

Screening of Conjugated Linoleic Acid Producing Lactic Acid Bacteria from Fecal Samples of Healthy Babies

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ABSTRACT : This study was carried out to obtain conjugated linoleic acid (CLA) producing lactic acid bacteria for further study on the enzymes related to the production of CLA which has gained considerable attention and on the development as a probiotic culture. Total 34 lactic acid bacteria were isolated from 19 feces samples of healthy babies. CLA forming ability was measured spectrophotometrically by the modification of linoleate 12-cis, 11-trans-isomerase activity measuring method, and CLA of the cultures were extracted, methylated, and examined by HPLC analysis. CLA methyl ester of only one culture showing the highest value of CLA forming ability could be detected by HPLC analysis. The culture was found to be Gram positive, rods and catalase negative. It grows at 45°C but not at 15°C, and was identified to be *Lactobacillus fermentum* on the basis of the biochemical characteristics and the utilization of substrates. These results provide an efficient experimental method to screen CLA producing lactic acid bacteria. (*Asian-Aust. J. Anim. Sci.* 2002, Vol 15, No. 7: 1031-1035)

Key Words : Conjugated Linoleic Acid, Lactic Acid Bacteria, *Lactobacillus Fermentum*

INTRODUCTION

Conjugated linoleic acid (CLA) is a general term for positional and geometrical isomers of linoleic acid (LA). *cis*-9, *cis*-12 octadecadienoic acid, in which the double bonds are conjugated instead of being in the typical methylene interrupted configuration. CLA has gained considerable attention because of its anticarcinogenic (Ha et al., 1987; Ip et al., 1991; Pariza and Hargraves, 1985), antioxidative (Parodi, 1994), cholesterol-depressing (Huang et al., 1994), and growth-promoting (Chin et al., 1994) properties.

Of the individual isomers of CLA, *cis*-9, *trans*-11-octadecadienoic acid (c9, t11-18:2) has been suggested to be the most important in terms of biological activity because it is the major isomer. CLA can be produced by alkaline isomerization, but there are as many as 16 isomers which are not fully characterized (Nichols et al., 1951; Sehat et al., 1998). Therefore, there is increasing interest in CLA producing microbes and enzymes.

CLA has been shown to be produced from polyunsaturated fat by certain rumen microorganisms such as *Butyrivibrio* species (Forgety et al., 1988). More recently, it was reported that *Propionibacterium freudenreichii*, commonly used as a dairy starter culture, was able to produce CLA from free linoleic acid (Jiang et al., 1998). Pariza and Yang (2000) screened 45 cultures from whole intestinal tract of two conventional rats by measuring the conversion of linoleic acid to CLA with HPLC and GC, and

developed the method of producing CLA with the selected strain in Tris-HCl buffer. However, Ogawa et al. (2001) insisted after 4 days of reaction with washed cells of *L. acidophilus* transformed more than 95% of the added linoleic acid into CLA, and cells themselves could be used as sources of CLA. Because of its beneficial physiological effects, there is a potential demand for CLA and CLA producing lactic acid bacteria as a starter culture for making milk products, a source of enzyme systems for the production of CLA, or a probiotic culture.

To obtain a lactic culture which can produce CLA, lactic acid bacteria were isolated from feces of healthy babies, and were compared CLA forming ability by the modification of the method of linoleate 12-*cis*, 11-*trans* isomerase activity test. The experiment described here sought to provide screening method for CLA producing lactic acid bacteria.

MATERIALS AND METHODS

Isolation of lactic acid bacteria from the feces of healthy babies

Isolation of lactic acid bacteria was carried out by inoculating one loop of the feces samples into De Man-Rogosa-Sharpe (De Man et al., 1960) broth (Difco Laboratories, USA) containing 0.02% sodium azide. After incubation at 37°C for 1 day, one loop of culture was streaked on MRS agar plate containing 0.02% NaN₃, and then colonies were picked up and grown on MRS agar slant.

