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ARTICLE

Production of Conjugated Linoleic Acid (CLA) by *Bifidobacterium breve* LMC520 and Its Compatibility with CLA-Producing Rumen Bacteria

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This study was performed to characterize the ability of an active *Bifidobacterium* strain to produce conjugated linoleic acid (CLA) and to test its possible utilization as a probiotic compatible to the ruminal condition. *Bifidobacterium breve* LMC520 can actively convert linoleic acid (LA) to *cis*-9, *trans*-11-CLA, which is a major isomer derived from microbial conversion. LMC520 showed reasonable tolerance under acidic conditions (pH 2.5 with 1% pepsin) and in the presence of oxgall (0−3%). The growth and CLA production of LMC520 were tested under ruminal conditions and compared with those of *Butyrivibrio fibrisolvens* A38, which is a major CLA producer in the rumen as an intermediate in the biohydrogenation (BH) process. LMC520 converted 15% of LA to CLA under ruminal conditions, which was 2 times higher activity than that of A38, and there was no decline in CLA level during prolonged incubation of 48 h. The BH activity of LMC520 was comparable to that of A38. When LMC520 was cocultured with A38, even with slight decrease of CLA due to high BH activity by A38, but the level of CLA was maintained by the high CLA-producing activity of LMC520. This comparative study shows the potential of this strain to be applied as a functional probiotic not only for humans but also for ruminants as well as to increase CLA production.

KEYWORDS: *Bifidobacterium breve*; *Butyrivibrio fibrisolvens*; conjugated linoleic acid (CLA); biohydrogenation; probiotics; ruminant

INTRODUCTION

Probiotics are gradually becoming of interest for many human nutrition researchers as various studies have continually shown evidence of their benefits for the promotion of human health. Among the probiotics, bifidobacteria have been confirmed to have specific health benefits that can be mediated through any of the following: enhancement of vitamin synthesis and mineral bioavailability (*1*), improvement of immune function (*2*), reduction of gastrointestinal disturbances (*3*, *4*), and anticancer activity (*5*, *6*). Moreover, some strains of *Bifidobacterium breve* have been shown to produce conjugated linoleic acid (CLA), which has functional properties (*7*−*10*). CLA is a mixture of positional and geometric isomers of linoleic acid (LA; $C_{18:2}$, *cis*-9,*cis*-12-octadecadienoic acid) with conjugated double bonds. CLA is naturally found primarily in a variety of dairy foods and ruminant meats (*11*). Ruminant fats are the richest natural sources of CLA because it is a byproduct of microbial conversion in the rumen (*12*, *13*). The major CLA isomers in foods include the following: *cis*-9,*trans*-11-CLA (also called rumenic acid) > *trans*-7,*cis*-9-CLA > 11, 13-CLA (*cis*/*trans*) > 8,10-CLA (*cis*/*trans*) > *trans*-10,*cis*-12-CLA isomer > other isomers (*14*−*16*).

cis-9,*trans*-11-CLA is a major isomer produced by ruminants (*12*, *17*) and is mainly produced from LA by rumen bacteria such as *Butyrivibrio fibrisolvens* (*18*). *cis*-9,*trans*-11-CLA is an intermediate in the biohydrogenation (BH) of LA to stearic acid (SA) by the anaerobic rumen bacterium *B. fibrisolvens* (*19*). The reaction sequence of LA to SA involves at least three steps and begins with the isomerization of LA to *cis*-9,*trans*-11-CLA, followed by hydrogenation of the *cis*-double bond of the conjugated diene to yield *trans*-11-octadecenoic acid (*trans*-vaccenic acid). Vaccenic acid (VA) is known to be reduced to SA by microbial activity other than that of *B. fibrisolvens* in the rumen (*20*). VA can also be converted to *cis*-9,*trans*-11 CLA by the Δ9 desaturase enzyme in mammary tissue, providing another mechanism for CLA formation in milk (*18*). Typically, CLA as a dietary supplement is produced through organic synthesis, which includes various unidentified isomers, because natural occurrence is far below the physiologically effective level (*21*). On the basis of such reports, extensive trials have been performed to enrich CLA in ruminal tissue and dairy products and to maximize its health-promoting effects (*22*, *23*).

