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Production of a Conjugated Fatty Acid by *Bifidobacterium breve* LMC520 from α-Linolenic Acid: Conjugated Linolenic Acid (CLnA)

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ABSTRACT: This study was performed to characterize natural CLnA isomer production by *Bifidobacterium breve* LMC520 of human origin in comparison to conjugated linoleic acid (CLA) production. *B. breve* LMC520 was found to be highly active in terms of CLnA production, of which the major portion was identified as *cis*-9,*trans*-11,*cis*-15 CLnA isomer by GC-MS and NMR analysis. *B. breve* LMC520 was incubated for 48 h using MRS medium (containing 0.05% L-cysteine·HCl) under different environmental conditions such as atmosphere, pH, and substrate concentration. The high conversion rate of α-linolenic acid (α-LNA) to CLnA (99%) was retained up to 2 mM α-LNA, and the production was proportionally increased nearly 7-fold with 8 mM by the 6 h of incubation under anaerobic conditions at a wide range of pH values (between 5 and 9). When α-LNA was compared with linoleic acid (LA) as a substrate for isomerization by *B. breve* LMC520, the conversion of α-LNA was higher than that of LA. These results demonstrated that specific CLnA isomer could be produced through active bacterial conversion at an optimized condition. Because many conjugated octadecatrienoic acids in nature are shown to play many positive roles, the noble isomer found in this study has potential as a functional source.

KEYWORDS: *Bifidobacterium breve LMC520, conjugated linolenic acid (CLnA), conjugated linoleic acid (CLA), α-linolenic acid, NMR*

INTRODUCTION

Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of linoleic acid (LA; *cis*-9,*cis*-12-octadecadienoic acid) that have two conjugated double bonds, and it is an intermediate that naturally occurs during microbial biohydrogenation processes. CLA has been well-characterized as a potentially beneficial factor as indicated in various animal studies.[1] It has been shown that CLA has anticarcinogenic effects against a variety of cancers,[2,3] as well as antiatherogenic effects[4] and antiobesity effects.[5] Thus, there have been continuous attempts to increase the CLA concentration in dairy products and beef by manipulating microbial activity.[6−8]

Conjugated linolenic acid (CLnA), which has octadecatrienoic fatty acid isomers with at least two conjugated double bonds in different positional (8,10,12−18:3; 9,11,15−18:3; 10,12,14−18:3; 11,13,15−18:3, etc.) and geometric (*ttt*, *ctt*, *ctc*, etc.) configurations, is present in several kinds of seed oils, and it can also be produced through organic synthesis from α-linolenic acid (α-LNA; C18:3, *cis*-9,*cis*-12,*cis*-15 octadecatrienoic acid). CLnA does not occur in significant amounts in any animal fats but occurs abundantly in some plant seed oils. Punicic acid (*cis*-9,*trans*-11,*cis*-13) is contained at about 70% in pomegranate seed oil.[9] α-Eleostearic acid (*cis*-9,*trans*-11,*trans*-13) is contained in bitter gourd oil and tung seed oil at about 60 and 70%, respectively.[9,10] Moreover, pot marigold seed oil contains calendic acid (*trans*-8,*trans*-10,*cis*-12), and catalpa seed oil contains catalpic acid (*trans*-9,*trans*-11,*cis*-13) at about 30%.[9] A mixture of CLnA isomers was reported to have effects on antioxidation,[11] antiobesity,[12,13] and anticarcinogenesis.[14,15] It was reported that each CLnA isomer has different biochemical activities.[9] Although CLnA isomers are known to be abundant in plant seed oils, its availability is limited, because it is very difficult to obtain an individual CLnA isomer that is responsible for the physiological activities. However, our study showed that a single isomer (>99%) could be produced by microbial conversion, and this could elucidate the physiological role of a specific isomer.

There have been scarce reports on the full conversion of LA to CLA without a further saturation process; thus, we have screened a wide spectrum of bacteria for conjugation activity for unsaturated fatty acids and came up with very active bifidobacterium, *Bifidobacterium breve* LMC520, which was originated from human intestine.[7] In our previous study, we found that this bacterium had very specific activity in the isomerization of LA. The objectives of this study were to identify the structure of CLnA isomer produced by *B. breve* LMC 520 and to optimize CLnA production from α-LNA in a comparison to CLA production by the same microorganism. The present findings will lead to further study on the mechanism of microbial fatty acid conjugation and isomer-specific effects in vitro and in vivo.

