Production of Free Conjugated Linoleic Acid by *Lactobacillus acidophilus* and *Lactobacillus casei* of Human Intestinal Origin¹

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ABSTRACT

A gas chromatographic procedure was used for analysis of conjugated linoleic acid (CLA) isomers *cis*-9, *trans*-11-octadecadienoic; *trans*-10, *cis*-12 octadecadienoic; and *trans*-9, *trans*-11-octadecadienoic (c9t11, t10c12, t9t11) produced by lactobacilli. Four different cultures, two strains each of *Lactobacillus acidophilus* and *Lactobacillus casei* were tested for their ability to produce CLA from free linoleic acid in MRS broth supplemented with linoleic acid. Different concentrations of linoleic acid (0, 0.05, 0.1, 0.2 and 0.5 mg/ml) were added to MRS broth, inoculated with the lactobacilli, and incubated at 37°C. Viable counts and amounts of individual isomers of CLA (c9t11, t10c12, t9t11) were measured at 0, 24, 48, and 72 h. All the cultures were able to produce free CLA in media supplemented with linoleic acid. Maximum production of CLA (80.14 to 131.63 µg/ml) was observed at 24 h of incubation in broth containing 0.02% of free linoleic acid. No significant ($P > 0.05$) increases in total CLA levels were observed after 24 h of incubation. The ability of the cultures to produce CLA in skim milk supplemented with 0.02% free linoleic acid also was studied. In this medium, the total amounts of free CLA after 24 h of incubation ranged from 54.31 to 116.53 µg/ml. The use of lactic acid bacteria able to form free CLA in cultured dairy products may have potential health or nutritional benefits. Free CLA in the products likely would be more readily available for absorption from the digestive tract than if it were incorporated into the cells of the starter culture.

(Key words: conjugated linoleic acid, gas liquid chromatography, lactobacillus, probiotics)

Abbreviation key: CLA = conjugated linoleic acid, c-9,t-11 = *cis*-9,*trans*-11, FAME = fatty acid methyl ester, NFM = nonfat milk, SM = skim milk, t-10,c12 = *trans*-10,*cis*-12, t9,t11 = *trans*-9,*trans*-11.

INTRODUCTION

Conjugated linoleic acid (CLA) is a generic name for a mixture of isomers of linoleic acid with conjugated double bonds. The double bonds can be in several possible positions either as *cis* or *trans* isomers. Double bonds of CLA are mainly found at positions 9 and 11, or 10 and 12 (Ha et al., 1987), while isomers having double bonds at other positions also have been reported (Christie et al., 1997). Several scientists, based on studies primarily in animal models, have suggested that CLA has potential health or nutritional effects, including anticarcinogenic activity (Ha et al., 1990), antiatherogenic activity (Lee et al., 1994; Pariza et al., 1996), the ability to reduce the catabolic effects of immune stimulation (Cook et al., 1993), and the ability to reduce body fat (Pariza et al., 1996). Of the individual isomers of CLA, *cis*-9, *trans*-11-octadecadienoic acid has been implicated as the most biologically active because it is the predominant isomer incorporated into the phospholipids of cell membranes (Ip et al., 1994). The *cis*-9, *trans*-11 CLA isomer also is the predominant isomer found in the diet.

Food products from ruminants, particularly dairy products, are the major dietary source of CLA for humans. They are intermediates in the biohydrogenation of linoleic acid, and it is generally accepted that CLA in ruminants originate from the incomplete biohydrogenation of the unsaturated fatty acid linoleic acid by rumen bacteria (Kelly et al., 1998). However, it has been demonstrated that cows also can synthesize CLA from *trans*-11-octadecenoic acid, another intermediate in the rumen biohydrogenation process (Kim and Liu, 2000).

Information is lacking on CLA levels required by humans, but it is estimated from animal studies that a

Received July 30, 2002.

Accepted November 5, 2002.

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¹Approved for publication by the Director, Oklahoma Agricultural Experiment Station. This project was supported under Project H-2293 and Sitlington Endowment Funds.

