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Synthesis of Conjugated Linoleic Acid by Human-Derived *Bifidobacterium breve* LMC 017: Utilization as a Functional Starter Culture for Milk Fermentation

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This study was performed to discover bifidobacteria isolated from human intestines that optimally convert linoleic acid (LA) to conjugated linoleic acid (CLA) and to optimize the culture conditions of milk fermentation. One hundred and fifty neonatal bifidobacteria were screened for CLA-producing ability, and *Bifidobacterium breve* LMC 017 was selected as it showed about 90% conversion of free LA in MRS broth. The selected strain showed resistance at 0.5% LA in microaerophillic conditions. When monolinolein (LA 90%) was used as a substrate for CLA production, the conversion rate was lower compared to free LA, but the growth rate was unaffected during the milk fermentation. There was no significant difference in CLA production between aerobic and anaerobic conditions, and little decline in CLA was shown after the maximal CLA level had been reached. CLA production increased by 80% with 24 h of incubation in milk containing additional skim milk (5%), where the proteins may have facilitated the production of CLA by enhancing the interaction of substrate with the bacteria. CLA production did not decline after 9 h of fermentation and an additional 12 weeks of storage with other commercial starters. This demonstrates the possibility of using this strain as a costarter in the production of CLA-enriched yogurt.

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

INTRODUCTION

Fermented milk consumption has long been considered to be beneficial to human health. Metchnikoff proposed that the longevity of Bulgarians may have been partially due to their regular consumption of yogurt (*1*). Also, epidemiological (*2*) and animal studies (*3*) have shown that the consumption of fermented milk products produced with lactic acid bacteria (LAB) can reduce a variety of tumor incidences. LAB introduce an acidic environment into the human intestine, thus altering the gut flora, reduce the production of mutagenic compounds, and facilitate their excretion by attachment to the bacterial surface (*4, 5*).

Bifidobacteria are often positively associated with several healthy and nutritional benefits in humans, and there has been increasing interest in their utilization within functional dairy products (*6*). Because bifidobacteria colonize the gastrointestinal tract after birth and remain constant in both infants and adults (*7*), it is feasible to add functional value to dairy products using suitable strains isolated from the human intestine.

Table 1. Contents of CLA Isomers Produced by Screened Bifidobacteria[a]

	strains [mg/mL (% of total CLA)]			
CLA isomer	LMC 220	LMC 052	LMC 021	LMC 017
cis-9,*trans*-11-CLA	0.384 (82.5)b	0.258 (90.2)c	0.238 (81.0)d	0.432 (91.1)a
total CLA	0.466b	0.286c	0.294c	0.474a

[a] The strains were incubated in mMRS broth with 0.06% LA for 24 h at 37 °C. Values within rows followed by different letters are significantly different ($p < 0.05$).