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MATERIALS AND METHODS

Chemicals. The LA, CLA mixture, heptadecanoic acid, L-cysteine·HCl, KCl, chloroform, H_2SO_4, NaCl, and buffers were obtained from Sigma Chemical Co. (St. Louis, MO), and α-LNA (99%) for standard was obtained from Lipozen (Pyongtaek, Korea). MRS broth was purchased from Difco (Detroit, MI). Ethanol, *n*-hexane, and methanol were analytical grade (Fisher, Springfield, NJ).

Substrate Preparation. For the incubation of activated bacterial cells with fatty acid substrates, concentrated LA (99%) and α-LNA (80 + 20% LA) solutions were dissolved in ethanol and added as a stock solution (1 M).

Microbial Production of Conjugated Fatty Acids. For the production of conjugated fatty acids, *B. breve* LMC520 was subcultured twice at 37 °C for 18 h in Bellco glass tubes (18 mm × 150 mm; Bellco, Vineland, NJ) containing 10 mL of MRS (MRS broth with 0.05% L-cysteine·HCl) medium, which were capped with septum stoppers (Bellco) and aluminum seals (Bellco) and had been flushed with O_2-free CO_2. The activated cultures were transferred to fresh MRS and then incubated under different conditions such as substrate, pH, and atmospheric conditions. The growth rates were estimated by a microplate reader at 600 nm for optical density (O.D.) (BIO-RAD, Hercules, CA). The pH value was measured with a Mettler Toledo MP-220K instrument (Mettler-Toledo AG, CH-8603 Schwerzenbach, Switzerland), which was calibrated using pH buffer (Mettler-Toledo) at pH 4.0 and 7.0, according to the operating manual. The time course of the batch fermentation by *B. breve* LMC 520 was performed in the MRS medium containing 2 mM α-LNA and LA and then incubated at 37 °C for 48 h under anaerobic conditions. The CLnA-producing activity of *B. breve* LMC 520 depending on different substrate concentrations was tested in MRS medium containing different concentrations of α-LNA (up to 10 mM) at 37 °C for 24 h under anaerobic conditions. To investigate the effects of different atmospheric conditions on the production of CLnA, *B. breve* LMC520 was incubated in MRS medium containing 2 mM α-LNA at 37 °C for 48 h under aerobic and anaerobic conditions. The effects of pH on conjugated fatty acid production were tested at different pH ranges using different buffers: acetate buffer (pH 3.5−5.5), potassium phosphate buffer (pH 6.0−7.0), Tris buffer (pH 7.5−8.5), and carbonate buffer (pH 9.0−10.0). *B. breve* LMC520 was grown in the 10 mL of MRS medium for 24 h at 37 °C under anaerobic condition, and the culture was immediately cooled in an ice bath and then centrifuged (3000*g*, 10 min, 4 °C). The cell harvest was washed twice with phosphate-buffered saline (PBS, pH 7.0) buffer and resuspended in different pH buffers of 1 mL containing 1 mg of α-LNA, and then, the mixtures were homogenized by precellys tissue homogenizers (precellys 24, Bertin technologies, Villeurbanne, France). Washed cells were used to avoid the inhibitory effect of free fatty acids on cell growth. The mixtures were incubated at 20 °C for 3 h under shaking at 100 rpm on a rotating incubator (JSSI-100C, JSR, Chungnam, Korea).

Ethylation for Fatty Acid Analysis. One milliliter of culture samples in the MRS medium or buffers was collected into the Bellco tube with septum stopper by a syringe and needle. The samples with heptadecanoic acid ($C_{17:0}$) added as an internal standard (IS) were extracted with 12 mL of chloroform/methanol (1:1, v/v). The lower layer was mixed vigorously with 2 mL of 0.88% KCl solution and was then evaporated with nitrogen until dryness. The extracted lipids were ethylated using 2% H_2SO_4 of 10 mL in ethanol at 80 °C for 60 min.[16] After 8 mL of saturated NaCl solution and 4 mL of *n*-hexane were added, fatty acid ethyl esters were obtained in the *n*-hexane layer and analyzed for total fatty acids using a 7890A gas chromatograph with a flame ionization detector (FID, Agilent Technologies, Wilmington, DE). The fatty acid ethyl esters were separated using a Supelcowax-10 fused silica capillary column (100 m × 0.32 mm i.d., 0.25 μm of film thickness; Supelco, Inc., Bellefonte, PA) with a 1.2 mL/min of helium flow. The oven temperature was increased from 190 to 240 °C at a rate of 4 °C/min. The temperatures of the injector and detector were both 260 °C. One microliter of sample was injected into the column in the split mode (50:1). The peak of each fatty acid was identified and quantified by comparison with the retention time and peak area of each fatty acid standard, respectively. IS was included as an internal reference before extraction to determine the recovery of the fatty acids in each sample. The conversion rates (%) for CLA and CLnA were calculated as ΣCLA/(LA + ΣCLA) × 100 and ΣCLnA/(LNA + ΣCLnA) × 100, respectively.

