Utilization of Monolinolein as a Substrate for Conjugated Linoleic Acid Production by *Bifidobacterium breve* LMC 520 of Human Neonatal Origin

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This study was designed to isolate bifidobacteria from human intestines that efficiently converts monolinolein, a monoglyceride form of linoleic acid, into conjugated linoleic acid (CLA), as well as to optimize culture conditions for improving CLA production during milk fermentation. Among 150 screened neonatal bifidobacteria, *Bifidobacterium breve* LMC 520 showed the highest CLA-producing ability and was tested with different types of fat substrates at various concentrations to determine the optimal conditions for CLA production. Monolinolein was tested as a substrate for CLA production. The incubation time optimized for CLA production was 24 h, and CLA production was proportionally increased with monolinolein concentration. The incubation of LMC 520 with commercial starter strains caused minimal reduction in CLA production. Our results demonstrate that the CLA-producing ability of *B. breve* LMC 520 could offer beneficial effects when utilized as a starter culture for the development of functional dairy products.

KEYWORDS: *Bifidobacterium breve*; conjugated linoleic acid; milk fermentation; monolinolein; starter

INTRODUCTION

Conjugated linoleic acid (CLA) is comprised of positional and geometric isomers of octadecadienoic acid ($C_{18:2}$) with conjugated double bonds and has been the focus of considerable research targeting its functional properties such as antioxidation, cancer inhibition, cholesterol reduction, growth promotion, etc. (*1−4*). Dairy products are major dietary sources of CLA, the formation of which mainly occurs through the isomerization of linoleic acid (LA) or linolenic acid (LNA) and via endogenous conversions from vaccenic acid (VA; *trans*-11 $C_{18:1}$); furthermore, the oxidation of LA to resonance-stabilized allyl

radicals can occur, followed by reprotonation of the radicals by proteins during processing (*5*). Among these, the isomerization of unsaturated fatty acids during ruminal biohydrogenation (BH) process has long been suggested as the major origin of CLA in dairy products (*6−8*).

Ever since the enzymatic activity of rumen bacteria, including *Butyrivibrio fibrisolvens*, was found to be capable of converting LA or LNA to *cis*-9,*trans*-11-CLA (*9*), many efforts have been made to study the mechanisms of microbial CLA production, where the level in dairy products, however, is not phenomenal, since it occurs as an intermediate product during the BH process (*10, 11*). Ruminal BH is composed of sequential but distinct enzymatic processes to yield saturated fatty acids: the spontaneous isomerization of LA to CLA, followed by the reduction of CLA into VA and stearic acid, which requires energy sources (*9, 11*). VA can also be converted to *cis*-9,*trans*-11-CLA by Δ-9 desaturase, which is thought to be present in a variety of animal tissues (*12*).

More recently, some strains of propionibacteria, lactobacilli, and streptococci used as dairy starter cultures were shown to be capable of producing *cis*-9,*trans*-11-CLA from free LA (*13−15*). Moreover, bifidobacteria, which are considered beneficial pro-

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Table 1. Screening of Bifidobacteria for their Capacity to Produce CLA[a]

strains		formation of CLA[b]	source[c]
B. bifidum	ATCC 11863	−	A
B. bifidum	KFRI 894	−	K
B. breve	ATCC 15700	−	A
B. breve	ATCC 15701	−	A
B. infantis	ATCC 15697	−	A
B. infantis	ATCC 25962	−	A
B. longum	ATCC 15707	−	A
B. longum	KFRI 747	−	K
Bifidobacterium sp.	LMC 520	++++	I
Bifidobacterium sp.	LMC 129	++	I
Bifidobacterium sp.	LMC 117	++	I
Bifidobacterium sp.	LMC 065	++	I
Bifidobacterium sp.	LMC 052	+++	I
Bifidobacterium sp.	LMC 044	+	I
Bifidobacterium sp.	LMC 037	++	I
Bifidobacterium sp.	LMC 032	++	I
Bifidobacterium sp.	LMC 021	+++	I
other bifidobacteria	screened	−	I

[a] Incubation was performed with 0.5 mg/mL free LA. [b] +, Formation of *cis*-9,*trans*-11-CLA was 0.01−0.02 mg/mL; ++, 0.02−0.05 mg/mL; +++, 0.05−0.1 mg/mL; ++++, >0.1 mg/mL; and −, not detected (<0.005 mg/mL). [c] Source: A, Korean Collection for Type Culture; K, Korea Food Research Institute; and I, Isolate from Korean breast-fed infants and men.

biotics, have been investigated as a source of natural CLA production (*16, 17*). The use of bifidobacteria, as a commercial dairy starter, has been suggested for the production of dairy products with functional properties.

