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Conjugated linoleic acid concentration as affected by lactic cultures and additives

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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 thermophilus* (CCRC12257) were tested for the effects of additions of 60 g l^{-1} sucrose, lactose, fructose, and 10 g l^{-1} sodium chloride. The levels of c9,t11-conjugated linoleic acid (c9,t11-CLA) formed were determined by gas chromatography. A significant decrease in c9,t11-CLA level was observed in cultures with sucrose, lactose, fructose, and sodium chloride added, except for *Lc. lactis* subsp. *cremoris*. Inoculation of *L. acidophilus* into 60 g l^{-1} sweeteners and 10 g l^{-1} sodium chloride-treated skim milk medium under aerobic conditions for 24 h incubation was most effective in promoting c9,t11-CLA formation. Lactose and fructose are suggested for use in maintaining a high CLA level.

Keywords: Conjugated linoleic acid; Lactic culture; Sucrose; Lactose; Fructose; Sodium chloride

1. Introduction

Conjugated linoleic acid (naturally occurring isomers of octadecadienoic acid with conjugated double bonds at 9 and 11, 10 and 12, or 11 and 13 positions) has gained considerable attention because of its anticarcinogenic (Ha, Grimm & Pariza, 1987; Ip, Chin, Scimeca & Pariza, 1991; Pariza & Hargraves, 1985), antioxidative (Parodi, 1994), cholesterol-depressing (Huang, Luedecke & Schultz 1994), and growth-promoting (Chin, Storkson, Liu, Albright & Pariza, 1994) properties.

Foods of ruminant origin generally contain more CLA than foods of non-ruminant origin (Chin, Liu, Storkson, Ha & Pariza, 1992; Chin, Storkson, Liu & Pariza, 1991; Ha, Grimm & Pariza, 1987; Shantha, Crum & Decker, 1994) due to the presence of linoleic acid isomerase in ruminal bacteria (Kepler & Tove, 1967), which catalyzes the isomerization of linoleic acid into CLA. Processing methods, such as heat treatment (Ha, Grimm & Pariza, 1989; Parodi, 1994) and addition of oxidants (Shantha & Decker, 1993) enhance linoleic acid isomerization. Inoculation with various lactic cultures affects the CLA level in linoleic acid added to skim milk medium *in vitro* (Lin, Lin & Lee, 1999). The presence of whey protein (Shantha, Decker & Ustunol, 1992) and different polarities of lipids (Shantha, Ram, O'Leary, Hicks & Decker, 1995) also influence the CLA formation in fermented dairy products.

Some fermented dairy products contain higher levels of CLA than non-fermented milk. Ha et al. (1989) reported a higher CLA level in Cheese Whiz (8.81 mg CLA/g fat) than in unprocessed milk (0.83 mg CLA/g fat). Dahi, an Indian equivalent of yogurt, also contains more CLA (26.5 mg CLA/g fat) than the raw material (5.5 mg CLA/g fat) (Aneja & 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. Shantha et al. (1995) also 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. More recently, Jiang, Bjröck and Fondén (1998) reported enhanced levels of CLA in certain fermented dairy products. While at report indicated that CLA increased during fermentation, the data of Werner, Luedecke and Shultz

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

Since most fermented milks are sweetened in the West or salted in the Middle East and sugar or sodium chloride can affect the metabolism of starter cultures, it would be interesting to know how these additives might affect the production of CLAs.

In the previous study, we observed that the yield of CLA in the skim milk medium was primarily dependent on the concentration of linoleic acid added and the species of lactic acid bacteria inoculated (Lin et al., 1999). In order to examine the effects of sweeteners on CLA formation, the goal of this research was to determine the effect of sucrose, lactose, fructose, and sodium chloride on c9,t11-CLA production in the linoleic acid added to skim milk medium *in vitro*.

2. Materials and methods

2.1. Bacterial strains

Six strains of lactic acid bacteria: *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 thermophilus* (CCRC12257), purchased from Culture Collection and Research Center (CCRC), Food Industrial Research Institute, Shin Chu, Taiwan, were tested in this study. All the strains were activated in Lactobacilli MRS (Man-Rogosa-Sharpe) broth (Difco Lab., MI, USA) under aerobic condition for 12 h at 37°C except *Lc. lactis* subsp. *cremoris* which was activated at 26°C. The activated cultures were transferred to the medium containing 120 g l^{-1} skim milk powder (w/v). After incubation for 24 h at the activation temperatures, the cultures were stored at 2°C for further use.