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In this study, previously isolated *B. breve* LMC520 that can actively convert LA to *cis*-9,*trans*-11-CLA was used to test its potential as a probiotic for both ruminants and humans and to increase CLA production in vitro.

MATERIALS AND METHODS

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

Substrate Preparation. For the addition of substrate to bacterial cells after activation, concentrated LA (99% purity; Sigma) and CLA (95% purity; Lipozen, Pyongtaek, Korea) solutions (100 mg/mL water with 200 mg of bovine serum albumin) were sterile filtered (pore size = 0−22 μm) before use for incubation and analysis. The substrates were dissolved in ethanol and added as a stock solution.

Bacterial Growth and Sampling. The study employed *B. breve* LMC520, which has the highest CLA-producing activity among screened bifidobacteria in our previous study (*24*). For the production of CLA, *B. breve* LMC520 was subcultured twice at 37 °C for 18 h in tubes (18 × 150 mm; Bellco, Vineland, NJ) containing 10 mL of modified MRS (MRS broth with 0.05% L-cysteine·HCl; Sigma) medium that were capped with septum stoppers (Bellco) and aluminum seals (Bellco) and had been flushed with O_2-free CO_2. *B. fibrisolvens* A38 (ATCC 19171) was activated anaerobically at 37 °C for 18 h in 10 mL of basal medium for rumen bacterial culture containing (per liter), 292 mg of K_2HPO_4, 292 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4000 mg of Na_2CO_3, 600 mg of cysteine hydrochloride, 10 g of Trypticase (BBL Microbiology Systems, Cockeysville, MD), 2.5 g of yeast extract, and branched-chain volatile fatty acids (1 mM each of isobutyrate, isovalerate, and 2-methylbutyrate), plus hemin, vitamins, and trace minerals (*25*). Glucose (2 mg/mL, final concentration) was prepared as a separate solution and was added after autoclaving. Then 2 mL of activated cultures was transferred to fresh a basal medium in serum bottles (100 mL) and incubated with different substrates (LA and CLA) at 37 °C for 48 or 72 h in the shaking incubator at 100 rpm. For the coculture, *B. fibrisolvens* A38 and *B. breve* LMC520 were subcultured in 10 mL of basal medium, respectively, and then inoculated at the same OD value into the 100 mL basal medium. Coculture of *B. fibrisolvens* A38 and *B. breve* LMC520 was activated for 18 h, and then substrate was added. Growth rate was estimated by microplate leader for optical density (OD) at 600 nm (Bio-Rad, Hercules, CA). Culture samples (1 mL) were collected through the butyl rubber stopper with a syringes and needles at 0, 3, 6, 9, 12, 24, and 48 h of incubation. In the comparative study of two bacteria on the BH activity, sampling was performed at 6, 12, 18, 24, 48, and 72 h of incubation.

Acid Tolerance. The acid tolerance of *B. breve* LMC520 was determined at pH 2.5 (pH adjusted with HCl) with 1% pepsin (Sigma P7000). Briefly, activated bacteria were harvested by centrifugation, washed with phosphate-buffered saline (pH 7.2), and incubated in acid solution (pH 2.5) at 37 °C for 3 h. Cell numbers were determined by plate counts after incubation.

Growth of *B. breve* LMC520 in Bile-Containing Medium. *B. breve* LMC520 was precultured anaerobically in mMRS at 37 °C for 24 h. These cells were inoculated at 2% into mMRS with 0, 0.5, 1, 2, and 3% oxgall (Difco) as a bile source and anaerobically incubated at 37 °C for 24 h with 2 mM (0.56 mg/mL) free LA. Cell growth was measured by a microplate reader at 600 nm.

