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Effect of High Hydrostatic Pressure on the Production of Conjugated Fatty Acids and Trans Fatty Acids by *Bifidobacterium breve* LMC520

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ABSTRACT: This study was performed to investigate the effect of high hydrostatic pressure (HHP) on the conversion of linoleic acid, conjugated linoleic acid (CLA), and α-linolenic acid (α-LNA) as substrates by *Bifidobacterium breve* LMC520 and to optimize the HHP condition. Cell mixture were tested under HHP in a variety of conditions such as temperature, time, pressure, and pre- or post-treatment with substrates. The *cis*-9,*trans*-11 CLA producing activity of *B. breve* LMC520 was increased by HHP, whereas *trans*-9,*trans*-11 CLA producing activity was decreased. Optimal HHP conditions for the highest CLA production were obtained at 100 MPa for 12 h at 37 °C. Post-treatment groups showed higher conversion activity of substrates than pretreatment groups. Post-treatment groups decreased *trans*-9,*trans*-11 CLA and other CLnA, whereas the pretreatment groups increased them. It is concluded that HHP treatment could be an important factor to enhance CLA and CLnA production and for reducing *trans*-fatty acids.

KEYWORDS: *high hydrostatic pressure (HHP), conjugated linoleic acid (CLA), conjugated linolenic acid (CLnA), trans-fatty acid, Bifidobacterium breve LMC520*

INTRODUCTION

Some strains of bifidobacteria produce conjugated fatty acids including conjugated linoleic acid (CLA) and conjugated linolenic acid (CLnA). CLA is composed of positional and geometric isomers of linoleic acid (LA; $C_{18:2}$, *cis*-9,*cis*-12 octadecadienoic acid) with conjugated double bonds, and it has been the focus of considerable research targeting its functional properties such as antioxidation, cancer inhibition, cholesterol reduction, growth promotion, and immune enhancement.[1−4] CLnA refers to a group of octadecatrienoic acid ($C_{18:3}$) positional and geometric isomers that contains three conjugated double bonds, and it occurs abundantly in some plant seed oils.[5,6] Considerable interest has also been focused on CLnA isomers due to their physiological effects including antioxidation,[7] antiobesity,[8] and anticarcinogenesis.[9] Polyunsaturated fatty acid production has been reported for various lactic acid bacteria; however, the metabolic details and characteristics of the enzymes have not been described clearly. Further investigation on the fatty acid transformation in a wider range of anaerobic microorganisms is needed.

High hydrostatic pressure (HHP) is one of the most encouraging alternatives to traditional thermal treatments for sterilizing and preserving fluid food and provides an efficient method to extract food sources and to obtain native protein molecules from insoluble aggregates, and even covalently cross-linked aggregates.[10] Therefore, various biotechnological HHP applications have been developed. Among the applied biotechnology fields, the use of HHP in food technology has increased rapidly.[11] Food pasteurized by HHP is being marketed worldwide,[12,13] and HHP improves the safety of dairy products. The efficiency of HHP inactivating pathogenic microorganisms has been extensively studied.[14−17] Furthermore, the ability of HHP to inactivate viruses has been evaluated for vaccine development and virus sterilization.[18,19] In contrast, HHP can lead to misfolding and misassembly of proteins, which is necessary for biological function and recognition of proteins by other molecules, which leads to significant loss of biological activity.[20−23] Although HHP is an interesting and suitable tool to study protein functions, it has not been considered as a factor affecting the microbial production of conjugated fatty acid, and little information is available for industrial applications. It would be helpful to use HHP as a tool to detect subtle changes in the active site of important enzymes responsible for the microbial production of conjugated fatty acid.

The present study was conducted to characterize the substrates converting activity of *Bifidobacterium breve* LMC520 using HHP treatment. In particular, the effects of HHP treatment on the microbial production of conjugated fatty acids were studied.

