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Isolation and Characterization of *Bifidobacterium longum* subsp. *longum* BCBR-583 for Probiotic Applications in Fermented Foods

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Recent human gut microbiome studies have supported that the genus *Bifidobacterium* is one of the most beneficial bacteria for human intestinal health. To develop a new probiotic strain for functional food applications, fourteen fecal samples were collected from healthy Koreans and the strain BCBR-583 was newly selected and isolated from a 25-year-old Korean woman's fecal sample using the selective medium for *Bifidobacterium*. Subsequent fructose-6-phosphate phosphoketolase (F6PPK) test and 16S rRNA gene sequencing analysis of the strain BCBR-583 confirmed that it belongs to *B. longum* subsp. *longum*. The stress resistance tests showed that it has oxygen and heat tolerance activities (5- and 3.9-fold increase for 24 h at 60 and 120 rpm, respectively; 78.61 ± 6.67% survival rate at 45°C for 24 h). In addition, gut environment adaptation tests revealed that this strain may be well-adapted in the gut habitat, with gastric acid/bile salt resistance (85.79 ± 1.53%, survival rate under 6 h treatments of gastric acid and bile salt) and mucin adhesion (73.72 ± 7.36%). Furthermore, additional tests including cholesterol lowering assay showed that it can reduce 86.31 ± 1.85% of cholesterol. Based on these results, *B. longum* BCBR-583 has various stress resistance for survival during food processing and environmental adaptation activities for dominant survival in the gut, suggesting that it could be a good candidate for fermented food applications as a new probiotic strain.

Keywords: Bifidobacteria, probiotics, bile salt resistance, mucin adhesion, cholesterol lowering

Since the first isolation of the genus *Bifidobacterium* from the feces of breast-fed infants in 1899 by Henri Tissier [1], it has been known to be one of the major genera in the human gastrointestinal tract [2]. Bifidobacteria are Gram-positive, non-motile, and strictly anaerobic bacteria with bifid or irregular rod shapes. They have been widely recognized as beneficial bacteria for promotion of human intestinal health [3]. These potential health benefits of Bifidobacteria include prevention of diarrhea [4], establishment of a healthy microbiota in pre-matured infants [5], alleviation of constipation [6], lactose intolerance [7], cholesterol reduction [8], gut immune stimulation [9], and cancer prevention [10]. However, while the composition rate of bifidobacteria in infant fecal microbiota is up to 96%, it is lower, at 19%, in adult fecal microbiota, suggesting that supplementation of bifidobacteria is required to maintain the adult gut health [11]. Therefore, various commercial bifidobacteria have been developed and supplemented in many fermented dairy products. Interestingly, the clinical feeding study of commercial bifidobacteria revealed that they may not colonize and survive in the human intestinal environment, probably due to the strain attenuation by loss of competitive fitness against other intestinal bacteria during commercial fermentation [12]. To take advantage of the health-benefiting effects of bifidobacteria in the gut, *B. longum* BCBR-583 was newly isolated from a healthy Korean fecal sample and characterized by various evaluation tests to validate its probiotic effects, such as stress resistance tests, gut environment adaptation assays, and additional health promotion functions. This newly isolated and scientifically evaluated probiotic strain may be a good candidate for various food applications to enhance

the gut health of Koreans.

To isolate Korean-oriented bifidobacteria, fourteen fecal samples were collected from healthy Koreans and 753 strains were selected and isolated using BIM-25 bifidobacteria selective medium [13] under anaerobic incubation at 37°C for 24 h (data not shown). Among them, the strain BCBR-583 was selected by prescreening with simple oxygen tolerance test for further probiotic evaluation tests. To identify this strain, fructose-6-phosphate phosphoketolase (F6PPK) test [14] and 16S rRNA gene sequencing analysis with NCBI BLASTN program [15] were performed. Their results substantiated that this strain belongs to *B. longum* subsp. *Longum* (data not shown).

Bifidobacteria are one of the most important probiotics to endow the specific health promoting effects in fermented food products. However, food processing procedures are too tough for probiotics to survive in this condition. Therefore, stress tolerance activities are generally required for survival during food processing. The survival rate of the selected *B. longum* BCBR-583 was evaluated under oxygen and heat stress conditions. Oxygen tolerance test was conducted in two different ways with the incubation at 37°C for 24 h: Static incubation on MRS agar plate without anaerobic condition and broth culture incubation with shaking at 60 or 120 rpm [16]. After incubation, numerous colonies were observed on the agar plates. In addition, the optical density of each broth culture was monitored at 595 nm wavelength ($OD_{595\ nm}$). While initial $OD_{595\ nm}$ was 0.120 ± 0.02 at 0 h, it increased to 0.602 ± 0.07 at 60 rpm and 0.470 ± 0.06 at 120 rpm after 24 h shaking incubation, suggesting that *B. longum* BCBR-583 has oxygen tolerance activity (Fig. 1A). In addition, a heat tolerance test was performed as a previously published protocol [17]: (1) anaerobic incubation for 12 h (2) heat shock at 42°C, 45°C, and 60°C for 3 h, respectively (3) recovery at 37°C for 12 h (4) determination of absorbance at 595 nm wavelength. While >99% of BCBR-583 was recovered after both 42°C and 45°C heat shock conditions, only <30% of the strain survived after 60°C heat shock, suggesting that it may have moderate tolerance activity to temperature stress conditions. Based on this result, long-term heat tolerance activity of BCBR-583 at 45°C was determined. After incubation at 45°C for 24 h without recovery step, its optical densities at 595 nm wavelength were compared. Comparing $OD_{595\ nm}$ at 37°C for 24 h, BCBR-583 showed viability of 78.61 ± 6.67% at 45°C. This result suggests that it is stable and heat-tolerant even at long-term heat stress conditions, indicating that it may be suitable for further commercial applications (Fig. 1B).