Screening of CLA producing lactic acid bacteria

After subculturing twice in MRS broth (pH 6.0), the lactic acid bacteria were cultured in 5 mL MRS broth containing 9,12 linoleic acid (5 mg/mL) (Sigma Chemicals) with 1% between 80. The cells of a 4 mL culture were

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Received December 20, 2001; Accepted March 16, 2002

harvested by centrifugation (3,000 g for 10 min) and washed twice with 0.1 M potassium phosphate buffer (pH 7.0). After addition of 1.3 mL buffer, the cell suspension was disrupted by 3 min exposure to sonic oscillation. The disrupted cell mixture was centrifuged at 10,000 g for 15 min, and used as crude enzyme solution. CLA forming ability was assayed spectrophotometrically (Shimadzu UV1601, Japan) by measuring the highest appearance of the conjugated diene system at 233 nm according to the method described in Kepler and Tove (1969). An optically clear solution of 24 μ M linoleic acid is prepared by mixing 0.1 mL of the linoleic acid substrate solution with 2.7 mL of phosphate buffer and 0.2 mL of 1,3-propanediol in a silica cuvette. After preincubation at 35°C for 5 min, the reaction is initiated by the addition of 0.01 mL of enzyme solution, and the highest optical density at 233 nm was recorded. The HPLC analysis of CLA was based on the method described by Chin et al. (1992), sample preparation by Jiang et al. (1998). A 1 mL culture and 2 mL isopropanol were mixed vigorously; 1.5 mL of hexane were then added and the mixture was shaken for 3 min before being centrifuged at 2,520 g for 5 min at room temperature. The upper layer was then collected. The lower layer was extracted with 1.5 mL of hexane, twice, and the supernatant fluids were pooled with previous hexane layer in a screw-capped test tube. The extracted lipids were hydrolysed to free fatty acid by adding 2 mL of 2 mol KOH in ethanol and heating at 50°C for 30 min. Methyl esters were prepared with 2 mL of 3 N hydrochloric acid in methanol at 60°C for 20 min. The methylated sample was mixed with 1 mL water and 1 mL

hexane, and centrifuged at 2,520 g for 15 min. The organic layer was dried under a stream of nitrogen at room temperature and the residue was redissolved in 1 mL methanol. The CLA methyl ester in methanol (50 μ L) was directly injected into HPLC (HP Chemstation). Separations were performed on a reversed-phase analytical column (Eclipse XDB-C18, 250 \times 4.6(i.d.), ZORBAX). The isocratic mobile phase (95% acetonitrile:5% water) was delivered at a flow rate of 1.2 mL per minute. Eluent were monitored at 245 nm.

Identification of the CLA producing lactic acid bacteria

After a microscopic (Amarel CliniGold, USA) examination of the isolates, 49 kinds of substrates were tested for utilization by using API 50CHL kit (API bioMerieux, France). The colony on MRS agar was picked and transferred to filter paper, and fixed with 8% paraformaldehyde and 3% glutaraldehyde in cacodylate buffer overnight. After washing and dehydration, sample was coated with gold and observed under a Philips SEM (XL30CP). Genus and species of the lactic acid bacteria was determined by the API LAB PLUS system (BioMerieux, France).

RESULTS AND DISCUSSIONS

34 lactic acid bacteria were isolated from 19 feces samples, and CLA forming ability of them are presented in figure 1. The value was based on the linoleate 12-*cis*, 11-*trans* isomerase activity, and was ranged from 0.02 to 0.24.