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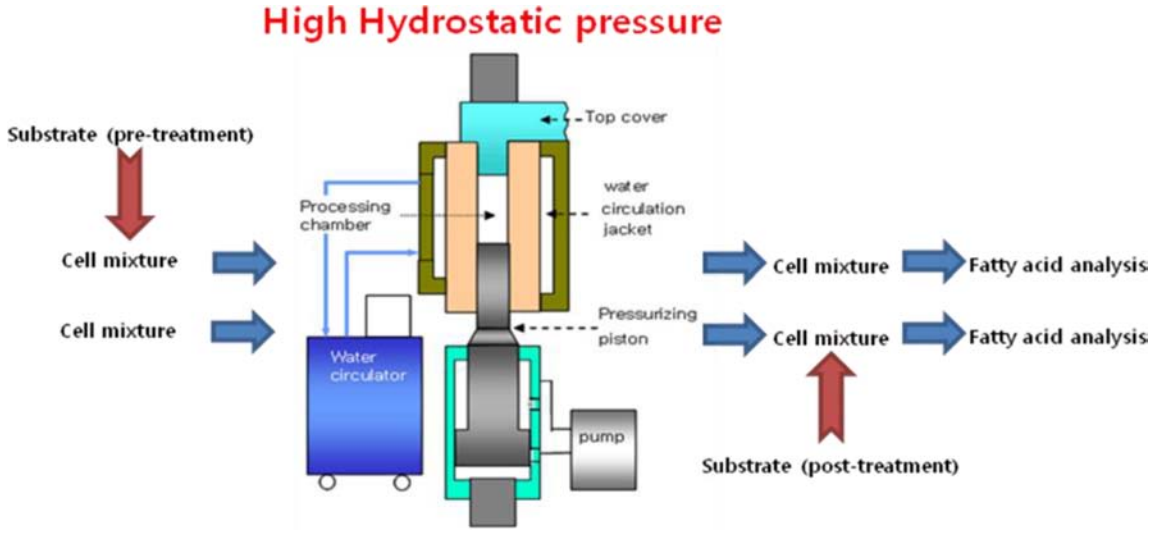

Figure 1. Scheme of pre- and post-treatment on the HHP treatment.

MATERIALS AND METHODS

Chemicals. Lipid standards were obtained from Sigma Chemical Co. (St. Louis, MO), and MRS broth was purchased from Difco (Detroit, MI). Passive lysis buffer (5×) was purchased from Promega (Madison, WI). All other chemicals used in the fatty acid analysis were analytical grade (Fisher-Scientific, Springfield, NJ).

Substrate Preparation. Concentrated LA (99% purity; Sigma), CLA (95% purity; Lipozen, Pyongtaek, Korea), and α-linolenic acid (90% α-LNA; Lipozen) solutions were dissolved in ethanol and added as a stock solution (1 M) to incubate the fatty acid substrates with bacterial cells after cultivation.

Bacterial Growth. This study employed *B. breve* LMC520, which showed the highest CLA-producing ability among the screened bifidobacteria in a previous study.[24] *B. breve* LMC520 was subcultured twice at 37 °C for 18 h in roll tubes (18 mm × 150 mm; Bellco, Vineland, NJ) containing 10 mL of 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.

High Hydrostatic Pressure Application for Conjugated Fatty Acid Production. *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 the cells (about 0.25 g) were resuspended in 1 mL of PBS buffer (pH 7.0) with 0.25 mL of passive lysis buffer and then disrupted with a sonicator in an ice bath for 10 min. The cell mixture was transferred onto polyethylene film and vacuum-packaged in a vacuum packer (BUSCH KOREA, Seoul, Korea). Pressure treatments were performed using a high pressure liquefy extractor (DFS-2L, TOYO KOATSU, Tokyo, Japan). Distilled water was used as the pressure medium, and targeted pressure was achieved in 3 min, whereas depressurization took <1 min. For the HHP experiment, the prepared pouches were subjected to various combinations of high pressure treatment (0−100 MPa) with different holding times (0−48 h) and temperatures (4 and 37 °C). The substrates (LA, CLA, and α-LNA) were added as substrates into the cell mixture (1 mL) before (pretreated-) or after (post-treated-) HHP treatment (Figure 1). The cells were not viable after treatment of lysis buffer, sonication and HHP. Except Figures 5 and 6, substrates were added after HHP treatment. The cell mixture treated with substrates was incubated at 20 °C for 3 h while shaking at 100 rpm on a rotating incubator. Total protein content was determined by the Bradford assay,[25] using pure bovine serum albumin as the standard.