2.2. Medium preparations and incubation conditions

Aliquots of 16 ml medium containing 150 g l^{-1} skim milk powder (Bonlac Food Ltd., Australia) and 20 mg linoleic acid (Sigma Chemical Co., St. Louis, MO) were autoclaved in test tubes at 121°C for 15 min. Solutions of 300 g l^{-1} sucrose, lactose, and fructose and 50 g l^{-1} sodium chloride (Sigma Chemical Co., St. Louis, MO) were filtered through 0.22 μm membrane filters by vacuum-filtration to reduce microorganisms. Four ml of each solution were mixed with 16 ml medium to make a final volume of 20 ml containing 120 g l^{-1} skim milk powder and 60 g l^{-1} sweetener or 10 g l^{-1} sodium chloride. After inoculation with 1% lactic culture (v/v), the medium was then incubated under aerobic conditions without shaking for 24 h at temperatures described above.

2.3. Lipid extraction

Following incubation, the culture medium was mixed with 200 ml chloroform: methanol (2:1, v/v), and 5 mg heptadecanoic acid (Sigma Chemical Co., St. Louis, MO) was added as the internal standard for GC analysis. After homogenizing in a Nihon Seiki universal homogenizer (Tokyo Nihon Seiki Seisakusho Co., Tokyo, Japan) 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, Tokyo, Japan). 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).

2.4. Lipid hydrolysis and preparation of fatty acid methyl esters

The residue was saponified with 2 ml of 1.0 N sodium hydroxide in methanol in a screw-capped test tube at 100°C for 15 min, then cooled at room temperature (25°C) for 10 min. The free fatty acids were methylated with 6 ml of 4% hydrochloric acid in methanol at 60°C for 20 min (Chin et al., 1992). The methylated sample was mixed with 2 ml hexane:water (1:1, v/v) and centrifuged at 500×g for 15 min at 4°C. The organic layer was dried under a stream of nitrogen at room temperature and the residue was redissolved in 1 ml hexane for further quantification of c9,t11-CLA by capillary GC.

2.5. 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×0.53 mm i.d., 1.00 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 1.0 μl. The temperature of the GC oven was programmed at 150°C for 7 min and from 150 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 and the flow rates were 2 and 30 ml/min, respectively. The split ratio was set at 1:50. The c9,t11-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 mg c9,t11-CLA/20 ml medium using heptadecanoic acid as internal standard.

2.6. Statistical analysis

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, 1986) and a significance level of 0.05 was used. Each treatment was performed in three replications.

3. Results and discussion

The retention time of c9,t11-CLA observed in the gas chromatogram of either standard fatty acid methyl esters or fatty acid methyl esters of cultured media was 48.0 min, as shown in Fig. 1. The peak of t10,c12-CLA appearing at 49.1 min in the chromatogram of standard fatty acid methyl esters was very small in the cultured media and the concentration was not calculated.

The effects of addition of three sweeteners on c9,t11-CLA level of skim milk medium after incubation varied with lactic culture (Fig. 2). C9,t11-CLA, produced by *Lactobacillus acidophilus*, was strongly inhibited by the additions of sucrose, lactose, and fructose. The strongest inhibitory effect was observed in the addition of sucrose and the level of CLA produced was 28% lower than the control. This stronger inhibitory effect was probably due to the inverting of sucrose into glucose and fructose, which increases the number of solute molecules (Banwart, 1989) in the skim milk medium and subsequently lowers the CLA isomerizing activity of the cultures. All three sweeteners inhibited the level of CLA produced by *L. delbrueckii* subsp. *bulgaricus*. However, no significant difference ($P > 0.05$) was detected among them. Since some lactic acid bacteria have a tolerance to lactic acid produced by lactose fermentation (Banwart, 1989), CLA inhibitory property was less severe with lactose addition, and only two lactic strains, *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus*, were inhibited. While lactose addition did not lower CLA level in the medium inoculated with *L. delbrueckii* subsp. *lactis* after incubation, both sucrose and fructose additions showed a definite inhibition on CLA produced by this culture, and sucrose was stronger than fructose in inhibition of CLA formation in this treatment. The level of CLA produced in the medium inoculated with *Lactococcus lactis* subsp. *cremoris* was not inhibited by lactose. However, it decreased significantly ($P < 0.05$) with the addition of sucrose, and the reduction rate was 33%. CLA level was also inhibited by sucrose in the treatment of *Lc. lactis* subsp. *lactis*, whereas it was inhibited by fructose in the treatment of *Streptococcus thermophilus*. The addition of fructose showed an increase in CLA level of 22% in the medium with *Lc. lactis* subsp. *cremoris* inoculated, the only treatment with higher CLA level than the control. Sucrose is a non-reducing sugar, whereas both fructose and lactose are reducing