Ethylation for Fatty Acid Analysis. One milliliter of medium with heptadecanoic acid ($C_{17:0}$) added as an internal standard was 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. The lower layer was evaporated with nitrogen until dry. The extracted lipids were ethylated using 10 mL of 2% H_2SO_4 in ethanol at 80 °C for 60 min (*26*). After the addition of 8 mL of saturated NaCl solution and 4 mL of *n*-hexane, fatty acid ethyl esters were obtained in the *n*-hexane layer and analyzed for total fatty acids, including CLA isomers, using a 7890A gas chromatograph with a flame ionization detector (Agilent Technologies). Fatty acid ethyl esters were separated using a Supelcowax-10 fused silica capillary column (100 m × 0.32 mm i.d., 0.25 μm film thickness; Supelco, Inc., Bellefonte, PA) with a 1.2 mL/min helium flow. The oven temperature was increased from 190 to 240 °C at a rate of 4 °C/min. The temperature of the injector and detector was 260 °C. One microliter of sample was injected into the column in the splitless mode (50:1). The peaks of each CLA isomer and other fatty acids were identified and quantified by comparison with the retention time and peak area of each fatty acid standard, respectively. Internal standard was included as an internal reference before extraction to determine the recovery of the fatty acids in each sample.

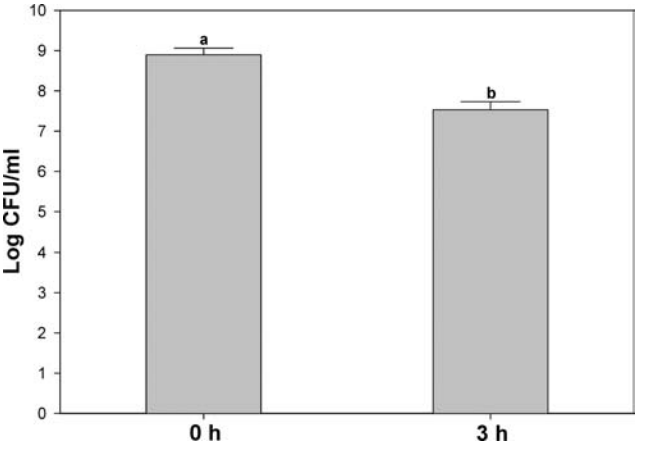

Figure 1. Acid tolerance test of *B. breve* LMC520. Bars with different letters are significantly different ($p < 0.05$). Error bars represent standard deviations from each data point ($n = 3$).

Statistical Analysis. All experiments were replicated at least three times, and statistical analysis was conducted using the SAS (SAS Institite Inc., Cary, NC). The results in the figures are presented as mean ± standard deviation ($n = 3$). Analysis of variance 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

Acid Tolerance and Bile Resistance of *B. breve* LMC520. There has been increasing interest in the effects of probiotic utilization and the incorporation of probiotics into various types of food products or as an adjunct. To be termed a probiotic, a micorganism has to maintain good viability because probiotics may come in contact with gastric juices and bile during intestinal transit. In general, optimal probiotic functionality can be obtained by sufficient doses (at least $\log^{9-10}$ cells per day) of living cultures (*27*). Therefore, acid tolerance and bile resistance are critical factors for probiotic application.

Although the cell growth of *B. breve* LMC520 decreased to some degree under acidic conditions (pH 2.5 with 1% pepsin) as compared to nonacidic conditions (**Figure 1**), it was thought to be clearly acid tolerant as shown in a previous study (*28*). Indeed, the survival of probiotics during intestinal transit should be estimated as approximately 10−40% of the administered total numbers (*29*), but the survival rate of LMC520 was found to be 84% higher. Because LMC520 is isolated from the human intestine, it was thought to be an acid-tolerant bacterium.

To be applied as a probiotic, a microorganism should be able to grow in medium containing 0.3% oxgall (*30*). When LMC520 was incubated with 0−3% oxgall, it grew at all tested oxgall concentrations and produced CLA (**Figure 2**). These results indicate that LMC520 has acid tolerance and bile resistance. Thus, LMC520 can be used as a probiotic source in food products or as an adjunct.