In this study, several strains of bifidobacteria were isolated from breast-fed infants, which could actively convert LA to *cis*-9,*trans*-11-CLA. The objectives of this study were to examine screened bifidobacteria for their utilization of different fat substrates and to provide insights into the CLA production mechanisms of the bacteria. We also suggest herein the potential use of the isolated strain as a dairy starter for CLA-enriched fermented dairy products.

MATERIALS AND METHODS

Chemicals. The lipid standards were obtained from Sigma Chemical Co. (St. Louis, MO), and MRS media was purchased from Difco (Detroit, MI). All other chemicals used in the fatty acid analysis were of analytical grade (Fisher, Springfield, NJ).

Subjects and Sampling. Fecal material was sampled from 20 Korean breast-fed infants, ages 3 weeks to 6 months, for screening. No neonates had undergone antibiotic treatment prior to sampling. In most cases, fecal swab samples were taken from the neonates and then stored at 4 °C prior to further laboratory processing. The swabs were vortex-mixed in a maximum recovery diluent (Oxoid, Ltd., Hampshire, United Kingdom) and serially diluted to isolate colonies for screening. Alternatively, the swabs were streaked directly onto bifidobacteria selective (BS) medium, which is made by BL agar (Difco) containing 15 g/L sodium propionate (Junsei Chemical, Tokyo, Japan), 50 mg/L paromomycin sulfate (Sigma), 100 mg/L neomysin (Sigma), and 3 g/L lithium chloride (Sigma). Sugar utilization was tested using API 50 kit strips (BioMerieux S.A., Marcy l'Etoile, France). Fructose-6-phosphate phosphoketolase assay was based on the ability of studied strains to transform fructose-6-phosphate into acetyl-phosphate and erythrose-phosphate by the enzyme phosphoketolase (*18*).

Microbial Production of CLA. The bacterial strains used in this study are listed in **Table 1**. The test strains included bifidobacteria isolated from Korean breast-fed infants and six strains from a culture collection. For the screening of the CLA-producing bifidobacteria, the strains were activated in MRS broth with added 0.05% L-cystein·HCl (mMRS) at 37 °C for 18 h under O_2-free CO_2 in Bellco tubes (18 mm × 150 mm; Bellco, Vineland, NJ) that were capped with septum stoppers (Bellco) and aluminum seals (Bellco). The activated cultures were transferred to culture medium containing 10% skim milk (Difco)

and then incubated with 0.5 mg/mL free LA (Sigma, 99% purity) at 37 °C for 48 h. The total viable counts were determined by plating serial dilutions in maximum recovery diluent using mMRS medium under anaerobic condition. For the production of CLA by the screened *Bifidobacterium* sp. during milk fermentation, each strain was subcultured twice in mMRS prior to being cultured in milk media (2% fat milk containing 3% skim milk and 1% sucrose) spiked with various substrates (LA; ML, monolinolein; DL, dilinolein; 50% MG:50% monoglyceride safflower oil; and 90% MG:90% monoglyceride safflower oil) and different concentrations of ML (95% purity, Ilshin Science, Chungju, Korea) at the same condition used for the screening otherwise indicated. The substrates were dissolved in ethanol and added as a stock solution. To test the compatibility with other starter strains for CLA production, commercial starter cultures used for yogurt production were cocultured, *Lactobacillus acidophillus* and *Streptococcus thermophillus*, which were selected from commercial starter cultures typically used in Korea for yogurt production (*19*).

Fatty Acid Analysis. Ten milliliters of the cultures or fermented milk with added hepta-decanoic acid (Sigma) as internal standard was extracted with 100 mL of chloroform/methanol (1:1, v/v). The lower layer was mixed vigorously with 34 mL of 0.88% KCl solution; then, the lower layer was evaporated with nitrogen until dry. The extracted lipids were ethylated using 2% H_2SO_4 in ethanol at 80 °C for 60 min (*20*). After the addition of 10 mL of saturated NaCl solution and 4 mL of *n*-hexane, fatty acid ethyl esters were obtained in the *n*-hexane layer and analyzed on a Supelcowax-10 fused silica capillary column (60 m × 0.32 mm, 0.25 μm film thickness) using a gas chromatograph (3800; Varian, Harbor City, CA) equipped with a flame ionization detector. The analysis conditions were as follows: 2.4 mL/min helium flow; injector, 250 °C; detector, 260 °C; the column chamber temperature was initially 190 °C (1 min) and then increased to 240 at 4.5 °C/min and held for 30 min. Samples (1 μL) containing 0.5−5 μg of LA or CLA were injected into the column using a split/splitless mode (split ratio of 1:50). *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA were used as CLA standards (Lipozen, Pyongtaek, Korea; >98% purity). The recovery of CLA was 83%, and that of $C_{17:0}$ was 80%. A known standard mixture (fatty acid ethyl ester standard mixture; Cayman Chemical, Ann Arbor, MI) of fatty acids was used to identify the other fatty acids.