Fatty Acid Analysis. One milliliter of cell mixture, with heptadecanoic acid ($C_{17:0}$) added as an internal standard (IS), 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, 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.[26] After adding 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 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 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 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. For the identification of CLnA, we compared this peak with previous study using GC-MS.[27] The IS was included as an internal reference before extraction to determine the recovery of fatty acids in each sample.

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

HHP Reaction Conditions. First of all, in order to confirm the effect of temperature for HHP treatment on the CLA production, HHP was applied to *B. breve* LMC520 at 4 and 37 °C after cell disruption (Figure 2). All treatment groups showed higher CLA production than that in the control group, except the 10 min, 4 °C group, and CLA production increased with HHP reaction time (Figure 2a). No significant difference was found between the two temperature conditions except the 10 min groups. These results indicate that CLA production during HHP treatment could be affected by reaction time and temperature. *trans*-9, *trans*-11 CLA decreased significantly following the HHP treatment, indicating that pressure may be a key factor for reducing undesirable *trans*-fatty acids (Figure 2b). It was apparent that *trans*-fatty acid production decreased as the HHP treatment was prolonged for up to 16 h. Indeed,

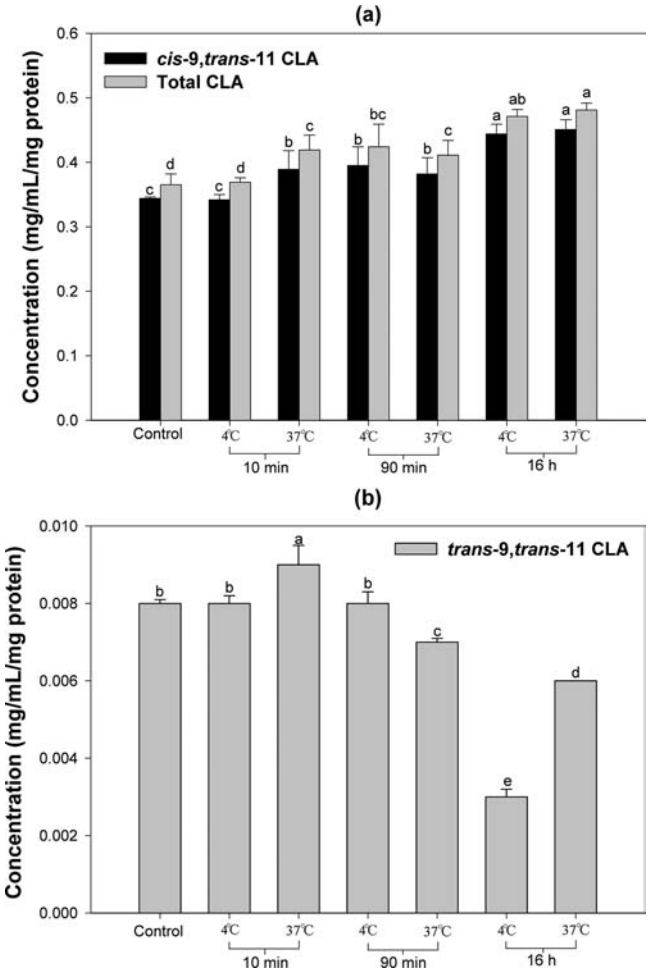

Figure 2. Effects of high hydrostatic pressure on conjugated linoleic acid (CLA) production. (a) Total CLA and *cis*-9,*trans*-11 CLA, (b) *trans*-9,*trans*-11 CLA. Reaction time: 10 and 90 min and 16 h. Temperature: 4 and 37 °C. Pressure: 100 MPa. Data was expressed as (mg/mL)/mg protein. Bars with different letters are significantly different ($p < 0.05$).