Coculture of *B. breve* LMC520 with Rumen Bacteria. As previously described, CLA is produced mainly as an intermediate product by microbial metabolism in the rumen, and there are

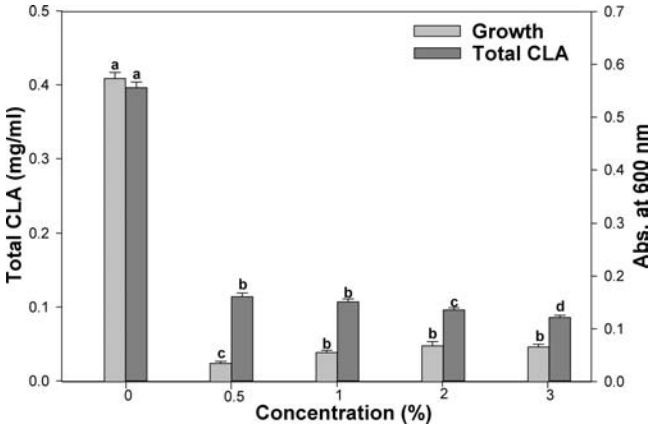

Figure 2. Bile resistance test of *B. breve* LMC520. Bars with different letters are significantly different ($p < 0.05$). Error bars represent standard deviations from each data point ($n = 3$).

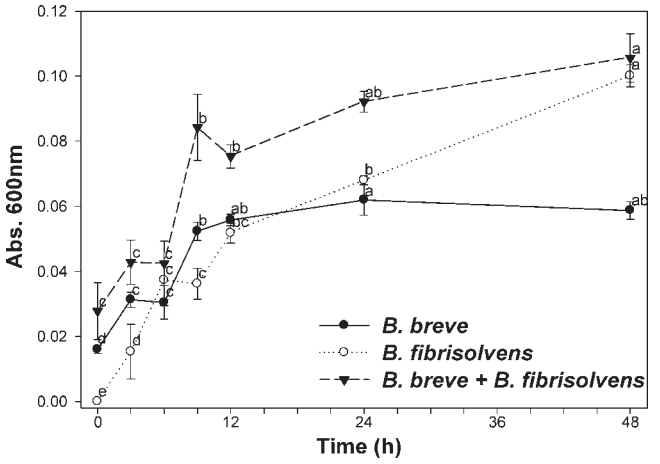

Figure 3. Growth of *B. breve* LMC520 and *B. fibrisolvens* A38 under ruminal condition alone and in the presence of *B. fibrisolvens* A38. Bacteria were inoculated into a basal medium for rumen bacterial culture and incubated at 37 °C for 48 h under anaerobic conditions. Values having different letters are significantly different for each column ($p < 0.05$). Error bars represent standard deviations from each data point ($n = 3$).

various microbes involved in CLA production (*20*, *31*). Among the microorganisms, *B. fibrisolvens* A38 was used in this study because it is found dominantly in the rumen of cattle and is a major CLA producer with high BH activity. CLA-producing activity was tested under ruminal condition for 48 h at 37 °C, and 1 mM free form of LA, which was the optimal concentration in a previous study (*32*), was used.

The growth of LMC520 declined after 24 h, but A38 showed a continued increase until the end of incubation (48 h) (**Figure 3**). When the two bacteria were cocultured, the total growth was similar to that of A38. Overall, except for stearic acid, the pattern of all fatty acid changes was similar and peaked at 6 h by A38 (**Figure 4**). After a decrease at 12 h of incubation, there was no further decrease during prolonged incubation until 48 h. To be more specific, the CLA produced from the LA by A38 was reduced to VA and to SA by the BH process in an early stage of incubation. This result indicates that A38 has high BH activity and low CLA-producing activity, as shown in our previous study (*20*). When LMC520 was incubated in a basal medium for rumen bacterial culture, the concentration of *cis*-9,*trans*-11-CLA significantly increased up to 2-fold as compared to that by