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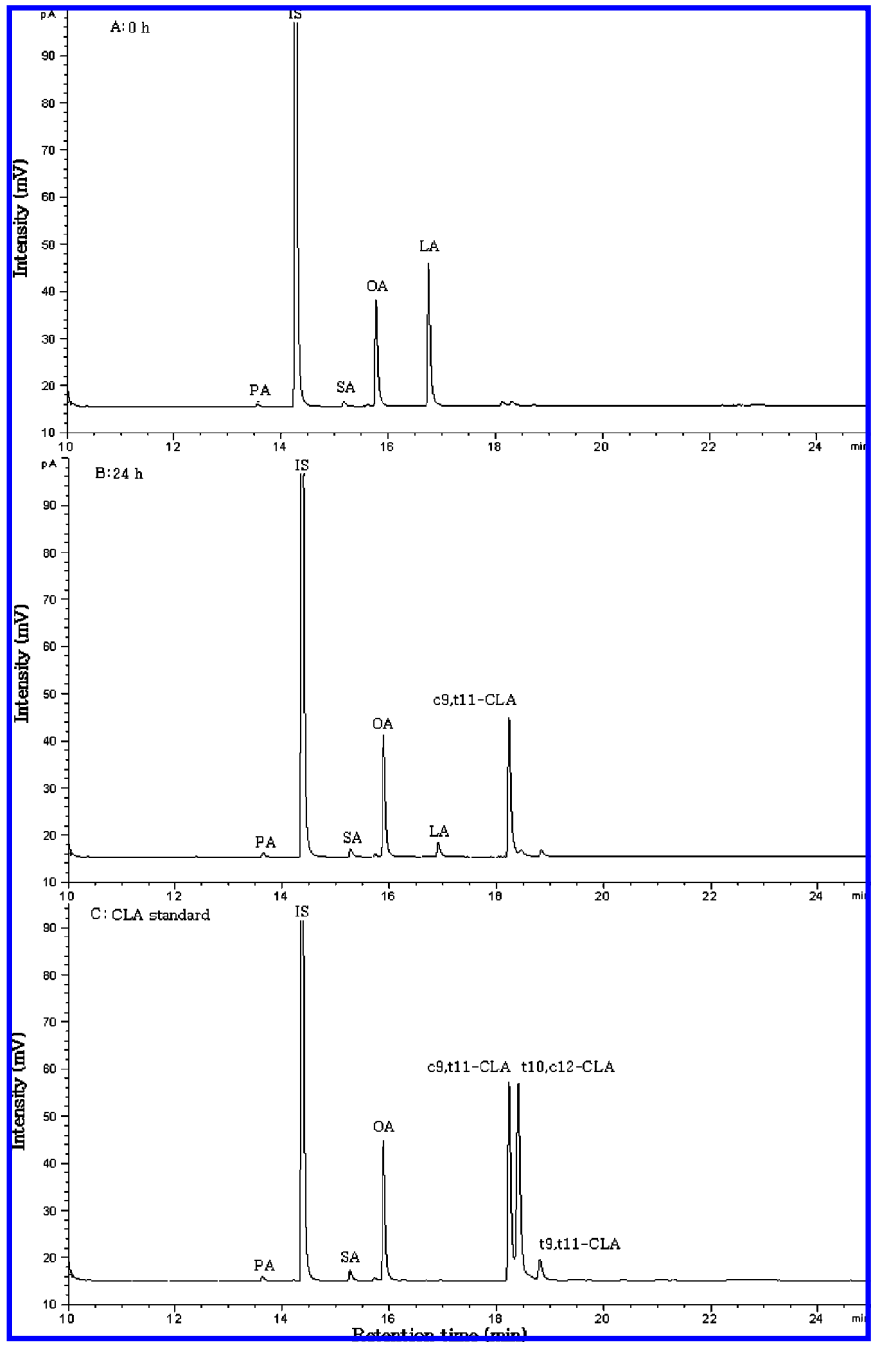

Figure 1. Partial chromatograms in CLA range at 0 and 24 h and that of CLA standard mixture: (**A**) mMRS medium with 0.03% free LA; (**B**) incubation of *B. breve* LMC 017 in mMRS medium with 0.03% free LA for 24 h; (**C**) CLA standard mixture.

Conjugated linoleic acid (CLA), mainly found in dairy products, has shown cancer-preventive roles in numerous studies (*8, 9*). CLA refers to a group of positional and geometric isomers of octadecadienoate ($C_{18:2}$) with conjugated double bonds and is known as a functional compound in dairy products. Strong cancer-preventive roles of CLA were shown in earlier studies (*8–11*). CLA occurs mainly in dairy products because it is an intermediate during the biohydrogenation (BH) pathway, a characteristic biochemical process carried out by rumen microorganisms (*12, 13*). Moreover, some LAB are also able to produce CLA (*14*). The level of CLA in dairy products, however, is far lower than the level needed to play a physiological role in humans (*15*). Dairy products contain about 5 mg of CLA/g of fat, and 0.1% CLA in the diet is shown to be enough to participate in physiological functions (*16*). A 350 g rat fed a diet with 0.1% CLA would consume approximately 15 mg of CLA/day. By extrapolation, this would be equivalent to a daily CLA intake of 3 g for a 70 kg human, which is about 3 times higher than the estimated typical CLA consumption in Western countries (*17*). CLA consumption may be much less in Asian people due to their low intake of dairy products. Therefore, it would be desirable to concentrate CLA in yogurt to maximize the beneficial effects via its physiological roles in human. Consequently, much effort has been made to increase CLA in dairy and beef products (*18–20*) and to understand the key factors involved. Dietary lipids hydrolyzed into free fatty acids (FFAs) are subjected to microbial BH from which CLA is derived as an intermediate, and BH is known to be influenced by various environmental factors (*13, 21*). On this basis, many researchers have tried to increase the level of natural CLA in dairy products by manipulating the incubation conditions of starter cultures used for milk fermentation. The present study