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daily intake of 3 g/d may be effective for cancer prevention (Ip et al., 1994). The average estimated CLA intake has been reported to range from 0.35 to 1 g/d (Chin et al., 1992; Fritsche et al., 1998). Dietary sources of CLA include milk fat, natural and processed cheeses, meat products, and plant oil (Ha et al., 1989; Shanta et al., 1992). Animal sources are richer in CLA than plant sources, and in general, foods from ruminants contain more CLA than foods from non ruminants. Considerable research has been conducted on the CLA content and isomer distribution in cow's milk. A number of factors have been shown to influence CLA concentration in bovine milk fat, including stage of lactation (Stanton et al., 1997), dietary restriction (Ip et al., 1991), and feed allowance (Stanton et al., 1997; Lawless et al., 1998). Among the different isomers of CLA, *cis*-9, *trans*-11-octadecadienoic represents more than 90% of the total CLA in milk fat. The level of CLA in raw milk fat varies from 0.2 to 2% (wt/wt). The wide variation of CLA content in dairy products also may be due to processing parameters such as different heat treatment procedures during pasteurization. The CLA content in yogurt or cheese can be increased by action of the starter cultures (Lin et al., 1999). The finding of the production of CLA by food starter cultures opens interesting perspectives for the future in producing fermented products enriched in CLA.

Jiang et al. (1998) reported the formation of CLA from linoleic acid by *Propionibacterium freudenreichii*. Their study also included seven cultures of lactobacilli, none of which were found to form CLA. Lin et al. (1999), Lin (2000), and Ogawa et al. (2001) reported the production of CLA from free linoleic acid by *Lactobacillus acidophilus*. Each study included one strain, and in all cases the entire culture (i.e., growth medium plus cells) was assayed for CLA. Kishino et al. (2002) found that washed cells of *Lactobacillus plantarum* formed high levels of CLA from free linoleic acid upon extended incubation. They indicated most of the CLA for this culture was associated with the bacterial cells.

The objective of this study was to evaluate the capacity of *L. acidophilus* and *L. casei* of human intestinal origin to produce free CLA in MRS broth and in skim milk.

MATERIALS AND METHODS

Cultures, Media, and Growth Conditions

Two strains of *L. acidophilus* (L1 and O16) and two of *L. casei* (E5 and E10) isolated from human intestinal sources were used in this study (Buck and Gilliland, 1994; Brashears et al., 1998). The strains were obtained from the stock culture collection of the Food Microbiology Laboratory at the Food and Agricultural Product

Research and Technology Center, Oklahoma State University. The cultures were subcultured at least three times in MRS broth or 10% nonfat milk (NFM) immediately before experimentation using 1% inocula and an 18-h incubation at 37°C. The cultures were held at 2°C between subcultures.

Production of CLA in Broth

Lactobacilli MRS broth (Difco Laboratories, Detroit, MI), supplemented with different concentrations of linoleic acid (0, 0.05, 0.1, 0.2 and 0.5 mg/ml), was used in this study. The linoleic acid (Sigma Chemical Co., St. Louis, MO) was added as aqueous solution in 1% (vol/vol) of Tween 80. The broth medium was inoculated at 1% and incubated at 37°C for 0, 24, 48, and 72 h. After each incubation period, a tube of culture from each concentration of linoleic acid was placed in an ice-water bath. The pH was measured and viable count was determined by plating appropriate dilutions on MRS agar. (The plates were incubated 48 h at 37°C.) Then samples were centrifuged at $23,500 \times g$ for 10 min at 5°C. The pellet was discarded, and the supernatant was recovered for CLA analysis. The complete experiment, including all cultures, was replicated three times on different days.

Production of CLA in Milk

NFM was reconstituted at 10% (wt/vol) in deionized water and supplemented with 0.2 mg/ml linoleic acid. The milk was pasteurized at 80°C for 30 min and then inoculated at 1% and incubated at 37°C for 24 h. After incubation, viable count was determined by plating in MRS agar. Then samples were centrifuged at $23,500 \times g$ for 10 min at 5°C. The pellet was discarded, and supernatant (whey) was kept for CLA analysis. The complete experiment involving all cultures was replicated three times on different days.