GC-MS Analysis. The CLnA from the medium was prepared using ethylation; the obtained CLnA was filtered through anhydrous sodium sulfate. GC-MS analysis of the CLnA was performed on an Agilent Technologies 6890N GC with a 5973N mass-selective detector (MSD, HP 5973, Hewlett-Packard Co.). The column oven temperature was maintained at 150 °C for 1.0 min, increased to 200 at 15 °C/min, increased to 230 at 2 °C/min, and then held at 230 °C for 5.0 min. The temperatures of the injector and the interface were both 250 °C. The column was a DB-23 (60 m × 0.25 mm I.D., 0.25 μm, Agilent Technologies), with the flow pressure of helium set at 1.0 mL/min. The split ratio was 2:1. The ionization potential of the MS was 70 eV, and the scan range was 40−450. The identification of volatiles was achieved by comparing the mass spectral data of samples with those of the Wiley Library (Hewlett-Packard Co.) as well as those of the standards. The area of each peak was integrated using ChemStation software.

NMR Analysis. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were taken on a Varian Unity Inova AS 400 FT-NMR spectrometer (Varian, Palo Alto, CA).[17]

Statistical Analysis. All experiments were replicated at least three times, and statistical analysis was conducted using the SAS (SAS Inst., Inc., Cary, NC). The results in the figures are presented as means ± standard deviations (n = 3). Analysis of variance (ANOVA) was performed by ANOVA procedures. Duncan's multiple-range test was used to determine differences between the means, and $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Identification of CLnA Isomers by GC-MS and NMR. The extract obtained from the culture medium incubated with α-LNA was analyzed for CLnA production. GC-MS analyses of the ethyl esters of the extract revealed several fatty acid peaks. Among the converted α-LNA isomers, "a" was the major peak with a retention time of 15.7 min (Figure 1). Seven ethyl esters

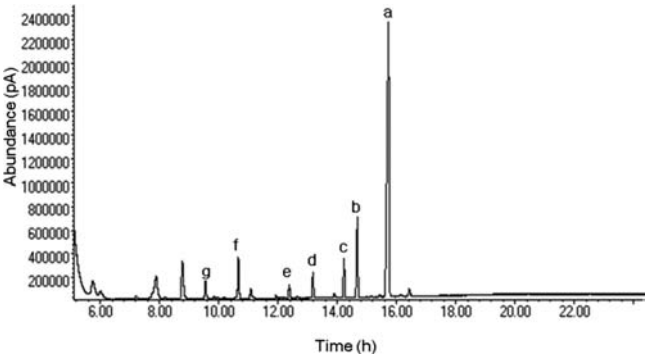

Figure 1. GC-MS separation of fatty acids produced by *B. breve* LMC520 after incubation with α-LNA. (a, c) Octadecatrienoic acid ethyl ester (C18:3, MW 306), (b, d) octadecadienoic acid ethyl ester (C18:2, MW 308), (e) octadecenoic acid ethyl ester (C18:1, MW 310), (f) heptadecanoic acid ethyl ester (C17:0, MW 298), and (g) hexadecanoic acid ethyl ester (C16:0, MW 284).

in the medium were identified by GC-MS, and the peaks "a" and "c" had the same molecular weight (MW 306) with an octadecatrienoic acid ethyl ester. The peaks "b", "d", "e", "f", and "g" were identified as ethyl esters of CLA (MW 308), LA (MW 308), oleic acid (MW 310), IS (MW 298), and palmitic acid (MW 284), respectively. The CLA was converted from

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Table 1. ^{1}H NMR, ^{13}C NMR, gCOSY, and gHMBC Data of CLnA [(9*Z*,11*E*,15*Z*)-LNA] (in $CDCl_3$)[a]