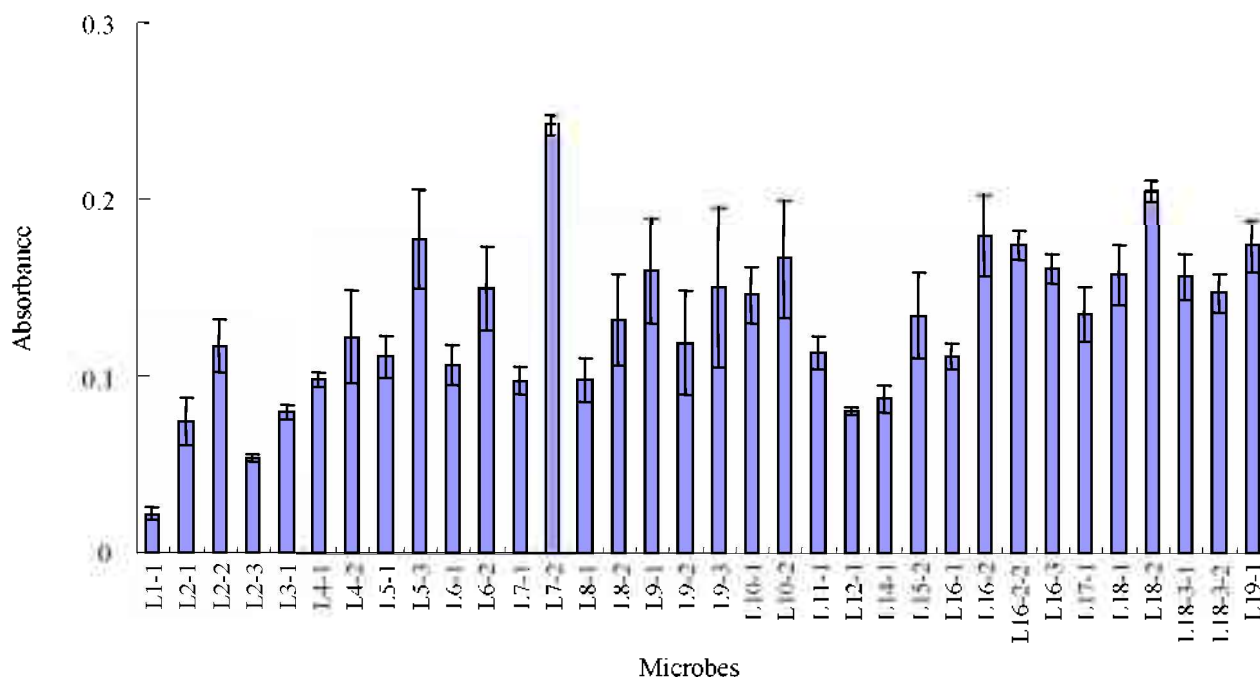


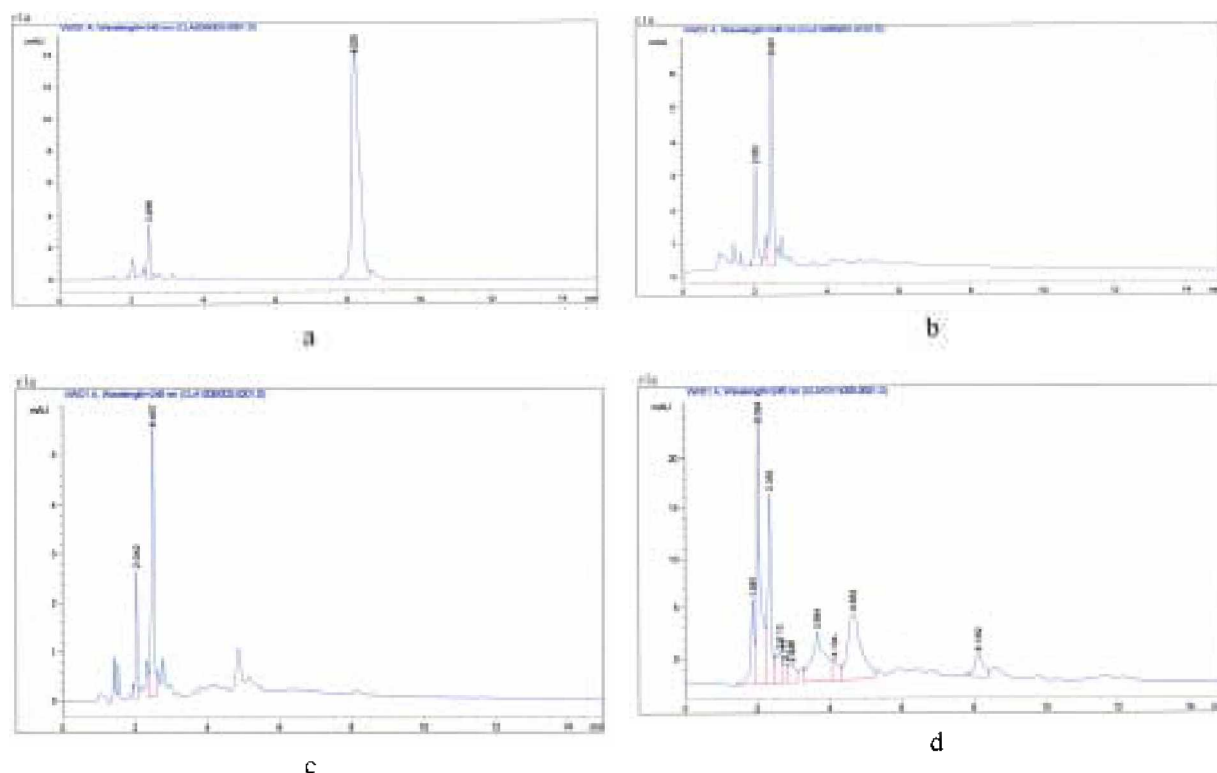
Figure 1. Absorbance at 233nm after the addition of disrupted cell into phosphate buffer containing linoleic acid.