Lipid Extraction

Six milliliters of spent broth or whey was mixed with 60 μ l of heptadecanoic (Sigma Chemical Co., St. Louis, MO), which served as internal standard (64.4 mg of heptadecadienoic/10 ml of hexane), and 12 ml of isopropanol and shaken vigorously; 9 ml of hexane were then added, and the mixture was shaken 3 min before being centrifuged at $1900 \times g$ for 5 min at 5°C. The upper layer was then collected by aspiration and filtered through sodium sulphate. After filtration, the sodium sulfate was washed with an additional 7 ml of hexane. The lipid fraction of the sample was collected in a 25-ml, pear-shaped flask and dried under nitrogen gas at 70°C

Figure 1. Production of total conjugated linoleic acid (CLA) by *Lactobacillus acidophilus* (L1) in MRS broth supplemented with 0% (x), 0.005% (◆), 0.01% (■), 0.02% (▲) and 0.05% (●) linoleic acid after 0, 24, 48 and 72 h of incubation at 37°C.

in a Zymark Turbo Vap LV evaporator (Zymark Corp., Hopkinton, MA). The dried sample was redissolved with 500 μ l hexane and transferred to a microtube. Methanolic sodium hydroxide (1 N) 100 μ l, was added to the tube containing the extracted lipid fraction, and the mixture was vortexed for 1 min, then held at 70°C in a water bath for 15 min. The free fatty acids in the mixture were methylated by adding 200 μ l of 14% boron trifluoride in methanol (Sigma Chemical Co. St. Louis, MO) at room temperature for 30 min. Then 0.2 ml of hexane and 0.1 ml of water were added, samples were vortexed thoroughly, and centrifuged at $200 \times g$ for 3 min. The organic layer was recovered and stored in a vial at -20°C until being analyzed by GLC. Uninoculated broth or whey (collected from milk acidified to pH 4.5 with lactic acid) were extracted in a like manner to provide background levels of CLA isomers that might be present.

Gas Chromatographic Analysis

Methyl esters of CLA were analyzed as described by Alonso et al (2000). The specific equipment and materials included a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector. Analyses were performed with a CP Sil 88 column (100 m $\times$ 0.25 mm i.d) containing 100% cyanopropyl siloxane stationary phase, 0.20- μ m film thickness (Chrompack, Varian, Inc., CA). The conditions used were 190°C isothermal temperature, 250°C injector temperature, and 250°C detector temperature. The split ratio was 1:50, and the carrier gas was helium with a head pressure of 28 psi (1.97 kg/cm²). The injection volume was 1 μ l, and the CLA peaks were identified by comparison with

the retention times of the reference standards (Matreya, Inc., State College, PA). With the Millennium 32 Workstation SS (500) software (Waters Corp., Milford, MA), the amounts of CLA isomers in each sample were calculated (μ g CLA/ml) based on the areas of individual CLA peaks.

Statistical Analysis

Data were subjected to analysis of variance for a one-factor completely randomized design and Duncan's multiple range tests (SAS User's Guide, 1995) procedure using a significance level of 0.05. Concentration of linoleic acid and incubation time were the main effects studied.

RESULTS AND DISCUSSION

Analyses of CLA

The GLC technique using a long capillary column has been successful in quantitating total amount of CLA as FAME. This method separates with a good resolution, *cis/trans* and *trans/trans* isomers of CLA based on the chain length and number of double bonds. The advantages of methylating CLA are that the FAME derivatives allow for GLC identification and quantification of individual CLA isomers. The FAME, including the CLA, can be quantitated by GLC by adding an internal standard such as heptadecanoic acid (C17:0) into the lipid mixture before methylation (Yurawecz et al., 1996). The methylation procedure chosen will depend on the type and chemical composition of material to be methylated. In our study, we used a two-stage process for methylation, first an alkaline-catalyzed hydrolysis using NaOH/methanol for esterified CLA isomers, followed by boron trifluoride/methanol catalyzed methylation of the free CLA isomers. This method has an advantage over other methods for methylating CLA isomers in that it does not isomerize conjugated double bonds (Koritala and Rohwedder, 1972; Yurawecz, 1997). The retention times for the FAME of the three isomers of CLA (c9t11, t10c12, and t9t11) detected in this study were 43.9, 45.1, and 48.0 min, respectively, which are indications of the separation obtained. Under conditions used in our study, this method produced a good repeatability as indicated by low coefficients of variation (CV = 4.62 to 8.20). Sterile unsupplemented MRS broth contained 4 to 9 μ g/ml of the three CLA isomers. Known amounts of the individual CLA isomers were added to MRS broth and assayed using heptadecanoic acid as internal standard. Recovery of CLA isomers was in the range of 87.22 to 89.57%. The recovery values found in this study were slightly higher than those reported by Lin et al. (1999) using a KOH/ethanol and

Table 1. Production of individual isomers and total conjugated linoleic acid (CLA) by *Lactobacillus acidophilus* and *Lactobacillus casei* ssp. *casei* in MRS broth supplemented with 0.02% of linoleic acid after 24 h of incubation at 37°C.