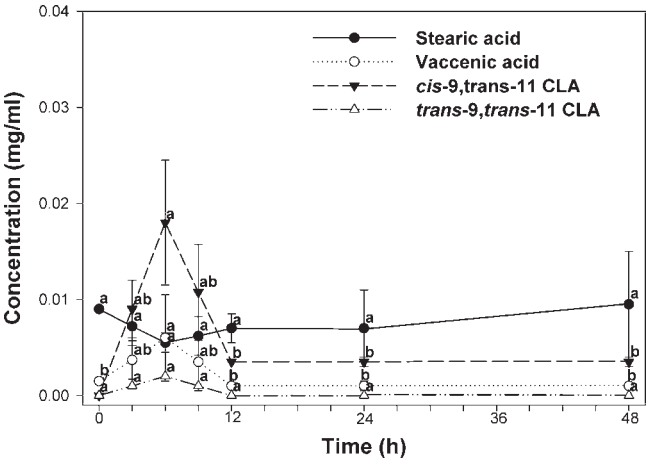

Figure 4. CLA production from free linoleic acid by *B. fibrisolvens* A38 under ruminal condition. Two percent (v/v) *B. fibrisolvens* A38 cultures were inoculated into a basal medium for rumen bacterial culture and incubated at 37 °C for 18 h under anaerobic conditions, and then 1 mM free linoleic acid was added. After the addition of free linoleic acid, the culture was incubated at 37 °C for 48 h under anaerobic conditions. Values having different letters are significantly different for each column ($p < 0.05$). Error bars represent standard deviations from each data point ($n = 3$).

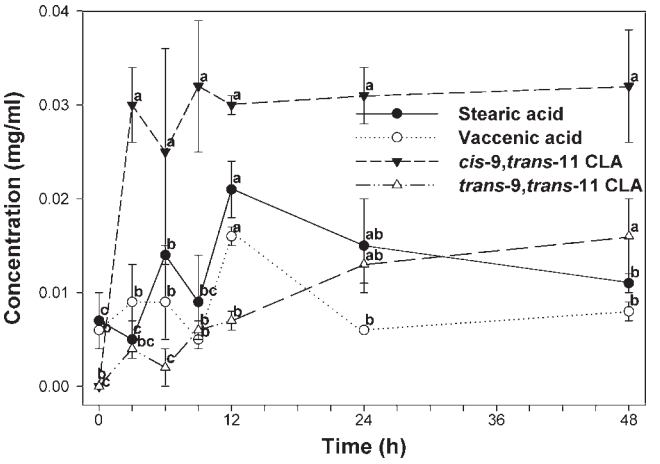

Figure 5. CLA production from free linoleic acid by *B. breve* LMC520 under ruminal condition. Two percent (v/v) *B. breve* LMC520 cultures were inoculated into a basal medium for rumen bacterial culture and incubated at 37 °C for 18 h under anaerobic conditions, and then 1 mM free linoleic acid was added. After the addition of free linoleic acid, the culture was incubated at 37 °C for 48 h under anaerobic conditions. Values having different letters are significantly different for each column ($p < 0.05$). Error bars represent standard deviations from each data point ($n = 3$).

A38 and did not decline during prolonged incubation (**Figure 5**). The concentration of *trans*-9,*trans*-11-CLA steadily increased for 48 h. This shows that LMC520 has less BH activity and higher CLA-producing activity as compared to A38.

A38, a natural inhabitant found in the ruminal environment of cattle, was tested for compatibility with LMC520, and the influence of each microbe on CLA production was examined in a medium for rumen bacterial culture. When LMC520 was cocultured with A38, the concentration of *cis*-9,*trans*-11-CLA reached a maximum level at 6 h and then steadily decreased but not significantly ($p > 0.05$) (**Figure 6**). The concentration of *trans*-9,*trans*-11-CLA increased gradually during the incubation for 48 h. The final concentration of *trans*-9,*trans*-11-CLA was