Statistical Analysis. All data were in triplicate and expressed as the mean ± standard deviation. Analysis of variance (ANOVA) was performed by ANOVA procedures. Duncan's multiple-range test was used to determine the difference of means, and $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Screening and Identification. The bifidobacteria strains from different sources were assessed for their ability to convert free LA to CLA (**Table 1**). These strains were isolated from the stool samples of breast-fed neonates. Among the 150 screened bifidobacteria, nine strains showed CLA-producing capacity in the skim milk medium (10%). No strain from the tested culture collection was capable of producing CLA. Among the tested bifidobacteria, *B. breve* LMC 520 showed the highest CLA-producing ability (0.113 mg/mL; **Table 1**).

The screened bacteria with CLA-producing ability underwent procedures for identification, followed by culture in BS medium for the isolation. All strains grown on BS media were morphologically identified as bifidobacteria and showed fructose-6-phosphate phosphoketolase activity, which is a key enzyme for bifidobacteria selection. None of the strains showed fermentation ability with L-(+)-arabinose and D-(+)-xylose; however, they did ferment D-(+)-manose, salicin, D-(−)-mannitol, and D-(−)-sorbitol, which is characteristic of *B. breve*. The tested strains were able to ferment melezitose and threhalose, whereas the control strain, *B. breve* ATCC 15700, showed no activity with melezitose and threhalose. Thus, we speculated that the isolated strains were new *B. breve* strains worth further
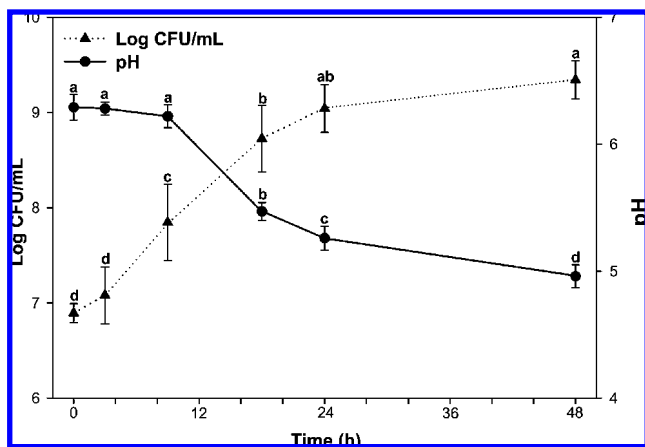
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Figure 1. Viable counts and pH changes during yogurt fermentation by *B. breve* LMC 520. The 2% (v/v) *B. breve* LMC 520 cultures were inoculated into milk media containing 0.05% free LA and incubated at 37 °C for 48 h under anaerobic conditions. Values having different letters are significantly different ($p < 0.05$).

characterizing. Furthermore, all of the strains showed an ability to produce CLA that had originated from the neonatal gastrointestinal tracts and were identified as *B. breve*. Coakley et al. (*16*) also reported that nearly all of the CLA-producing bifidobacteria from the human intestine belonged to *B. breve*. Because bifidobacteria colonize the gastrointestinal tract after birth, some *B. breve* may be able to colonize throughout the later stage of life, and its CLA production in the human intestine could be increased by enrichment through the consumption of dairy products fermented with active *B. breve* strains.

Conditions for CLA Production. When *B. breve* LMC 520 was incubated in the milk media containing 0.05% free LA, the total plate counts from 0 to 24 h of incubation increased significantly from 6.89 to 9.04 log CFU/mL (**Figure 1**). When the pH level was lowered to pH 5, there was an increase in total viable counts during the 48 h of incubation. In fact, a pH of 5−5.5 was the optimal range for the growth of *L. acidophilus*, which is a typical starter strain for milk fermentation (*21*). Therefore, this acidic condition could be advantageous for milk fermentation. Moreover, an optimal incubation time for CLA production in the milk media was obtained at 24 h (0.3 mg/mL), whereas prolonged incubation until 48 h did not appear to further enhance CLA production (**Figure 2**).