Culture	pH	Log ₁₀ cfu/ml ²	Concentration µg/ml ¹			
			c9t11	t10 c12	t9t11	Total CLA
<i>L. acidophilus</i> L1	3.70	9.17 ± 0.04	115.1 ± 3.36	13.23 ± 2.20	7.3 ± 0.56	131.63 ± 5.82 ^a
<i>L. acidophilus</i> O16	3.70	9.49 ± 0.03	54.77 ± 0.04	5.7 ± 0.56	0.39 ± 0.06	60.86 ± 0.30 ^b
<i>L. casei</i> E5	3.70	9.21 ± 0.16	93.9 ± 2.25	14.14 ± 0.65	3.14 ± 0.76	111.18 ± 2.32 ^c
<i>L. casei</i> E10	3.70	9.51 ± 0.06	70.66 ± 2.39	7.03 ± 0.45	2.45 ± 0.35	80.14 ± 2.31 ^d

^{a,b,c,d}Means in a column with different letter are significantly different ($P > 0.05$).

¹Each value is a mean from three trials (the µg/ml of each isomer was corrected for the small amounts present in uninoculated broth); values in parentheses are standard deviations.

²Log₁₀ colony forming units per ml on MRS agar at end of incubation.

HCL as methylation reagent. They found 83% recovery of CLA and coefficient of variation CV = 6.6 for repeatability using a capillary GLC analysis. However, Jiang et al. (1996) reported the recovery of the CLA for the analytical procedure was 89.4%, and the coefficient of variation for the overall reproducibility of the CLA quantification method was 3.6%, using KOH/ethanol and HCL as reagent for methylation with HPLC-GLC analysis. Therefore, it was concluded that under the given conditions, the GLC method used in our study does not appreciably underestimate for individual CLA isomers (free plus esterified).

CLA Production

Two cultures each of *Lactobacillus acidophilus* (O16, L1) and *Lactobacillus casei* (E5, E10) were able to convert free linoleic acid to CLA. Of the possible isomers of CLA, c9t11, t10c12, and t10t12 were formed, of which c9t11 comprised more than 90% of the total CLA. No significant increases ($P > 0.05$) in total amounts of CLA were formed for any of the four cultures as incubation time increased beyond 24 h in broth containing added linoleic acid. Figure 1 shows the production of total CLA by the *L. acidophilus* L1 in MRS broth supplemented

with different amounts of free linoleic acid during 24, 48, and 72 h of incubation time at 37°C. The highest level of CLA production was observed after 24 h of incubation in the broth supplemented with 0.02% linoleic acid. Similar patterns of formation of CLA were obtained with the other cultures included in this study. It appeared that addition of greater than 0.02% linoleic acid exerted an inhibitory effect on CLA production in that less was produced in the presence of 0.05% than in 0.02% linoleic acid, 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). Table 1 shows the production (amounts for each were corrected for background levels in uninoculated broth) of the individual isomers c9t11, t10c12, t9t11, and total CLA in MRS broth supplemented with 0.02% linoleic acid after 24 h for the four different cultures of lactobacilli studied. The highest amount of c9t11, t10c12, and t9t11 was produced by *L. acidophilus* L1 with values of 115.1, 13.23, and 7.3 µg/ml, respectively, and 131.63 µg/ml for total CLA. The lowest amounts were produced by *L. acidophilus* O16 with values for c9t11, t10c12, and t9t11 of 54.77, 5.7, and 0.39 µg/ml, respectively, and 60.86 µg/ml for the total CLA. A similar pattern of

Table 2. Production of individual isomers and total conjugated linoleic acid (CLA) by *Lactobacillus acidophilus* and *Lactobacillus casei* ssp. *casei* in skim milk supplemented with 0.02% of linoleic acid after 24 h of incubation at 37°C.