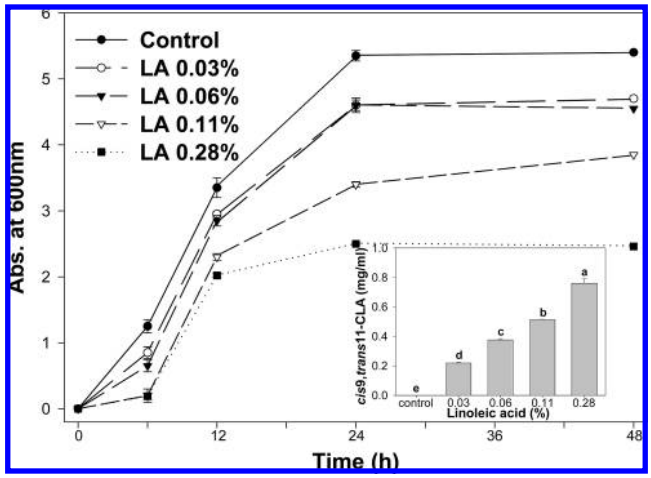

Figure 2. CLA production and growth of *B. breve* LMC 017 in mMRS broth at different concentrations of linoleic acid (FFA). The culture (2%, v/v) was incubated in mMRS broth containing different concentrations of LA for 48 h under anaerobic conditions. Control was the culture without a substrate. Bars with different letters are significantly different at 24 h ($p < 0.05$).

was performed to isolate and investigate an active bifidobacterium that could convert linoleic acid (LA) into CLA during milk fermentation, in conjunction with other starter cultures, and to investigate the optimal fermentation conditions.

MATERIALS AND METHODS

Chemicals. Lipid standards for fatty acid analysis were obtained from Sigma Chemical Co. (St. Louis, MO). The MRS medium was purchased from Difco (Detroit, MI). All other chemicals used for 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, with permission from their parents for screening. None of the infants had been on antibiotic treatment prior to sampling. In most cases, fecal swab samples were taken from the infants and then stored at 4 °C prior to laboratory processing. The swabs were Vortex-mixed in a maximum recovery diluent (Oxoid, Ltd., Hampshire, U.K.) and serially diluted to isolate colonies for screening. Alternatively, the swabs were streaked directly onto MRS agar plates.

Species Identification of Representative Strains. Bifidobacteria were identified by their cell morphologies on BS medium and by their fructose-6-phosphate phosphoketolase activity. Species identification of the *Bifidobacterium breve* was obtained by using API 50 kit strips (bioMerieux S.A., Marcy l'Etoile, France) and then referring to the manufacturer's databank.

Microbial Production of CLA. The test strains used for this study included bifidobacteria isolated from Korean breast-fed infants and six from the Korean Type Culture Collection (KTCC, Seoul, Korea). Among the tested bacteria, some strains showed high CLA-producing abilities (**Table 1**). To screen for CLA-producing bifidobacteria, the strains were subcultured twice in mMRS broth that consisted of MRS supplemented with 0.05% L-cystein·HCl and were cultured in 10% skim milk media (Difco) and then incubated with 0.06% free LA (Lipozen, Pyoungtaek, Korea, 99% purity) or monolinolein (Ilshin Science, Chungju, Korea; 95% purity) at 37 °C for 24 h. The total viable counts were determined by plating serial dilutions of the culture in maximum recovery diluent using MRS medium. For the production of CLA by the screened *Bifidobacterium* species during milk fermentation, each strain was anaerobically subcultured twice in mMRS broth before inoculation (2%, v/v) and spiked with different concentrations of LA. The LA and monolinolein were dissolved in ethanol and added as a stock solution. Cell growth was measured at 600 nm at room temperature. To create anaerobic conditions, mixed anaerobic gas (N_2/CO_2/H_2 = 80:15:5) was used and 0.05% L-cystein·HCl was added to MRS broth.