To find the most efficient substrate for CLA production, *B. breve* LMC 520 was tested with various fat substrates (**Figure 3**). Among the tested substrates, ML and 90% MG demonstrated the most efficient CLA production ability, 0.41 and 0.39 mg/mL, respectively, which was greater than the production with the free LA (0.28 mg/mL), in agreement with studies on the antimicrobial effect of free LA for propionic acid bacteria reported by Boyaval et al. (*22*) and Jiang et al. (*13*). The 50% MG substrate showed a similar result (0.25 mg/mL) to that of the free LA. The degree of CLA production from DL was the lowest (0.05 mg/mL) among the test groups. Because free LA is not allowed to be a food additive due to its potential toxicity at high levels (*23, 24*), the addition of free LA as a substrate was considered undesirable for commercial yogurt production. Overall, these data led us to consider the monoglyceride forms such as ML, 90% MG, and 50% MG to be efficient for CLA-enriched yogurt production. Because *B. breve* LMC 520 showed a relatively higher conversion activity with the monoester form of LA as compared to the free form (**Figure 3**), we can conclude that this strain could isomerize LA in esterified forms. This is surprising in that other microbes showed preferential substrate
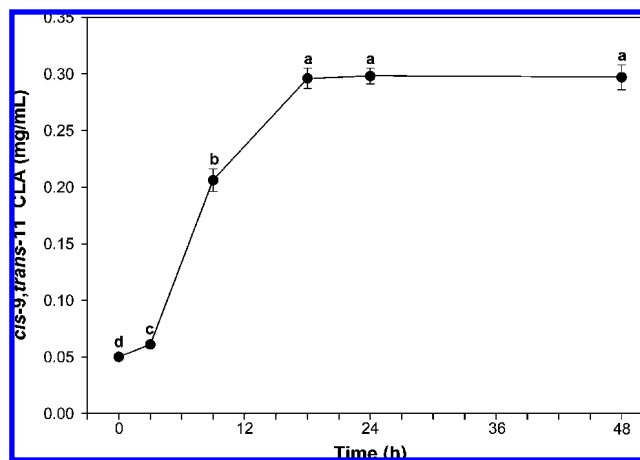

Figure 2. CLA production by *B. breve* LMC 520 in milk media containing free LA. The culture (2%, v/v) was incubated in milk media containing 0.05% free LA at 37 °C for 48 h under anaerobic conditions. Values having different letters are significantly different ($p < 0.05$).
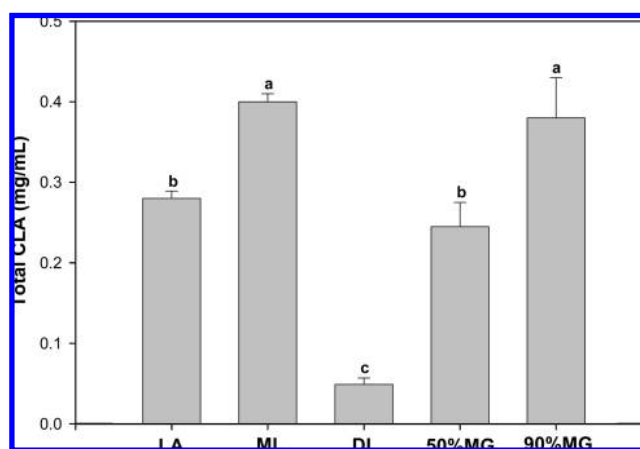

Figure 3. CLA production by *B. breve* LMC 520 depending on various substrates. The culture was incubated in milk media containing 0.05% various substrates at 37 °C for 18 h under anaerobic conditions. Bars with different letters are significantly different ($p < 0.05$).

specificity toward the free form of LA in the lipoxygenase pathway (*25*). We found the DL was not preferred by *B. breve* LMC 520 as compared to ML. Hall et al. (*25*) also showed a lower preference to DL or TL than to ML. This difference might be due to the ease of entry of fat substrates into the active site of the enzyme.