According to Kepler and Tove (1969), the reaction was very rapid and reached equilibrium in a few minutes. However, the value showed rapid or slow decrease according to microbes. It was thought an undesired reductase activity of the microbes might convert CLA to other compounds. A unit of linoleate 12-cis, 11-trans isomerase enzyme is the amount that catalyzes the isomerization of 1 millimicromole of linoleic acid per minute, equivalent to a change in optical density of 0.008 per minute in a 1 cm cuvette (Kepler and Tove, 1969). The yield of enzyme from twelve liters of *B. fibrisolvens* culture was about 5,500 units with a specific activity of about 20 units per milligram of protein (Kepler and Tove, 1969). The enzyme needs to be purified and characterized for cloning and further utilization.

Only the CLA methyl ester of L7-2 culture which showed the highest value in figure 1 was detected by HPLC (figure 2), and others was not. Consistent retention time of CLA methyl ester cannot be obtained by the HPLC analysis condition described in Chin et al. (1992). It may be caused from difference of column. Mobile phase (85% acetonitrile:15% water) was changed to 95% acetonitrile:5% water with consistent column temperature (40°C), 2 mL of flow rate was changed to 1.2 mL because of high pressure. The retention time of CLA methyl ester was about 8.2 under the condition. The L7-2 culture was found to be Gram positive, rods (figure 3), and catalase negative, and grown at 45°C but not at 15°C. It was identified to be *Lactobacillus*

fermentum (96.1%, API LAB PLUS) on the basis of the biochemical characteristics and the utilization of substrates (table 1). There are few reports on CLA producing lactic acid bacteria. Pariza and Yang (2000) isolated *L. reuteri* (ATCC 55739) from conventional rats after being fed 5% linoleic acid for 4 to 8 weeks. *L. reuteri* is related to *L. fermentum*, and can be distinguished by G+C content.

Pariza and Yang (2000) developed a method for forming CLA by the grown cells in 0.1 M tris-HCl buffer (pH 8.5). In addition to Ogawa et al. (2001) insisted that after 4 days of reaction with washed cells of *L. acidophilus* transformed more than 95% of the added linoleic acid (5 mg/mL) into CLA in 100 mM potassium phosphate buffer (pH 6.5) with microaerobic condition, and cells could be used as sources of CLA because it was accumulated as intracellular or cell-associated lipids in free form. CLA production by microbes may more expensive but more safe than by alkali isomerization. Lactic acid bacteria is more easy to grow than *B. fibrisolvens* and bifidobacteria (Yoon, 2001) which are strictly anaerobes. CLA producing lactic acid bacteria can be useful as a starter culture for making milk products, a source of enzyme systems for the production of CLA, or a probiotic culture. However, one of the most important requirement for the use as a probiotic culture is human origin, since some effects can be only species specific (Brassart and Schiffrin, 2000).