Carbon	δ_H[b]	δ_C[c]	gHMBC[d]
1	-	179.9	
2	2.32 (t, *J*=7.2 Hz, 2H)	34.1	H2↔C3 (J_2), H2↔C1 (J_2)
3	1.61 (tt, *J*=7.2, 7.2 Hz, 2H)	24.7	H3↔C1 (J_3), H3↔C2 (J_2)
4	1.20~1.38 (m, 8H)	29.1	
5		29.2	
6		29.7	
7		31.7	
8a	2.13 (tdd, *J*=7.2, 14.8, 10.8 Hz, 1H)	27.7	H8↔C10 (J_3)
8b	2.14 (tdd, *J*=7.2, 6.8, 10.8 Hz, 1H)		
9	5.33 (ddd, *J*=7.2, 14.8, 10.8 Hz, 1H)	130.1	H9↔C11 (J_3), H9↔C8 (J_2), H9↔C10 (J_2)
10	5.92 (dd, *J*=10.8, 10.8 Hz, 1H)	128.5	H10↔C12 (J_3), H10↔C8 (J_3), H10↔C11 (J_2), H10↔C9 (J_2)
11	6.29 (dd, *J*=14.8, 10.8 Hz, 1H)	125.8	H11↔C13 (J_3), H11↔C9 (J_3), H11↔C12 (J_2), H11↔C10 (J_2)
12	5.64 (dt, *J*=14.8, 7.2 Hz, 1H)	133.8	H12↔C14 (J_3), H12↔C10 (J_3), H12↔C13 (J_2), H12↔C11 (J_2)
13	2.12 (td, *J*=7.2, 7.2 Hz, 2H)	33.0	H13↔C11 (J_3), H13↔C15 (J_3)
14	2.03 (td, *J*=7.2, 7.2 Hz, 2H)	27.1	H14↔C12 (J_3), H14↔C16 (J_3)
15	5.29 (td, *J*=7.2, 10.8 Hz, 1H)	128.1	H15↔C17 (J_3), H15↔C13 (J_3),
16	5.38 (td, *J*=7.2, 10.8 Hz, 1H)	132.0	H16↔C18 (J_3), H16↔C14 (J_3)
17	1.24 (qt, *J*=7.6, 7.2 Hz, 2H)	22.7	H17↔C15 (J_3), H17↔C18 (J_2)
18	0.86 (t, *J*=7.6 Hz, 3H)	14.2	H18↔C17 (J_2), H18↔C16 (J_3)

[a]s, singlet; d, doublet; t, triplet; and q, quartet. [b]^{1}H NMR (400 MHz). [c]^{13}C NMR (100 MHz). [d]^{1}H−^{13}C gradient heteronuclear multiple bonding connectivity: mainly, J_3 correlation.

20% LA in the original fat substrate by *B. breve* LMC520, and the oleic and palmitic acids were carried over from the MRS medium.

The structure determination using NMR was carried out by the signal of the major component. The ^{1}H and ^{13}C NMR data suggested that the major component signal was an unsaturated fatty acid. The ^{1}H NMR spectrum of the major component signal showed six olefin methine protons [δ_H 6.29 (dd, *J* = 14.8, 10.8 Hz, H-11), δ_H 5.92 (dd, *J* = 10.8, 10.8 Hz, H-10), δ_H 5.64 (dt, *J* = 14.8, 7.2 Hz, H-12), δ_H 5.38 (td, *J* = 7.2, 10.8 Hz, H-16), δ_H 5.33 (ddd, *J* = 6.8, 14.8, 10.8 Hz, H-9), and δ_H 5.29 (td, *J* = 7.2, 10.8 Hz, H-15)], eight allyl methylene protons [δ_H 2.32 (t, *J* = 7.2 Hz, H-2), δ_H 2.14 (tdd, *J* = 7.2, 14.8, 10.8 Hz, H-8b), δ_H 2.13 (tdd, *J* = 7.2, 14.8, 10.8 Hz, H-8a), δ_H 2.12 (td, *J* = 7.2, 7.2 Hz, H-13), δ_H 2.03 (td, *J* = 7.2, 7.2 Hz, H-14)], 12 methylene protons [δ_H 1.61 (tt, *J* = 7.2, 7.2 Hz, H-3), δ_H 1.20−1.38 (m, H3~H7, H17)], and a terminal methyl moiety of the fatty acid at δ_H 0.86 (t, *J* = 7.6 Hz, H-18), indicating that peak "a" was a fatty acid with three olefin groups. The ^{13}C NMR spectrum yielded a carbonyl carbon signal at δ_C 179.9 (C-1), six olefin methane carbon signals [δ_C 133.8 (C-12), δ_C 132.0 (C-16), δ_C 130.1 (C-9), δ_C 128.5 (C-10), δ_C 128.1 (C-15), and δ_C 125.8 (C-11)], 10 methylene carbon signals [δ_C 34.1 (C-2), δ_C 33.0 (C-13), δ_C 31.7 (C-7), δ_C 29.7 (C-6), δ_C 29.2 (C-5), δ_C 29.1 (C-4), δ_C 27.7 (C-8), δ_C 27.1 (C-14), δ_C 24.7 (C-3), and δ_C 22.7 (C-17)], and a terminal methyl carbon signal at δ_C 14.2 (C-18). Totally, these 18 carbon signals indicated unsaturated octadecanoic acid including three olefins. Additionally, the locations of the three olefin groups were verified based on gradient correlation spectroscopy (gCOSY) and gradient heteronuclear multiple bond coherence (gHMBC) (Table 1). On the gCOSY spectrum, the olefin methine proton signal of H-9 (δ_H 5.33) showed J_3 correlation peaks with H-8a (δ_H 2.13)/8b (δ_H 2.14) and J_3 correlation peaks with H-10 (δ_H 5.92), with the chemical constant as 10.8 Hz, indicating zusammen-conformation (*Z*). H-10 showed J_3 correlation peaks with H-11 (δ_H 6.29) with the chemical constant as 10.8 Hz indicating (*Z*)-conformation, and H-11 showed J_3 correlation peaks with H-12 (δ_H 5.64) with the chemical constant as 14.8 Hz, indicating entgegen-conformation (*E*). Therefore, two olefin groups were coupled in order, such as *Z*−*Z*−*E*. H-16 (δ_H 5.38) showed J_3 correlation peaks with H-15 (δ_H 5.29) coupled with the chemical constant as 10.8 Hz, indicating (*Z*)-conformation. Determination of the final structure of the major component signal, including the location of the three olefin groups, was accomplished by gCOSY and gHMBC spectra. On the basis of these data, the major component signal was identified as 9*Z*,11*E*,15*Z*-octadeca-9,11,15-trienoic acid, comprising conjugated 9*Z*,11*E*,15*Z*-LNA (Figure 2).