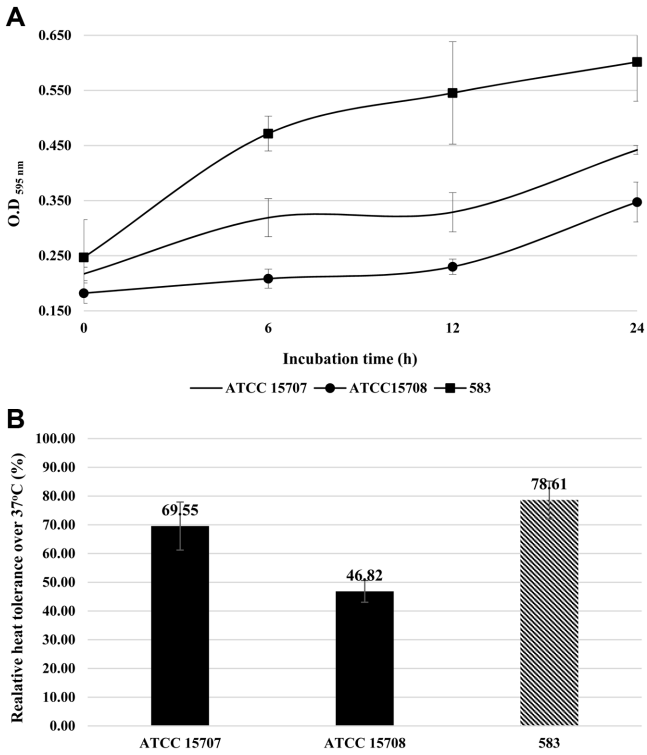

Fig. 1. Oxygen and heat tolerance tests for stress resistance with *B. longum* BCBR-583 and *B. longum* subsp. *longum* type strains, ATCC 15707 and ATCC 15708.

(**A**) Growth curves of three strains with shaking of 60 rpm at 37°C for 24 h. (**B**) Comparative 24 h heat tolerance test between 45°C and 37°C. Error bars indicate standard deviations.

To survive in the human intestinal environment, gastric acid/bile salt tolerance and mucin layer adhesion may be important. Therefore, gastric acid/bile salt tolerance and mucin adhesion activities of BCBR-583 were determined. Gastric acid/bile salt test were conducted using a previously published procedure [18]: (1) bile salt exposure at 37°C for 3 h (2) PBS washing (3) gastric acid exposure at 37°C for 3 h (4) viable cell count after incubation on MRS agar plate at 37°C for 24 h. The survival rate of BCBR-583 was 85.79 ± 1.53% in gastric acid/bile salt, which is similar to type strains, *B. longum* ATCC 15707 and 15708 (Fig. 2A). This result indicates that BCBR-583 may survive in the passages of the stomach and intestinal environments. In addition to survival in the digestive organs, adhesion to mucin surface layers is also important for colonization and propagation of bifidobacteria in the intestinal environment. After attachment of mucin (Sigma, Germany) on the surface of 96-well plates at 4°C for 12 h, BCBR-583 cells were added to the plate and incubated at 37° C for 3 h. The number of attached cells was

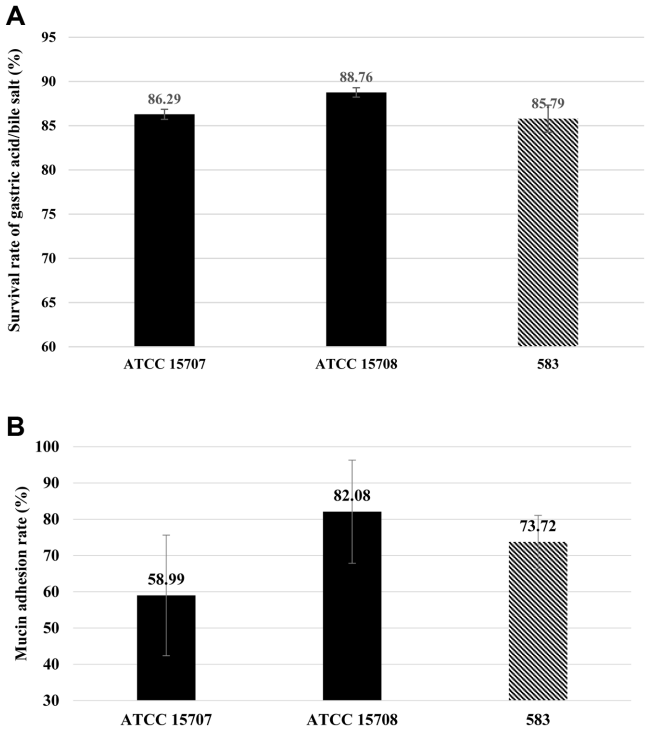

Fig. 2. Intestinal adaptation test of gastric acid/bile salt tolerance and mucin adhesion test with *B. longum* BCBR-583 and *B. longum* subsp. *longum* type strains, ATCC 15707 and ATCC 15708.