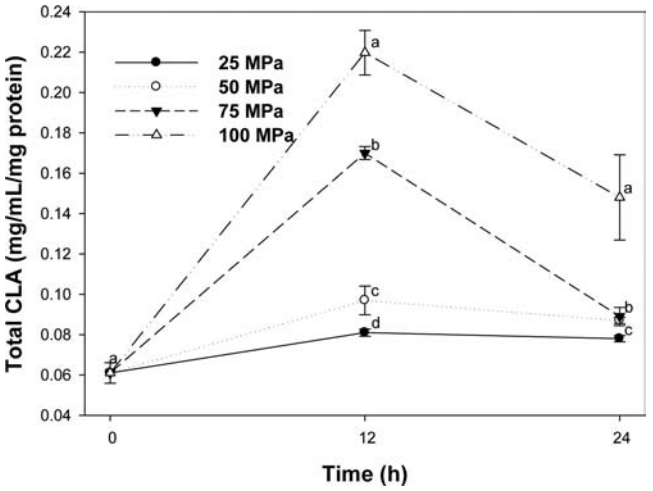

Figure 3. Effects of high hydrostatic pressure at different pressures. Reaction time: 24 h. Temperature: 37 °C. Pressure: 0−100 MPa. LA preincubated cells were used instead of the original LMC 520 cells. Data was expressed as (mg/mL)/mg protein. For letters a−d, values followed by different superscripts are significantly different at each time point ($p < 0.05$).

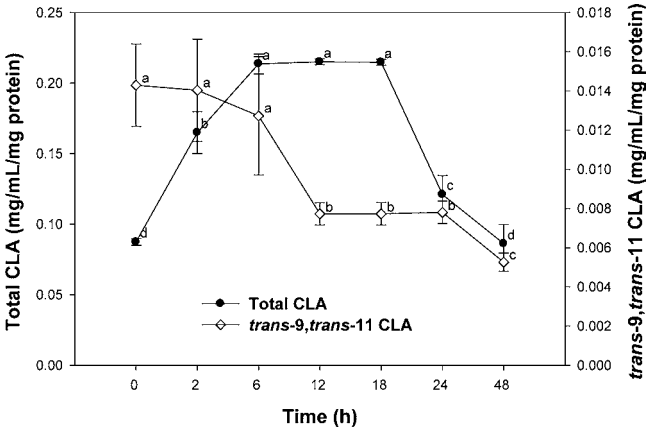

Figure 4. Effects of high hydrostatic pressure depending on reaction time. Reaction time: 0−48 h. Temperature: 37 °C. Pressure: 100 MPa. LA preincubated cells were used instead of the original LMC 520 cells. Data was expressed as (mg/mL)/mg protein. For letters a−d, values followed by different superscripts are significantly different for each column ($p < 0.05$).

trans-fatty acids decreased more than 2-fold at 4 °C after 16 h of treatment.

A preincubation with LA seemed to decrease enzymatic activity for CLA production in our previous study. To better show the difference, LA preincubated cells, which were incubated with 2 mM of LA for 7 d with daily passage, were used instead of the original LMC 520 cells (Figures 3 and 4), as LMC 520 cells showed almost complete conversion of LA into CLA.[28] The fatty acids in the LMC520 cells preincubated with LA were used as a blank. When the cells were incubated under different pressure conditions, CLA production increased nearly 3-fold in 12 h under the HHP condition (Figure 3). This result indicates that CLA-producing ability increased based on pressures up to 100 MPa.

CLA production increased steadily with duration of HHP reaction time up to 12 h, and then decreased significantly after 24 h (Figure 4). Notably, *trans*-9,*trans*-11 CLA production tended to decrease substantially with increased reaction time up to 48 h. The optimal HHP reaction time for CLA production occurred at 6−18 h, and *trans*-fatty acids decreased proportionally with HHP treatment.

Conjugated Fatty Acid Production Mechanism Using HHP. To confirm the changes in conversion of substrates during HHP treatment with cell mixture, the pretreatment groups were compared with the post-treatments (Figure 1). First, substrates were treated under HHP without cell mixture at 100 MPa to confirm whether the profile of substrates is changed by HHP treatment. None of the substrate treatments changed the fatty acid profile after HHP treatment (data not shown). This result indicates that the increase of CLA production in the previous results was not based on physical change by HHP. When LA was used as the substrate, the post-treatment group showed higher *cis*-9,*trans*-11 CLA production compared to that in the pretreatment group (Figure 5a). Interestingly, the *cis*-9,*trans*-11 CLA decreased in the pretreatment group, whereas the *trans*-9,*trans*-11 CLA level increased compared to the post-treatment group. This result was thought to be due to the alteration of enzymatic activity influenced by HHP, as the fatty acid profile was not changed when substrates were treated under HHP without cell mixture. This result was