Data from GC-MS and NMR showed that the major isomer was *cis*-9,*trans*-11,*cis*-15 CLnA (99%) converted from α-LNA by *B. breve* LMC520. It is known that some microorganisms can convert unsaturated fatty acids to conjugated counterparts.

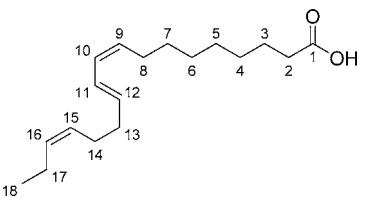

Figure 2. Chemical structure of 9*Z*,11*E*,15*Z*-LNA converted from α-LNA by *B. breve* LMC520.

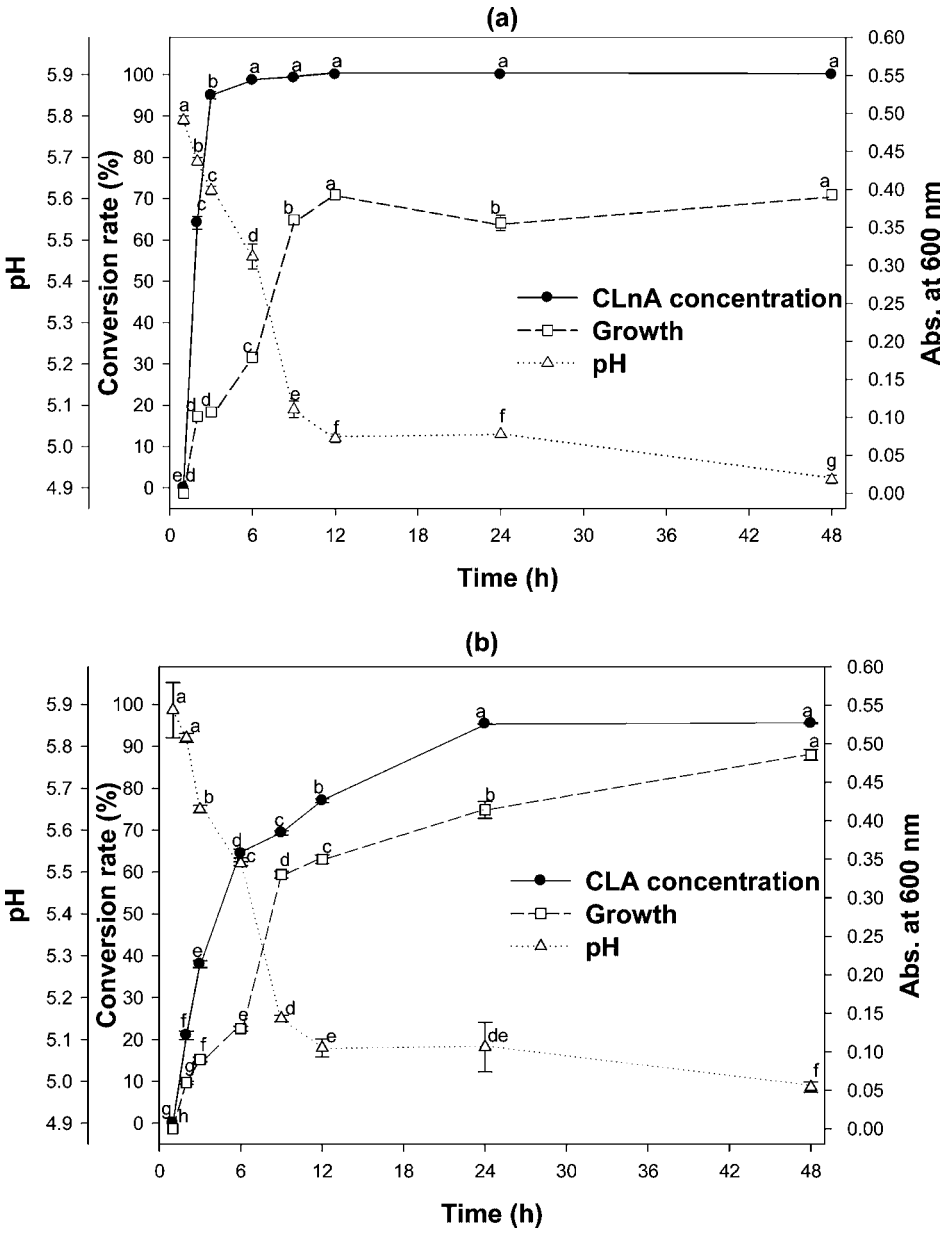

Figure 3. Conjugated fatty acid production by *B. breve* LMC520. *B. breve* LMC520 was inoculated into MRS medium containing 2 mM α-LNA (a) and 2 mM LA (b) and then incubated at 37 °C for 48 h under anaerobic conditions. The samples were analyzed by GC/FID. For letters a–h, values followed by different superscripts are significantly different for each column ($p < 0.05$).

Indeed, Ogawa et al.[18] reported that the α-LNA was converted to *cis*-9,*trans*-11,*cis*-15 CLnA by *Lactobacillus plantarum* AKU 1009a with about a 60% maximum conversion rate. In our previous study, *cis*-9, *trans*-11 CLA was produced by *B. breve* LMC520 with higher than 90% yield through the optimization of key environmental factors.[19] On the basis of these results, the characteristics of CLnA production were investigated as compared to those of CLA.

CLnA Production by *B. breve* LMC520. The production of CLnA by *B. breve* LMC520 was performed with 2 mM α-LNA under optimal culture conditions for CLA production from our previous study.