When *B. breve* LMC 520 was incubated at 37 °C for 9 h in milk media with different concentrations of ML, *cis*-9,*trans*-11-CLA and total CLA concentrations increased proportionally to 1.24 and 1.44 mg/mL, respectively, and this pattern of CLA production was similar between the *cis*-9,*trans*-11-CLA and the total CLA, which indicated that *cis*-9,*trans*-11 is the major isomer produced by the strain (**Figure 4**). A significant increase in CLA was obtained between 0.2 and 0.3% ML, and the highest CLA production was observed at 0.5%. This pattern was similar to the previous studies of lactobacillus strains (*15, 26*). The addition of LA was effective in enhancing CLA production during milk fermentation.

CLA Production by Coculture with Other Strains. As shown in **Figure 5**, the two strains, which have little CLA production activity, were selected for coculture with *B. breve* LMC 520. There was an approximate 20% decrease in CLA production by coculturing with the *L. acidophilus* starter strain, but a minimal decline was shown in the coculture with *S.*
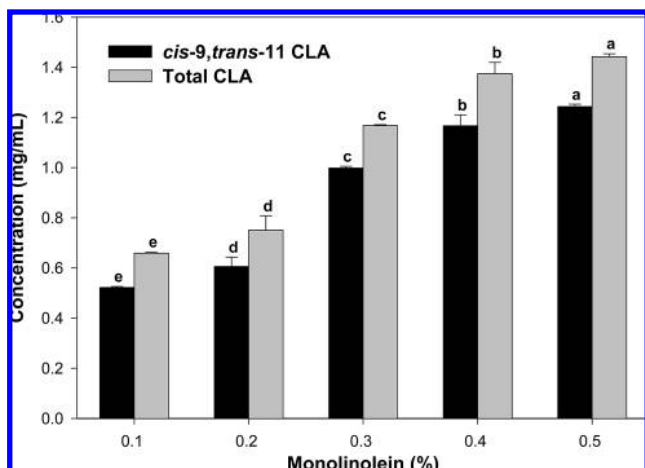
Figure 4. CLA production by *B. breve* LMC 520 depending on different concentrations of ML. The culture was incubated in milk media containing different concentrations of ML at 37 °C for 9 h under anaerobic conditions. Bars with different letters are significantly different ($p < 0.05$).
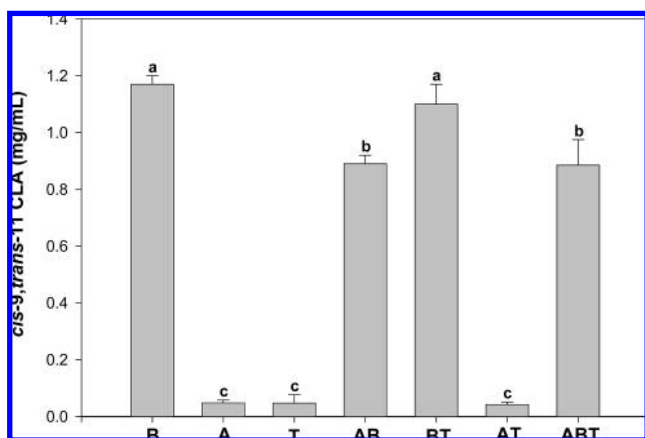

Figure 5. Compatibility of *B. breve* LMC 520 with other starter cultures for CLA production. The culture was incubated with different starter strains in milk media containing 0.5% ML at 37 °C for 9 h under anaerobic conditions. Bars with different letters are significantly different ($p < 0.05$). B, *B. breve* LMC 520 only; A, *L. acidophillus* only; T, *S. thermophillus* only; AT, *L. acidophillus* + *S. thermophillus*; BT, *B. breve* LMC 520 + *S. thermophillus*; AB, *L. acidophillus* + *B. breve* LMC 520; and ABT, *L. acidophillus* + *B. breve* LMC 520 + *S. thermophillus*.

thermophillus. When *B. breve* LMC 520 was cocultured with both starter strains, there was no significant difference as compared to the level shown in the coculture with *L. acidophilus*. At the 0.5% LA concentration, the coincubation of *B. breve* LMC 520 with both starter strains resulted in a higher level of CLA as compared with other *Lactobacillus* and *Streptococcus* strains in previous studies (*15*). Thus, it appeared that *B. breve* LMC 520 can be utilized as a CLA-producing starter strain for milk fermentation.

In conclusion, the production of CLA during fermentation was principally dependent on the growth conditions for *B. breve* LMC 520 as well as substrate choice. Additions of the esterified forms of LA were effective for enhancing CLA production during milk fermentation. During the incubation of *B. breve* LMC 520, the pH level decreased until the optimum pH level of costarter cultures. On the basis of these results, it is possible that *B. breve* LMC 520 can be used to develop new functional dairy products.

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