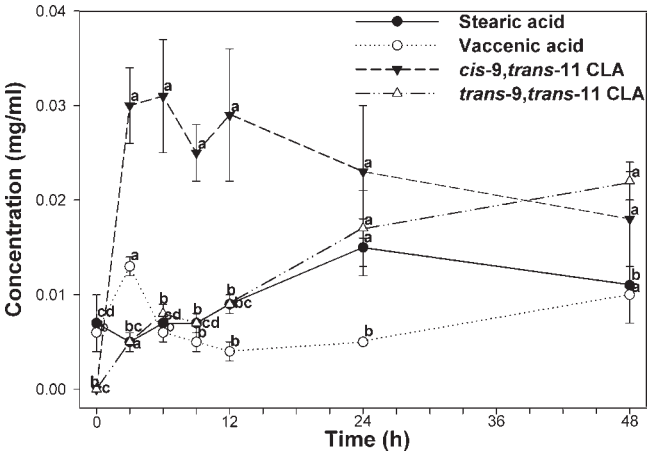

Figure 6. Compatibility of *B. breve* LMC520 with *B. fibrisolvens* A38 for CLA production under ruminal condition. Two percent (v/v) *B. breve* LMC520 and *B. fibrisolvens* A38 (1:1) cultures were inoculated into a basal medium for rumen bacterial culture. The cultures were incubated at 37 °C for 18 h under anaerobic conditions, and then 1 mM free linoleic acid was added. After the addition of free linoleic acid, the cultures were incubated at 37 °C for 48 h under anaerobic conditions. Values having different letters are significantly different for each column ($p < 0.05$). Error bars represent standard deviations from each data point ($n = 3$).

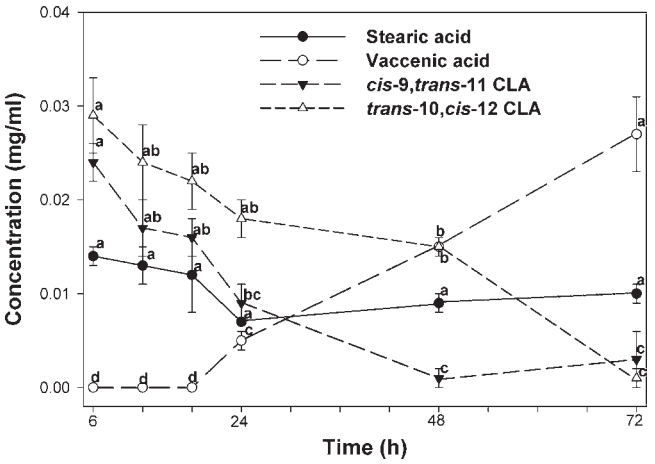

Figure 7. Biohydrogenation activity of *B. fibrisolvens* A38 under ruminal condition. Two percent (v/v) *B. fibrisolvens* A38 culture was inoculated into a basal medium for rumen bacterial culture. The culture was incubated at 37 °C for 18 h under anaerobic conditions, and then 0.06 mg/mL of CLA was added. After CLA addition, the culture was incubated at 37 °C for 72 h under anaerobic conditions. Values having different letters are significantly different for each column ($p < 0.05$). Error bars represent standard deviations from each data point ($n = 3$).

slightly higher than that of *cis*-9,*trans*-11-CLA. Both CLA isomers may have been decreased to some degree by the BH activity of A38, but there were no significant differences from the CLA profile of LMC520 alone. It was notable that this bifidobacterim with complex nutritional requirements for its growth could produce CLA in the minimal condition for the growth of rumen bacteria. Thus, CLA levels could be enhanced in the natural ruminal environment by LMC520 supplementation.

Subsequent studies were performed at the same conditions for 72 h with 0.06 mg/mL of CLA, which is the optimal concentration for the conversion by A38 (**Figure 7**), to compare the BH activity of the two bacteria. As shown in the previous experiment, when