[19] CLnA production was significantly increased in the middle of the logarithmic growth phase (6 h) and then reached a maximum level in the early stationary growth phase and prolonged incubation until 48 h did not appear to further increase CLnA production (Figure 3a). CLA production steadily increased until 24 h and was then maintained until the end of incubation (48 h; Figure 3b). Bacterial growth no longer increased after 12 h of incubation during CLnA production, whereas the growth was prolonged until 48 h of incubation during CLA production. The patterns of pH changes in two different fatty acid groups were similar during incubation. Overall, CLnA production was significantly higher than CLA production during the whole incubation period. CLA production proportionally increased with bacterial growth, whereas CLnA production significantly increased at the early logarithmic growth phase (from 0 to 6 h) without a correlation to bacterial growth, the pattern of which was similar between the two groups until 12 h. Moreover, most of the α-LNA was converted to CLnA, which was produced at its highest rate even before reaching maximum bacterial growth. In addition, Ogawa et al.[18] observed the conversion of *cis*-9,*trans*-11,*cis*-15 CLnA to *trans*-10,*cis*-15 CLA, whereas the *cis*-9,*trans*-

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11,*cis*-15 CLnA produced by *B. breve* LMC520 was not converted to other fatty acids.

When *B. breve* LMC520 was incubated at different concentrations of α-LNA up to 10 mM, CLnA production proportionally increased (Figure 4). The conversion rate was