Fatty Acid Analysis. Ten milliliters of the cultures or fermented milk with added heptadecanoic acid ($C_{17:0}$) 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. 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 (*22*). 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; column chamber temperature was initially 190 °C (1 min) and then increased to 240 °C 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 in the splitless mode. *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA were used as the CLA standard (Lipozen, >98% purity). **Figure 1** shows the fatty acid peak of mMRS medium with 0.03% free LA (**A**), CLA produced by *B. breve* LMC 017 for 24 h (**B**), and CLA standard mixture (**C**). 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 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 of Active Strains. One hundred and fifty neonatally derived bifidobacteria were screened for CLA production ability. The infants were breast-fed and did not receive any antibiotics during the sampling. Among the tested strains, four strains showed conversion rates greater than 80% for the conversion of free LA into CLA. The *cis*-9,*trans*-11 isomer was the major form of CLA produced by all four strains (>80%; **Table 1**). This is the typical pattern of CLA biosynthesis by most CLA-producing microorganisms shown in other studies with LAB (*23, 24*). Among the active strains for CLA production, we identified *B. breve* LMC 017 as a bacterium that converted 91.1% of the free form of LA and was therefore selected for further mechanistic studies. Bifidobacteria are known to colonize the gastrointestinal tract after birth and remain in high numbers in neonates. Among them, *B. breve* LMC 017 has been shown to be an active CLA producer in previous studies (*14, 25*).

Substrate Resistance and Utilization. The microbial growth of *B. breve* LMC 017 proportionally decreased as free LA concentration increased to 0.28% of the culture (**Figure 2**). After 24 h of incubation, there was no further increase in cell density, and maximal cell growth at the free LA level of 0.28% was approximately half that of bacteria grown without substrate. Furthermore, CLA production increased proportionally to 0.767 mg/mL when the free LA was increased from 0.03 to 0.28%.

Because long-chain FFAs are known to be inhibitory to microbial growth and FFAs are not allowed as food additives due to their potential toxicity at high levels (*26, 27*), adding FFA was not considered to be safe as a substrate for milk fermentation. To overcome such potentially detrimental effects of FFAs, the monoglyceride form of LA (monolinolein), which can be used as a food additive, was tested as a substrate for CLA production by *B. breve* LMC 017, up to a 0.5% level; however, the CLA production was lower with monolinolein (substrate conversion rate = 78.8% CLA) as compared to free LA (substrate conversion rate = 94.8% CLA). The inhibition effect of FFAs on microbial growth was higher as compared to

Table 2. CLA Production and pH Changes Depending on Monolinolein Concentration by *B. breve* LMC 017[a]

	concentration of added monolinolein (mg/mL)				
fatty acid	0.1%	0.2%	0.3%	0.4%	0.5%
$C_{16:0}$	2.839 ± 0.007d	3.385 ± 0.009c	3.584 ± 0.118b	3.682 ± 0.009b	4.641 ± 0.088a
$C_{16:1}$	0.120 ± 0.000d	0.142 ± 0.000c	0.147 ± 0.003b	0.150 ± 0.001b	0.191 ± 0.005a
$C_{18:0}$	1.723 ± 0.004c	2.093 ± 0.024b	2.146 ± 0.041b	2.133 ± 0.001b	2.592 ± 0.058a
$C_{18:1}$	3.192 ± 0.007e	3.871 ± 0.033d	4.023 ± 0.059c	4.146 ± 0.001b	4.975 ± 0.082a
$C_{18:2}$	0.535 ± 0.001e	0.634 ± 0.006d	1.053 ± 0.059c	1.952 ± 0.015b	2.027 ± 0.031a
cis-9,*trans*-11-CLA	0.299 ± 0.000e	0.393 ± 0.008d	0.602 ± 0.012c	0.669 ± 0.005b	0.881 ± 0.025a
trans-9,*trans*-11-CLA	0.087 ± 0.000e	0.108 ± 0.003d	0.130 ± 0.005c	0.137 ± 0.001b	0.175 ± 0.003a
total CLA	0.386 ± 0.000e	0.502 ± 0.010d	0.731 ± 0.018c	0.806 ± 0.006b	1.056 ± 0.027a
pH	4.92 ± 0.05e	5.13 ± 0.11c	5.10 ± 0.07c	5.31 ± 0.06b	5.45 ± 0.05a

[a] Incubation was performed in 10% skim milk media with monolinolein for 9 h at 37 °C. The isomers of $C_{16:1}$ and $C_{18:1}$ were not separated. Values within rows followed by different letters are significantly different ($p < 0.05$).