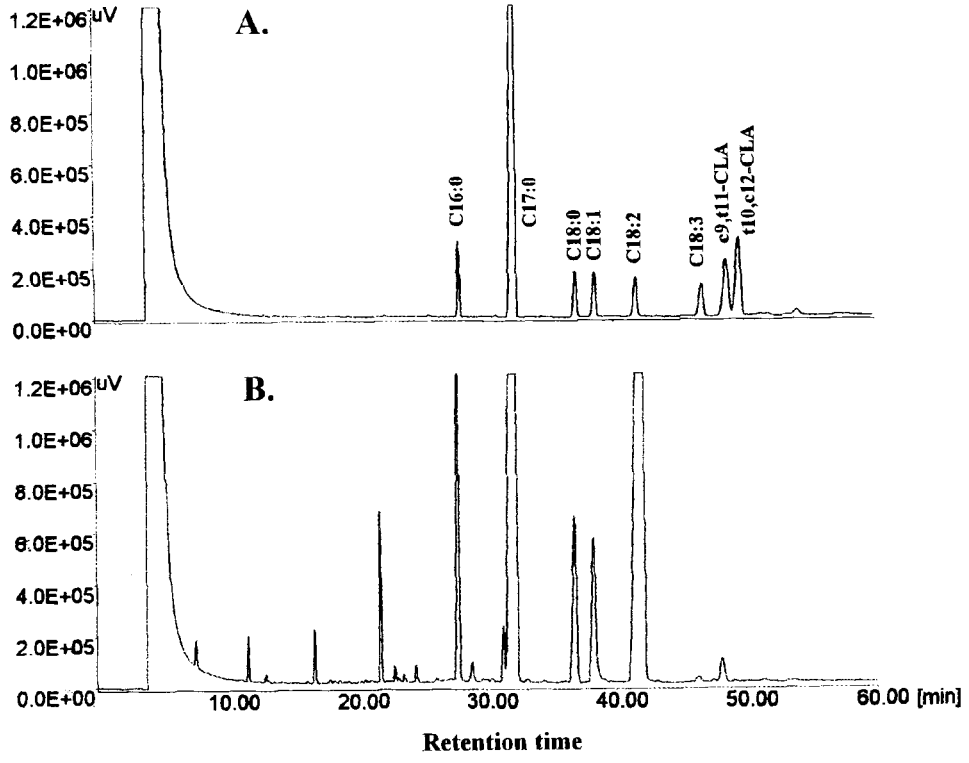

Fig. 1. Gas chromatograms of standard fatty acid methyl esters (A), and fatty acid methyl esters of cultured media (B).

sugars. However, the reducing powers are different among those sweeteners. The inhibitory effect did not decrease with increase in the reducing power, which indicated that the oxidation of linoleic acid into CLA was little affected by the differences in reducing power.

Though c9,t11-CLA produced in the skim milk medium varied with sweetener and lactic culture, *L. acidophilus* which was strongly inhibited by sucrose produced the highest amount of c9,t11-CLA with or without sweetener addition among six cultures due to the highest level of CLA produced in the control. Therefore, inoculation of *L. acidophilus* into 60 g l^{-1} lactose- and fructose-treated skim milk medium, under aerobic condition for 24 h incubation, was most effective in maintaining a high c9,t11-CLA level and is suggested for promoting CLA formation.

The addition of 10 g l^{-1} sodium chloride significantly lowered ($P < 0.05$) the levels of c9,t11-CLA in the skim milk media inoculated with six lactic cultures after 24 h incubation (Fig. 3), and the reduction rates were 13–32%. The highest rate was found in the treatment of *S. thermophilus* (32%), whereas the lowest rate was found in *L. lactis* subsp. *lactis* (13%). Though a 25% reduction in CLA level was observed in 10 g l^{-1} sodium chloride-treated medium inoculated with *L. acidophilus*, the CLA level of 1.58 mg formed in this treatment was the highest among six cultures due to the highest level of CLA produced in the non-sodium chloride-treated control. Therefore, inoculation of *L. acidophilus* into 10 g l^{-1} sodium chloride-treated skim milk medium under aerobic conditions for 24 h incubation was also most effective in maintaining a high c9,t11-CLA level.