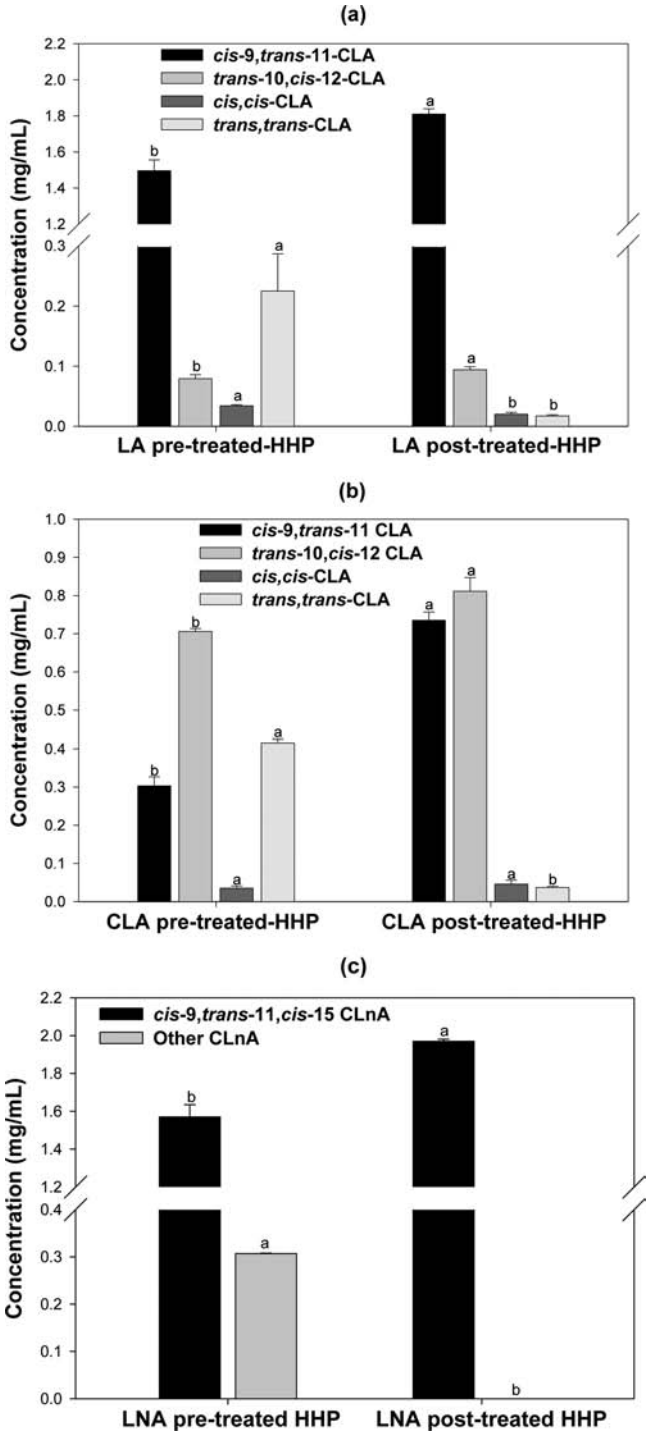

Figure 5. Change in conversion of LA (a), CLA (b), and α-LNA (c) as substrate by high hydrostatic pressure treatment (HHP). Reaction time: 12 h. Temperature: 37 °C. Pressure: 100 MPa. The substrates (2.0 mg/mL LA, 1.6 mg/mL CLA, and 2.0 mg/mL α-LNA) were added into the cell mixture (1 mL) before (pretreated-) or after (post-treated-) HHP treatment. Bars with different letters are significantly different ($p < 0.05$).

in a good agreement with the previous report of Coakley et al.[29] in which *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA were converted to *trans*-9,*trans*-11-CLA.

When CLA was used as the substrate, the result was similar to that of LA. In the pretreated-HHP group, *cis*-9,*trans*-11 CLA decreased, whereas *trans*-9,*trans*-11 CLA increased to the same degree compared with the post-treatment group (Figure 5b). The increase in *trans*-9,*trans*-11 CLA by pretreatment may be attributed to conversion from *cis*-9,*trans*-11 CLA. More importantly, *B. breve* LMC520 cells do not convert *cis*-9,*trans*-11 CLA under atmospheric pressure conditions.[28] Along with previous results that post-treatment of LA reduced *trans*-9,*trans*-11 CLA according to reaction conditions (Figures 2 and 4), these results clearly indicate that HHP treatment is a key factor for reducing *trans*-9,*trans*-11 CLA production.