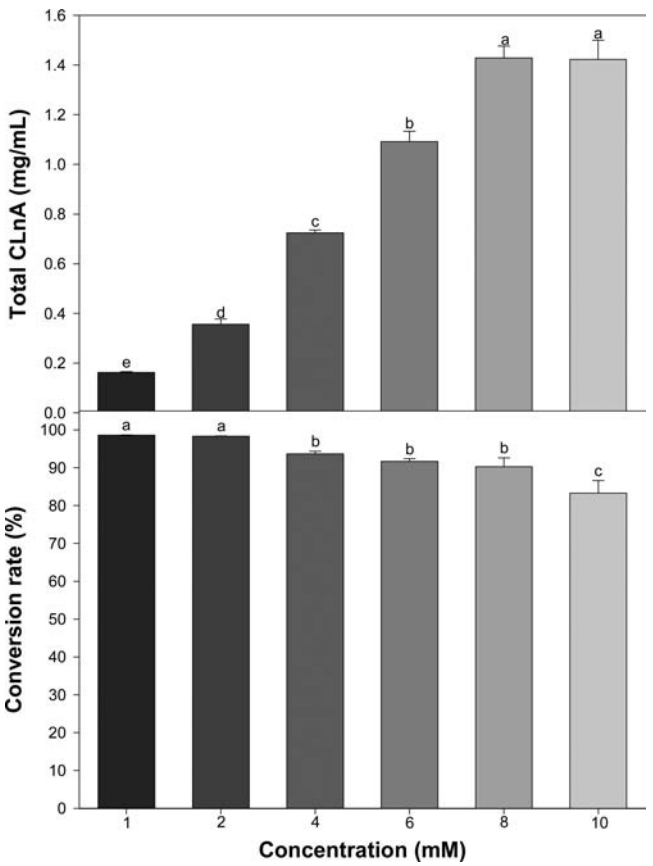

Figure 4. Conjugated fatty acid production at different α-LNA concentrations. *B. breve* LMC520 was incubated in MRS medium containing different concentrations of α-LNA at 37 °C for 24 h under anaerobic conditions. The samples were analyzed by GC/FID. Bars with different letters are significantly different ($p < 0.05$).

maintained at more than 90% of added α-LNA up to 8 mM. In our previous study,[19] the LA concentration for maximal CLA conversion was 1 mM, but CLnA production was not decreased up to 2 mM, and the yield was 30% higher as compared with CLA production at the same concentration (2 mM). The optimal LA concentration for CLA production was 1 mM, whereas the maximal conversion to CLnA was attained up to 2 mM α-LNA. This is noteworthy since fatty acids with higher degrees of unsaturation are typically inhibitory to the growth of most bacteria. In addition, the CLnA conversion for CLA production was 1 mM, whereas the maximal conversion to CLnA was attained at up to 2 mM a-LNA.

For bacterial growth and the activity of conjugated fatty acid-producing enzymes, pH is also an important factor. The highest level of CLA production (0.46 mg/mL) was obtained at pH 5.5 and decreased with pH (Figure 5b), but the highest CLnA production was attained between pH 5 and pH 9, which was higher than 98%, and no linear relationship was observed between pH and CLnA production (Figure 5a). CLnA production was relatively lower under acidic conditions (<pH 5.0) as compared to alkaline conditions. CLnA production was

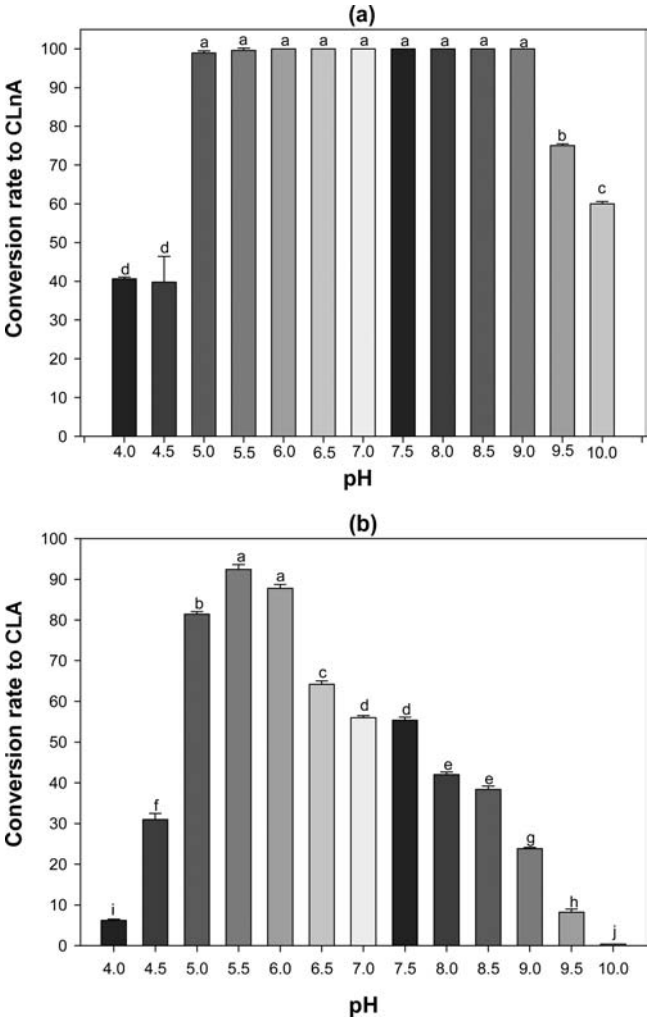

Figure 5. Effects of pH on conjugated fatty acid. The washed cells of *B. breve* LMC520 were incubated in different pH buffers with α-LNA (a) and LA (b) at 37 °C for 3 h under anaerobic conditions. The samples were analyzed by GC/FID. Bars with different letters are significantly different ($p < 0.05$). Graph (b) is adopted from our previous work (Park et. al.[19])

significantly higher than CLA production at all tested pH values. It was also notable that this strain had a very wide spectrum of optimal pH for CLnA production as compared to CLA production, which indicates that isomerase activity of this strain may be less pH-dependent for more highly unsaturated fatty acid substrates. Although CLnA production was significantly lower under acidic conditions than alkaline conditions, CLnA production was always greater than CLA production. This could be another advantage of using this strain as a functional starter culture.