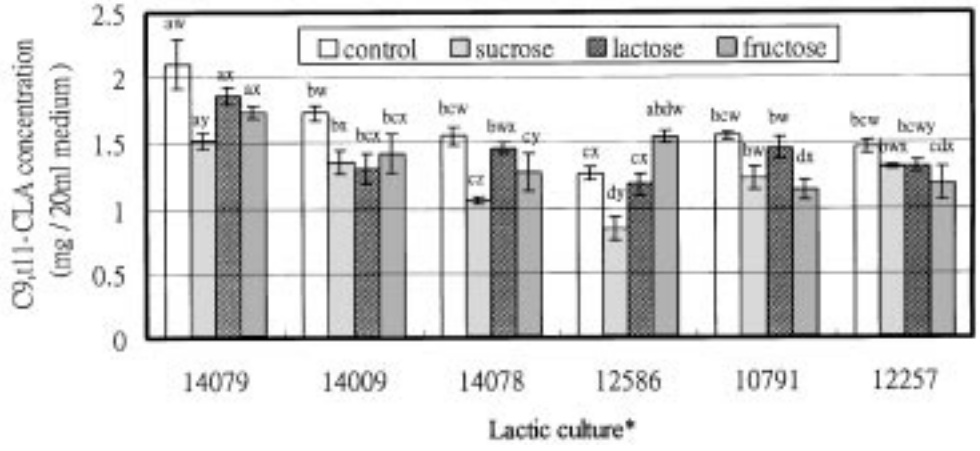

Fig. 2. Effect of lactic cultures and additions of sucrose, lactose and fructose on c9,t11-CLA concentration. * 14079: *Lactobacillus acidophilus*; 14009: *L. delbrueckii* subsp. *bulgaricus*; 14078: *L. delbrueckii* subsp. *lactis*; 12586: *Lactococcus lactis* subsp. *cremoris*; 10791: *Lc. lactis* subsp. *lactis*; 12257: *Streptococcus thermophilus*. [abcd]Different letters show significant differences ($P < 0.05$) as a function of lactic culture for a given sweetener. [wxyz]Different letters show significant differences ($P < 0.05$) in sweeteners for a given lactic culture. [I]Standard error bars are indicated.

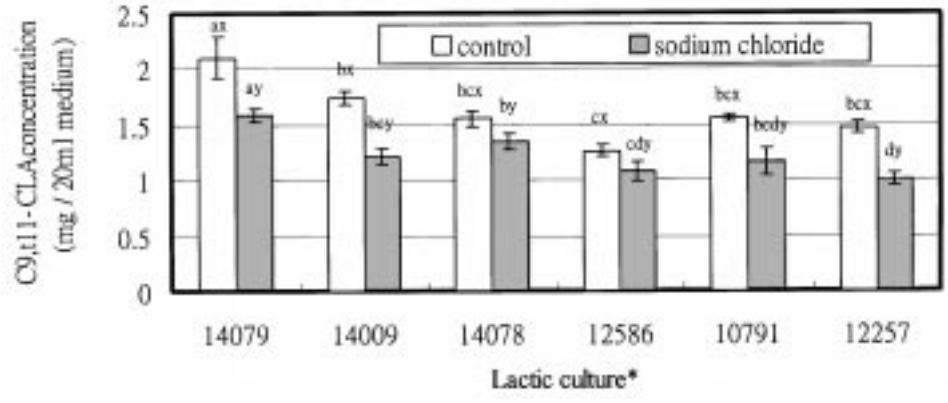

Fig. 3. Effect of lactic cultures and sodium chloride addition on c9,t11-CLA concentration. * 14079: *Lactobacillus acidophilus*; 14009: *L. delbrueckii* subsp. *bulgaricus*; 14078: *L. delbrueckii* subsp. *lactis*; 12586: *Lactococcus lactis* subsp. *cremoris*; 10791: *Lc. lactis* subsp. *lactis*; 12257: *Streptococcus thermophilus*. [abcd]Different letters show significant differences ($P < 0.05$) as a function of lactic culture. [wxyz]Different letters show significant differences ($P < 0.05$) as a function of sodium chloride addition. [I]Standard error bars are indicated.

4. Conclusion

Among six lactic cultures tested, inoculation of *L. acidophilus* into 60 g l^{-1} sweeteners and 10 g l^{-1} sodium chloride-treated skim milk medium under aerobic conditions for 24 h incubation was most effective in promoting c9,t11-CLA formation. The additions of lactose and fructose are suggested for use in maintaining a high CLA level.

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