. delbrueckii* subsp. *lactis* into 5000 μg/ml linoleic acid-added medium for 24 h appeared to have a similar enhancing effect on CLA production.

Increase in linoleic acid addition from 1000 to 5000 μg/ml did not show any significant increase ($p > 0.05$) in CLA concentration in the culture media after incubation. Instead, CLA level decreased significantly ($p < 0.05$) with increase in linoleic acid addition from 1000 to 5000 μg/ml in the media inoculated with *L. acidophilus* and *L. delbrueckii* subsp. *lactis* for 24 h incubation and *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, and *L. delbrueckii* subsp. *lactis* for 48 h incubation, respectively. It appeared that increase in linoleic acid addition from 1000 to 5000 μg/ml exerted a strong inhibitory effect on CLA production, which was in agreement with the studies of the antimicrobial effect of free linoleic acid on propionic acid bacteria, reported by Boyaval et al. (1995) and Jiang et al. (1998).

Based on our results, the yield of CLA after fermentation was primarily dependent on linoleic acid addition and strain of the starter culture inoculated. Addition of linoleic acid was effective in enhancing CLA conversion during fermentation and a level of 1000 μg/ml was recommended. Inoculation of *L. acidophilus* into 1000 μg/ml linoleic acid-added medium for 24 h incubation was most effective in promoting CLA formation. Increase in linoleic acid addition from 1000 to 5000 μg/ml in the culture medium showed little enhancement on CLA production after fermentation. CLA level did not rise in the culture medium as incubation time increased from 24 to 48 h.

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