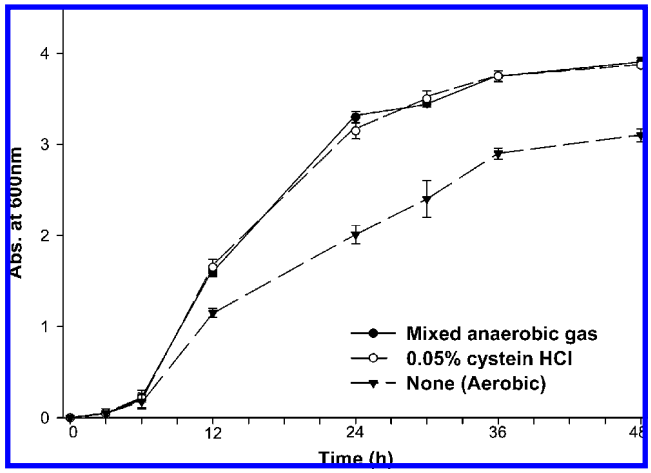

Figure 3. Growth of *B. breve* LMC 017 at different culture conditions. The culture was incubated in different culture conditions without substrate for 48 h. None (aerobic) = untreated MRS broth.

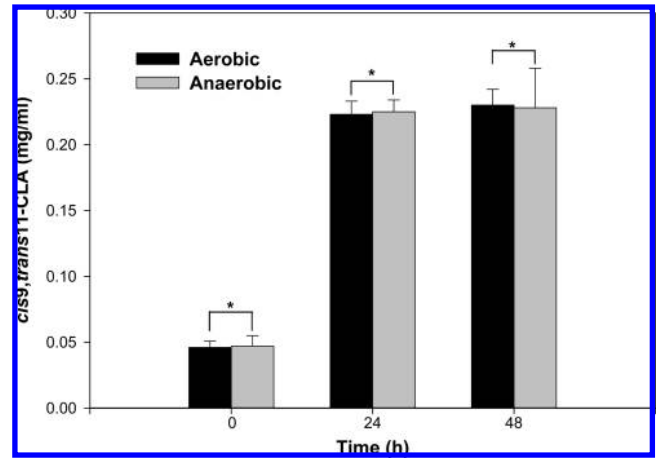

Figure 4. CLA production by *B. breve* LMC 017 depending on atmospheric conditions. The culture was inoculated into whole milk containing 0.1% (v/v) monolinolein and incubated for 48 h under anaerobic and aerobic conditions. ∗, values within each sampling time are not significantly different ($p > 0.05$).

that of monolinolein. In addition, the decrease in pH during *B. breve* LMC 017 incubation was inversely correlated with monolinolein concentration after 9 h (**Table 2**). Reduced pH is considered to be an indicator of microbial growth in LAB and, thus, a higher pH associated with increased concentrations of monolinolein indicates inhibition of growth of *B. breve* LMC 017. However, total CLA production was proportionally increased to the substrate concentration up to 0.5%. Therefore, monolinolein can be used as a good substrate in CLA production during milk fermentation

Table 2 shows the major fatty acid profile produced by *B. breve* LMC 017 during fermentation. When *B. breve* LMC 017 was incubated at 37 °C for 9 h and the monolinolein concentration increased from 0.1 to 0.5% of the media, CLA concentration proportionally increased nearly 3-fold. All other major long-chain fatty acids also proportionally increased, but this was due to the increased content in the substrate. This indicates that the activity of conversion of CLA into other fatty acids was not active enough to decrease the CLA level during fermentation. This characteristic offers the strain advantages as a CLA-producing starter culture in milk fermentation.

The growth of *B. breve* LMC 017 was inhibited by aerobic incubation, especially after 24 h of incubation, and the degree of inhibition was maintained until 48 h of incubation (**Figure 3**). There was no significant difference in cell growth between the incubation under mixed anaerobic gas and the incubation in MRS broth with 0.05% L-cystein·HCl, which was used for further analysis for convenience. The cystein supplementation was thought to create anaerobic conditions, which may be favorable for *B. breve* LMC 017 growth.

There was no significant difference in CLA production between the aerobic and anaerobic conditions during milk fermentation (**Figure 4**). Unlike other bifidobacteria, this strain may efficiently survive the microaerobic conditions that are created during fermentation. In fact, *Butyrivibrio fibriolvens*, a strict anaerobe, is known to be a major CLA-producing rumen bacterium, which has a higher rate of CLA accumulation in an aerobic condition compared to an anaerobic condition due to inactive BH in aerobic conditions. It was shown in a previous study that energy-requiring hydrogenation processes of CLA to vaccenic acid (VA) are significantly inhibited in aerobic conditions (*21*). In contrast, *B. breve* LMC 017 was shown to be inactive in BH, and thus the CLA level was not strongly influenced by atmospheric environment.