Culture	pH	Log ₁₀ cfu/ml ²	Concentration µg/ml ¹			
			C9t11	t10 c12	t9t11	Total CLA
<i>L. acidophilus</i> L1	5.19	7.89 ± 0.44	100.33 ± 3.22	9.97 ± 0.49	6.23 ± 0.56	116.53 ± 3.98 ^a
<i>L. acidophilus</i> O16	5.89	8.35 ± 0.08	45.30 ± 4.20	7.83 ± 0.42	1.02 ± 0.40	54.31 ± 4.13 ^b
<i>L. casei</i> E5	5.87	8.24 ± 0.02	85.03 ± 4.72	11.97 ± 0.15	2.90 ± 0.17	99.63 ± 4.48 ^c
<i>L. casei</i> E10	6.23	7.69 ± 0.06	61.00 ± 3.06	8.46 ± 0.57	1.90 ± 0.50	71.36 ± 2.75 ^d

^{a,b,c,d}Means in a column with different letter are significantly different ($P > 0.05$).

¹Each value is a mean from three trials (the µg/ml of each isomer was corrected for the small amounts present from acidified skim milk); values in parentheses are standard deviations.

²Log₁₀ colony forming units per ml in the cultured milk on MRS agar at end of incubation.

Figure 2. Production of total conjugated linoleic acid (CLA) by *Lactobacillus acidophilus* L1 (▲) and *Lactobacillus casei* sp. *casei* E5 (■) (A) and viable cell count (B) in MRS broth supplemented with 0.02% linoleic acid after 0, 6, 12, 18 and 24 h of incubation at 37°C.

formation of isomers of CLA was found by Jiang et al. (1998) using different cultures of *Propionibacterium* sp. This author found that the highest production for the isomers (c9t11 plus t9c11) was 246.4 μg/ml when 750 μg/ml free linoleic acid was added to the media. Lin et al. (1999) found that a culture of *L. acidophilus* produced 105.5 μg/ml of c9t11 in sterilized skim milk supplemented with 1000 μg/ml of free linoleic acid after 24 h of incubation at 37°C.

To determine the stage of growth at which the CLA was produced, we analyzed samples of MRS broth containing 0.02% linoleic acid inoculated with cultures *L. acidophilus* L1 and *L. casei* E5 after 0, 6, 12, 18, and 24 h of incubation at 37°C (Figure 2). Both cultures exhibited increases in CLA beginning at 6 h. The CLA production increased with each increment of incubation time throughout the 24 h. Growth of both cultures was similar during the 24-h incubation time (Figure 2). Most

CLA was formed after the cultures reached the stationary phase. These results are similar to those found by Lin et al. (1999) when they studied the production of CLA and growth of lactic cultures in skim milk supplemented with free linoleic acid.

The production of CLA by the four cultures of lactobacilli also was measured in skim milk (SM) supplemented with 0.02% linoleic acid. The variations among the cultures with respect to concentrations of the different isomers (c9t11, t10c12, and t9t11) produced in SM (Table 2) were similar to those formed in MRS broth. In skim milk *L. acidophilus* L1 again produced more CLA than the other cultures (116.53 μg/ml), and *L. acidophilus* O16 produced the lowest amount (54.31 μg/ml). The apparent differences in the amounts of CLA produced in MRS and skim milk supplemented with the same concentration of free linoleic acid could be due to the different amounts of growth and/or pH values observed in both media.

In previous studies (Jiang et al., 1998; Lin, 2000; Ogawa et al., 2001; Kishino et al., 2002) on CLA production by starter culture bacteria, the preparation of samples for analyses did not involve removal of the bacterial cells. Thus, there was no evidence presented showing that the CLA was not all contained in the bacterial cells. On the contrary, in our study, we removed the cells and assayed only the free CLA in the spent growth media. While this may not provide data for the total amounts produced (i.e., to include any associated with cells), it may be more important with respect to CLA in the products that would be available for absorption from the digestive tract when the product is consumed. Any CLA incorporated in the cells of the starter cultures may not be readily available in the digestive tract.

ACKNOWLEDGMENTS

L. Alonso thanks the Ministry of Education of Spain for their support during his time in the Food and Agricultural Product Research and Technology Center at Oklahoma State University.

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