A: Standard (CLA methyl ester) B: Blank (MRS broth) C: Blank (MRS broth+linoleic acid) D: 7-2 culture

Figure 2. HPLC analysis of CLA methyl ester.

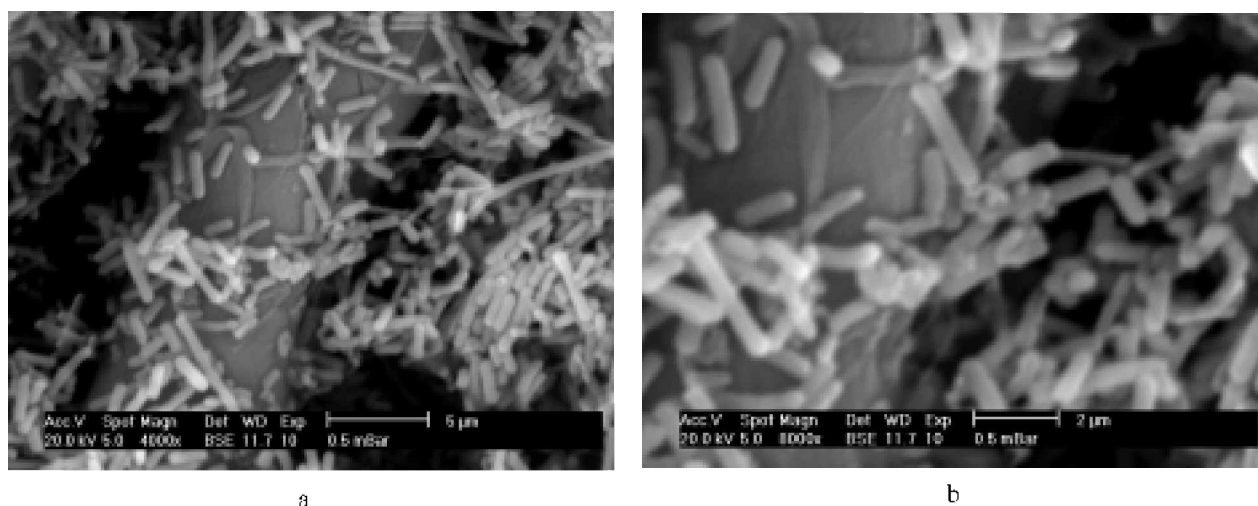


Figure 3. SEM micrograph of the CLA producing culture (A: $\times 4,000$, B: $\times 8,000$).

Table 1. Utilization of carbohydrate substrates

Glycerol	- Mannitol	- D-Raffinose	-
Erythritol	- Sorbitol	- Amidon	+
D-Arabinose	- α -Methyl-D-Mannoside	- Glycogen	-
L-Arabinose	- α -Methyl-D-Glucoside	- Xylitol	-
Ribose	- N acetyl glucosamin	- β -Gentiobiose	-
D-Xylose	- Amygdalin	- D-Turanose	-
L-Xylose	- Arbutin	- D-Lyxose	-
Adonitol	- Esculin	- D-Tagatose	-
β -Methyl-D-xyloside	- Salicin	- D-Fucose	-
Galactose	+ Cellobiose	- L-Fucose	-
D-Glucose	+ Maltose	+ D-Arabitol	-
D-Fructose	+ Lactose	- L-Arabitol	-
D-Mannose	- Melibiose	+ Gluconate	-
L-Sorbose	- Saccharose	+ 2-keto-gluconate	-
Rhamnose	- Trehalose	- 5-keto-gluconate	-
Dulcitol	- Inulin	-	-
Inositol	- Melezitose	-	-

This study demonstrated that screening method of CLA producing lactic acid bacteria from feces samples. Although the selected culture needs to be measured CLA production in tris-HCl or potassium phosphate buffer, and G+C content, it can be applicable as a CLA producing probiotic culture. The pathway and enzyme systems related to CLA production need to be studied further.

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