CLA production was higher when *B. breve* LMC 017 was incubated in whole milk as compared to skim milk, until 48 h of incubation (**Figure 5**). Although adding more skim milk (milk + skim milk) did not affect CLA production until the cells were in the log phase, it did have an effect after the log phase. The fats in the whole milk may have played roles as carriers of substrate FFAs to the microorganism, which would increase availability and accessibility of the substrate for the conversion of LA by *B. breve* LMC 017. The effect of adding more protein seemed to be minimal, because there was a relatively small boost when additional skim milk was given. Until 18 h of incubation, there was no further increase in CLA production with the additional skim milk. However, a boost in CLA production was evident after 24 h of incubation. Additional nutrients may have

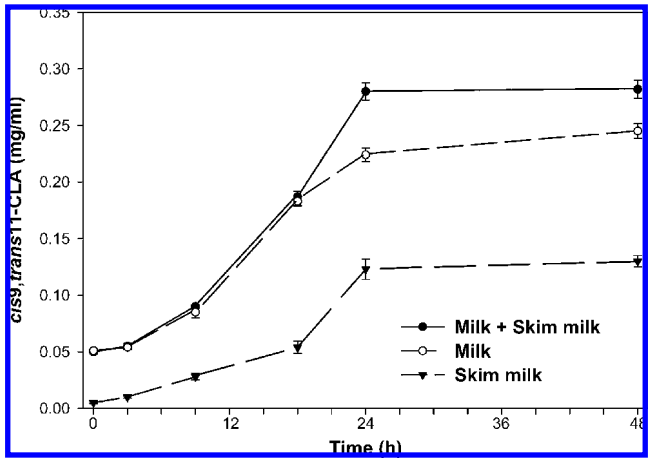

Figure 5. Production of CLA by *B. breve* LMC 017 depending on culture conditions. The culture was inoculated into media with various conditions containing 0.05% (v/v) LA and incubated for 48 h under anaerobic conditions. Milk = low-fat milk (2% fat); skim milk = 10% skim milk; milk + skim milk = low-fat milk + 5% skim milk.

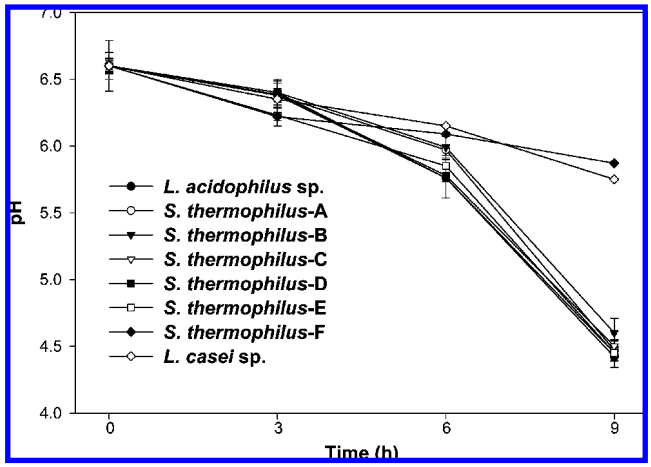

Figure 6. pH changes during milk fermentation by LA tolerant strains from commercial starter cultures (compatibility of *B. breve* LMC 017). Commercial starter cultures were inoculated into 10% skim milk media with 0.11% (v/v) LA and incubated for 9 h under anaerobic conditions.

contributed to an increase in cell numbers at the late stage of the log phase. In fact, environmental factors for maximizing cell numbers were shown to be important in CLA production (*18*), and this should be considered when CLA-enriched yogurt is produced.