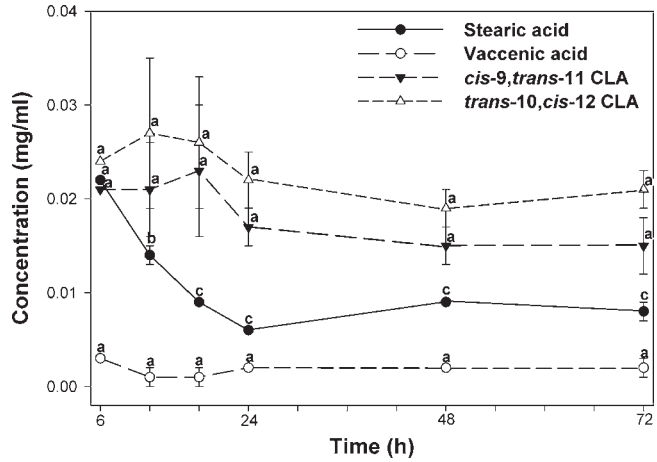

Figure 8. Biohydrogenation activity of *B. breve* LMC520 under ruminal condition. Two percent (v/v) *B. breve* LMC520 culture was inoculated into a basal medium for rumen bacterial culture. The culture was incubated at 37 °C for 18 h under anaerobic conditions, and then 0.06 mg/mL of CLA was added. After CLA addition, the culture was incubated at 37 °C for 72 h under anaerobic conditions. Values having different letters are significantly different for each column ($p < 0.05$). Error bars represent standard deviations from each data point ($n = 3$).

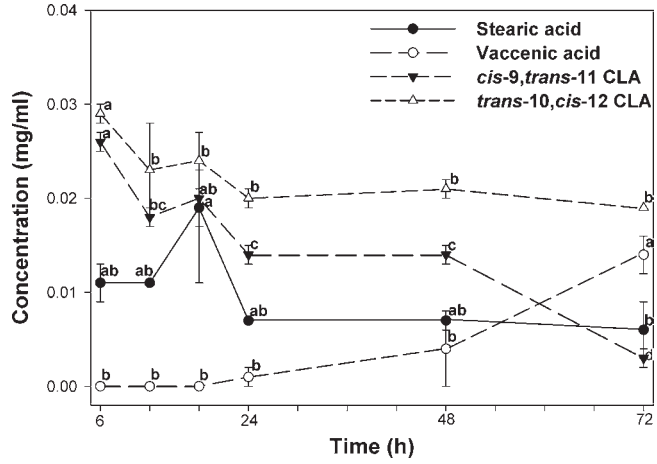

Figure 9. Compatibility of *B. breve* LMC520 with *B. fibrisolvens* A38 in terms of biohydrogenation activity under ruminal condition. Two percent (v/v) *B. breve* LMC520 and *B. fibrisolvens* A38 (1:1) cultures were inoculated into a basal medium for rumen bacterial culture. The cultures were incubated at 37 °C for 18 h under anaerobic conditions, and then 0.06 mg/mL of CLA was added. After CLA addition, the cultures were incubated at 37 °C for 72 h under anaerobic conditions. Values having different letters are significantly different for each column ($p < 0.05$). Error bars represent standard deviations from each data point ($n = 3$).

A38 was cultured with CLA, the two major CLA isomers almost disappeared after 48 h of incubation, and the concentration of VA increased proportionally. These results indicated that A38 is very active in BH, whereas LMC520 is not, which made it possible to maintain a high CLA level during long-term incubation (72 h) (**Figure 8**). When these two bacteria were co-incubated, CLA content slightly decreased by the BH of A38, but it was well maintained until the end of incubation by the high CLA-producing activity of LMC520 (**Figure 9**). These results indicate that LMC520 compensates for the BH activity of A38. Further investigation is underway with mixed rumen bacteria to test the compatibility as well as the possible

use of LMC520 as a feed additive to increase CLA content in ruminants.

In this study, we confirmed the possible utilization of *B. breve* LMC520 as a probiotic in the host (rumen) by testing the acid tolerance and bile resistance as the requirement for probiotic uses. To show functionality, which is the requirement of probiotics in the host, we confirmed CLA-producing activity of *B. breve* LMC520 in our previous study. *B. breve* LMC520 showed high CLA-producing activity and has low BH activity in a medium for rumen bacteria. The high BH activity of *B. fibrisolvens* A38 did not significantly affect CLA content primarily obtained from the activity of LMC520. On the basis of these results, we are testing the CLA-producing activity of *B. breve* LMC520 in the rumen fluid as mixed culture to confirm whether the produced CLA is stable in the ruminal environment. The conversion rate of this strain is very high at reasonably high concentration of linoleic acid, so relatively high CLA concentration could be maintained if this strain is stable at ruminal condition.

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