In our previous study, *B. breve* LMC520 cells mainly produced only a *cis*-9,*trans*-11, *cis*-15 CLnA isomer from α-LNA.[27] The *cis*-9,*trans*-11,*cis*-15 CLnA isomer was not converted to other isomers. In the present study, α-LNA was used as the substrate to identify the effect of HHP treatment. It was notable that a change in the two CLnA isomers was observed similar to the previous study (Figure 5a,b). When α-LNA was used as the substrate, other CLnA isomer was produced in the pretreatment group (Figure 5c). This result was also thought to be due to conversion from *cis*-9,*trans*-11, *cis*-15 CLnA.

When CLA and CLnA production was compared to the ratio of concentration/protein, the post-treatment group showed increased production compared to that in the pretreatment group for all tested substrates (Figure 6).

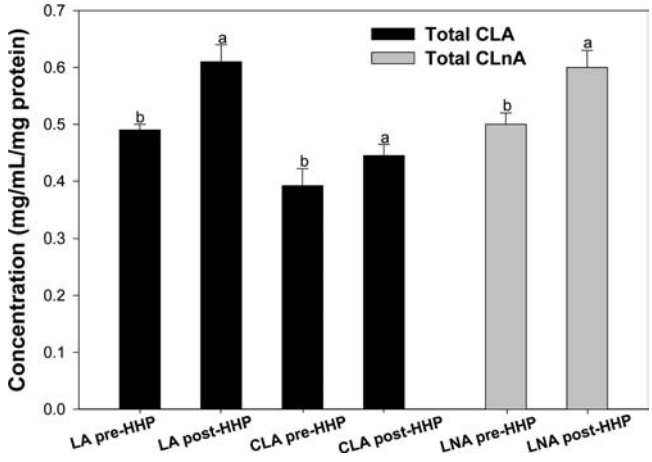

Figure 6. Comparison between pretreatment and post-treatment on the CLA and CLnA production. Data was expressed as (mg/mL)/mg protein. Bars with different letters are significantly different ($p < 0.05$).

In conclusion, it is tempting to suggest that HHP technology could be introduced to industrial applications to increase microbial CLA and CLnA production. The *cis*-9,*trans*-11 CLA production by *B. breve* LMC520 was increased after HHP treatment, whereas *trans*-9,*trans*-11 CLA producing activity was decreased. The optimal condition of HHP treatment for the CLA production was at 100 MPa for 12 h at 37 °C in our study. It was also notable that adding the substrates into cell mixture after HHP treatment (post-treatment group) reduced non-specific isomers, particularly *trans*-fatty acids, whereas adding the substrates into cell mixture during HHP treatment (pretreatment group) increased *trans*-fatty acid production. The increase of *trans*-9,*trans*-11 CLA and other CLnA in the pretreatment group may be by the conversion of *cis*-9,*trans*-11 CLA and *cis*-9,*trans*-11,*cis*-15 CLnA. Some reports indicate that environmental stresses induce changes in fatty acids composition.[30,31] On the basis of these results, stress exposure, such as pressure, may induce the overexpression of several genes

associated with the conversion of *cis*-9,*trans*-11 CLA and *cis*-9,*trans*-11,*cis*-15 CLnA in *B. breve* LMC520 or the alteration of enzyme activity by the subtle modification of structural configuration of the enzyme.

We have not yet determined which is the crucial factor directly responsible for the resulting fatty acid profiles (CLA, CLnA, *trans*-fatty acids, and other CLnA isomers) by HHP. Further studies are ongoing to clarify the mode of action by enzyme purification and to demonstrate whether this tool is widely applicable under optimal conditions considering pH, time, and temperature. Other physical factors that may affect enzyme activity should also be studied to reduce the production of *trans*-fatty acid during industrial processes.

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Author Contributions

¶The last two authors equally contributed to this work as corresponding authors.

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Notes

The authors declare no competing financial interest.

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