To test the compatibility of the commercial strains, resistant starters were selected by incubation at 0.11% LA. Resistance was indicated by the degree of pH reduction (**Figure 6**). *Streptococcus thermophilus* and *Lactobacillus acidophilus*, which are used as typical starter cultures for yogurt production, were tested for resistance against LA, and some resistant strains were selected as costarters. The two selected strains (*L. acidophilus* sp. and *S. thermophilus* D) had little CLA-producing activity, but did not inhibit the growth of *B. breve* LMC 017. When *B. breve* LMC 017 was incubated with the other LAB strains, *L. acidophilus* sp., and *S. thermophilus* D, CLA production increased up to 0.58 mg/mL at 0.5% (v/v) monolinolein (**Table 3**), which was lower compared to the level achieved when it was cultured alone (0.88 mg/mL; **Table 2**). Up to 0.2% (v/v) monolinolein, *cis*-9,*trans*-11-CLA concentration was not significantly different between the single and mixed cultures, but the increased rate of the *cis*-9,*trans*-11-CLA concentration of the mixed culture decreased with higher

Table 3. CLA Production and pH of Yogurt Fermented by *B. breve* LMC 017, *S. thermophilus*, and *L. acidophilus* during Fermentation and Storage[a]

	after fermentation		after storage	
monolinolein (%)	*cis*-9,*trans*-11-CLA (mg/mL)	pH	*cis*-9,*trans*-11-CLA (mg/mL)	pH
0.1	0.35d	4.35a	0.34d	4.46a
0.2	0.37c	4.33a	0.37d	4.42b
0.3	0.49b	4.31b	0.50c	4.38c
0.4	0.55a	4.31b	0.57b	4.40bc
0.5	0.58a	4.27c	0.61a	4.29d

[a] Fermentation was performed with monolinolein for 9 h at 37 °C and then stored for 12 h at 4 °C. Values in the column followed by different letters are significantly different ($p < 0.05$).

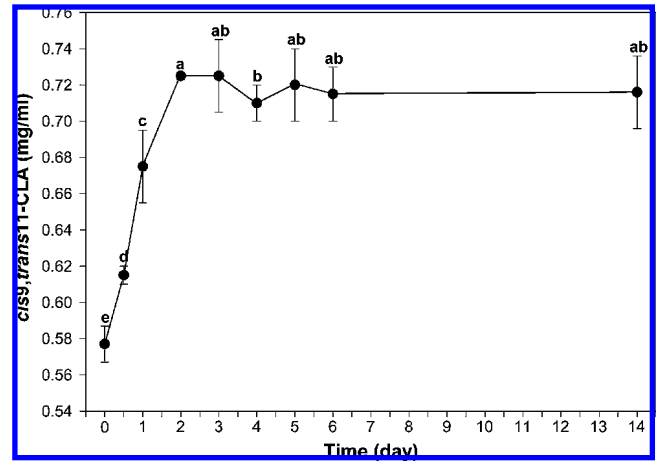

Figure 7. CLA content of yogurt by *B. breve* LMC 017, *S. thermophilus*, and *L. acidophilus* during storage. *B. breve* LMC 017 culture was inoculated with 0.5% (v/v) *S. thermophilus* and *L. acidophilus* into 10% skim milk, incubated with 0.5% (v/v) monolinolein at 37 °C for 9 h under anaerobic conditions, and then stored for 2 weeks at 4 °C. Values having different letters are significantly different ($p < 0.05$).

monolinolein concentration as compared to the single culture (**Tables 2** and **3**). There was no significant difference in pH during fermentation at all tested concentrations for 9 h at 37 °C, when *B. breve* LMC 017 was co-incubated with the other selected starter strains. Moreover, the CLA level did not decrease at any of the tested concentrations after 12 h of storage. This indicates that the strain is desirable as a CLA-producing starter culture for milk fermentation.

The level of CLA was monitored during storage at 4 °C for 2 weeks with two other selected starter culture strains, and some increase in CLA was observed up to 48 h of storage following fermentation; no significant change was observed thereafter (**Figure 7**). *B. breve* may not have the hydrogenation mechanism that is significant in certain rumen bacteria (*18*). This characteristic of *B. breve* LMC 017 made it possible to maintain the CLA level during fermentation and storage.

In conclusion, *B. breve* LMC 017 efficiently converted monolinolein into CLA under microaerobic fermentation conditions with the aid of protein carriers. There was no decrease in the CLA level during long-term incubation of *B. breve* LMC 017 alone and with commercial starter cultures. Additional studies are ongoing with the other CLA-producing bacteria to be used as costarter cultures for maximizing CLA production during milk fermentation. Further studies are needed to optimize the culture conditions for increasing CLA production in large-scale fermentation processes.

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