The conversion rate was also compared at different atmospheric conditions. Although there was a significant difference in CLnA production between the aerobic and the anaerobic conditions until 9 h of the incubation, there was no apparent difference shown afterward (Figure 6). However, CLA production was significantly higher in anaerobic conditions, and the growth pattern under both aerobic and anaerobic conditions was similar to the pattern of CLA production.[19] On the other hand, CLnA production was similar in both the aerobic and the anaerobic conditions after 12 h. Moreover, CLnA production was greater than CLA production in both

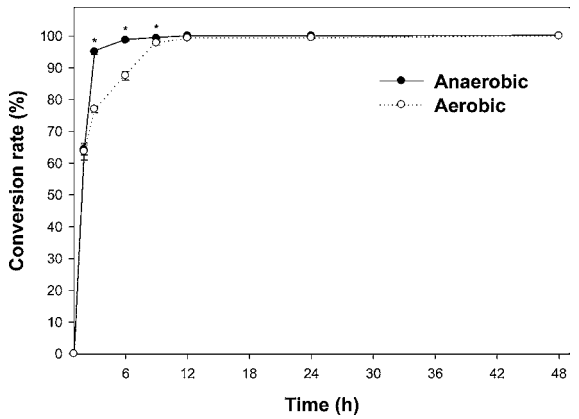

Figure 6. Conjugated fatty acid production at different atmospheric conditions. *B. breve* LMC520 was incubated in MRS medium containing 2 mM α-LNA at 37 °C for 48 h under anaerobic and aerobic conditions. The samples were analyzed by GC/FID. Asterisks indicate that the values at each time point are significantly different ($p < 0.05$).

atmospheric conditions. In general, *Bifidobacterium* species are considerably inhibited under aerobic conditions, but this was not the case for *B. breve* LMC520 and its CLnA production. In a previous study,[19] CLA production was significantly higher in anaerobic conditions than in aerobic conditions. On the other hand, there was no significant difference in CLnA production between aerobic and anaerobic conditions in the present study. This indicates that *B. breve* LMC520 had relatively high aerotolerance, and atmospheric conditions did not affect its production ability. *Butyrivibrio fibrisolvens* A38, which is known to be a major CLA-producing rumen bacterium, has a high rate of biohydrogenation that results in the saturation of most unsaturated fatty acid substrates.[20] This rumen bacterium is a strict anaerobe, and CLA production is higher under aerobic conditions due to the inhibition of biohydrogenation in aerobic conditions. However, *B. breve* LMC520 did not show biohydrogenation activity. This indicates that *B. breve* LMC 520 is a desirable starter strain for CLnA accumulation, especially during lactic acid fermentation.

Polyunsaturated fatty acids are converted to several conjugated fatty acids with various biological functions by some bacteria. Gorissen et al.[21] and Coakley et al.[22] reported that *B. breve* strains were able to convert α-LNA to CLnA with conversion rates varying from 55 to 66% and from 49 to 79%, respectively. In addition, *B. bifidum* and *B. pseudolongum* strains have been reported to show isomerase activity to convert α-LNA into CLnA.[21] On the basis of the reported data, *B. breve* LMC520 appears to have much greater conversion ability (>99%) to produce CLnA than other bacteria at similar substrate concentrations. In addition, *B. breve* LMC520 showed compatibility with CLA-producing rumen bacteria in terms of the growth and fat substrate conversion, which indicated that this bacterium could be useful as a functional probiotics for ruminant use.[23] Overall, the conversion of α-LNA to CLnA by *B. breve* LMC 520 was dependent upon certain environmental factors, including incubation time, substrate concentration, pH, and atmospheric conditions. *B. breve* LMC520 was more active in CLnA production as compared to CLA production. To make it clearer, we are currently working on the substrate specificity for the conjugation activity of this enzyme to find whether there are distinct enzymes for each fatty acid. This study showed optimal environmental conditions that can be applied to maximize the CLnA production and to investigate isomer-specific effects in vitro and in vivo. Further optimization study is required for large-scale fermentation process, and its potential for physiological activities are under investigation.

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