(**A**) Survival rate under 6 h treatments of bile salt (Pancreatin 0.1% (w/v), Bile 0.3% (w/v), final pH 8.0) and gastric juice (125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, 3 g/l pepsin, final pH 2.5 adjusted with 5 N HCl). (**B**) Mucin adhesion test with 10 mg/ml mucin (Type 3 porcine gastric mucin). Error bars indicate standard deviations.

determined with difference between viable cell number in the control culture and detached viable cell number in the PBS buffer after washing [17, 19]. The attachment rate of *B. longum* BCBR-583 was 73.72 ± 7.36%, which is a higher rate than that of ATCC 15707 (58.99 ± 16.65%), but a lower rate than that of ATCC 15708 (82.08 ± 14.22%) (Fig. 2B). This result suggests that BCBR-583 can survive during the passage of digestive organs and colonize in the mucosal cell surface layer of the human gut.

Because it is known that accumulation of excessive cholesterol in the blood stream may cause cardiovascular disease, concentration of cholesterol needs to be controlled. Interestingly, it was previously reported that bifidobacteria can assimilate cholesterol into their cell membranes [20]. To evaluate the cholesterol lowering activity of BCBR-583, it was cultured with 250 mM cholesterol in MRS broth medium and incubated at 37°C for 24 h. After incubation,

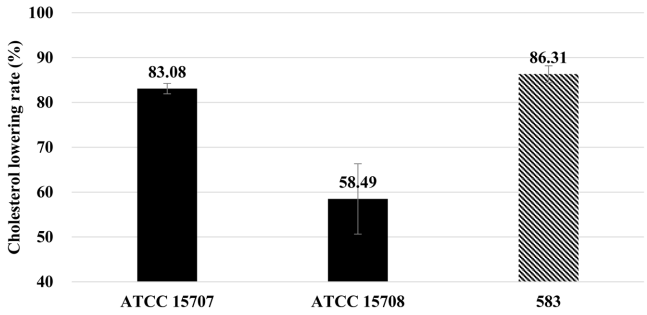

Fig. 3. Cholesterol lowering assay of *B. longum* BCBR-583 and *B. longum* subsp. *longum* type strains, ATCC 15707 and ATCC 15708 at 37°C for 24 h.

All experiments were performed in triplicate. Error bars indicate standard deviations.

the remaining cholesterol in the culture was determined using Total Cholesterol Assay Kit (Cell Biolabs Inc., USA). This result showed that *B. longum* BCBR-583 lowered up to 86.31 ± 1.85% of cholesterol in the culture, indicating its cholesterol lowering activity (Fig. 3). In addition, culture supernatant of BCBR-583 showed anti-*Helicobacter pylori* activity (diameter of inhibition zone, 16 ± 0.0 mm), comparing 15 mg/ml lactoferrin as a positive control (18.5 ± 2.12 mm) [21], suggesting that it can inhibit the growth of *H. pylori*. Furthermore, gut immune stimulation activity of BCBR-583 was determined using Caco-2 cells. To evaluate this activity, 10^8 CFU/ml of viable or dead BCBR-583 cells were added to Caco-2 cell culture and incubated for 24 h. After incubation, TNF-α, IL-6, IL-8, and IL-10 of Caco-2 cell culture supernatants were quantified using ELISA kit (Komabiotech, South Korea). These cytokines were mainly secreted by dead BCBR-583 cells (TNF-α, 10.55 ± 6.00 pg/ml; IL-6, 4.44 ± 2.76 pg/ml; IL-8, 44.28 ± 3.89 pg/ml; IL-10, 6.11 ± 1.7 pg/ml), suggesting that BCBR-583 has immune stimulation activity. Subsequent immune response assay with RAW 264.7 macrophage cells and dead BCBR-583 cells (10^8 CFU/ml) substantiated this activity (TNF-α, 57.32 ± 5.96 pg/ml; IL-6, 16.08 ± 3.7 pg/ml).

Therefore, the new probiotic strain, *B. longum* BCBR-583 revealed all required properties regarding survival and colonization abilities in the human gut environment as well as probiotic effects including cholesterol reduction, anti-*H. pylori* activity, and gut immune stimulation, suggesting that it can be a good candidate as a new probiotic strain of bifidobacteria for further probiotic applications in food industries. The GRAS (Generally Recognized As Safe) state of BCBR-583 support this. However, further in vivo evaluation tests of this strain to overcome various hurdles

may still be required for successful industrial applications as a new functional probiotic component in the near future.

Acknowledgements

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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