Identification of Potential *Bacillus subtilis* Probiotics from Korean Soybean Paste and Their Antimicrobial and Immune Activities

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Abstract

The potential probiotic of a total of 15 *Bacillus* species isolated from Korean soybean paste (*doenjang*) was evaluated. Among those tested, the CSY191 and CSY388 strains were selected as probiotic bacteria due to their acid and bile tolerance, respectively. These strains were classified as *Bacillus subtilis* based on morphological, physiological, and chemotaxonomic features as well as on phylogenetic analysis based on their 16S rDNA sequences. These strains CSY191 and CSY388 showed a significant survival with rate range of 30.0 to 58.3% and of 31.0% to 58.1%, respectively, under artificial gastric acidic conditions at pH 3.0. These CSY191 and CSY388 strains appeared to have high antimicrobial activity against *Salmonella* Typhimurium, *Bacillus cereus* and *Listeria monocytogenes*. Also, methanol extractions (surfactin-like compounds) of strain CSY191 and strain CSY388 activated RAW264.7 microphages and induced the production of nitric oxide (NO) in a concentration-dependent manner, respectively. Therefore, strain CSY191 and strain CSY388 can be used as potential probiotics.

Key words: probiotics, *Bacillus subtilis*, antimicrobial activity, immune activity

INTRODUCTION

Probiosis, although not a new concept, has recently begun to receive an increasing level of scientific interest. Probiosis is defined an association of two organisms that enhances the life processes of both. Probiotics are generally defined as live microbial feed supplements that can benefit the host by improving its intestinal balance (1) through immunomodulation, competitive exclusion of gastrointestinal pathogens, and secretion of antimicrobial compounds, which suppress the growth of harmful bacteria (1,2). Lactobacilli, streptococci, bifidobacteria, enterococci, and bacilli are the bacteria that are most often used in the production of probiotics (3). Although studies have demonstrated a direct probiotic effect of *Bacillus* spores, preliminary studies with poultry provided evidence of a competitive exclusion of *Escherichia coli* O78:K80 by *B. subtilis* (4), and a number of studies have demonstrated that harmful bacteria are suppressed by various *Bacillus* spore formers (2,5). Pinchuk et al. (6) reported the characterization of an antibiotic produced by the *B. subtilis* strain found in the commercial product Biosporin, which is known to inhibit the growth of *Helicobacter pylori*. Recently, Lim and Kim (7) reported the selection and characterization of

the bacteriocin produced by the *B. subtilis* BP6. In addition, a large number of *Bacillus* products are used as 'novel foods' or as dietary supplements, with claims of enhancing restoring the natural microflora to the gut and well-being of the consumer (8).

Fermented soybean foods, such as soybean pastes (*doenjang* and *cheonggukjang*), and soybean sauce (*kanjang*), have been served as side dishes for thousands of years, providing a major source of protein for Koreans. The production of Korean traditional soybean paste and sauce from the fermentation of soybeans depends predominantly on *Bacillus* species, notably *B. subtilis*, *B. pumilus*, *B. licheniformis*, and *B. amyloquefencies* (9). Foods derived from fermented soybeans have attracted considerable interest due to their excellent nutritional value and their beneficial impact on human health, such as acting as anticarcinogenic agents and as antioxidants (10,11). These foods are also known to play a role in both the prevention of heart attacks and the prevention of blood coagulation (12).

In the present study, the isolation of potential probiotics and classification of the isolates was performed by determining their morphological, physiological, and chemotaxonomic features, as well as by conducting phylogenetic analyses. In addition, the antimicrobial and im-

munological activities of potential *B. subtilis* probiotics were demonstrated.

MATERIALS AND METHODS

Isolation of *Bacillus* strains

Traditional Korean fermented soybean pastes (*deonjang*) were collected from both the local market and from Korean homes. Collected samples were diluted with 0.85% NaCl and, subsequently, 0.1 mL of the diluted suspension was plated on TSA (Difco, Detroit, MI, USA) plates. After the plates were incubated at 37^oC for 48 hr, a distinctive single colony was selected as a pure isolate on each TSA plate. Isolates were stored until needed at -80^oC with 20% sterile glycerol.

Determination of tolerance to acid, artificial gastric acid, and bile acid

The method described by Cho (13) was used to determine the tolerances to acid, artificial gastric acid, and bile acid. For determination of acid tolerance, isolated *Bacillus* strains were grown in TSB at 37^oC for 48 hr and were diluted to 10^6 CFU/mL in fresh TSB adjusted to pH 3.0 with hydrochloric acid (3 M). Diluted cells were incubated for 3 and 6 hr at 37^oC. After incubation, the cells were serially diluted in a phosphate buffer (0.1 M, pH 6.2) to neutralize the medium acidity, and the viable cells on the TSA plate were counted after 48 hr of incubation at 37^oC. The survival rate was calculated as the percentage of *Bacillus* sp. colonies grown on the TSA plate compared to the initial bacterial concentration.

For determination of the artificial gastric acid tolerance, an artificial gastric juice was created by adding 1% pepsin (Sigma-Aldrich, St. Louis, MO, USA) to the TSB at pH 3.0. Cells grown in TSB at 37^oC for 48 hr were diluted to 10^6 CFU/mL in fresh artificial gastric acids adjusted to pH 3.0. Diluted cells were incubated for 3 and 6 hr at 37^oC. After incubation, the cells were serially diluted in phosphate buffer (0.1 M, pH 6.2) to neutralize the acidity of the medium. The viable cells were then incubated on the TSA plate for 48 hr at 37^oC and were counted. The survival rate was calculated as the percentage of *Bacillus* sp. colonies grown on the TSA plate compared to the initial bacterial concentration.

For determination of bile tolerance, 15 μL of the cells grown in TSB at 37^oC for 48 hr (equivalent to 10^6 CFU/mL) was spotted on TSA plates containing oxgall bile (Sigma-Aldrich) at different concentrations (1~4% w/v). The plates were incubated at 37^oC for 5 days. The minimum inhibitory concentration (MIC) of the bile for the *Bacillus* strain was determined as the lowest concentration that completely inhibited cell growth as judged from visual examination.

The two potential probiotics *Bacillus* sp. CSY191 and CYS388 were screened from Korean soybean paste (*doenjang*) and compared to previously reported activities of the potential probiotic *B. subtilis* CS90 (13).

Morphological and physiological characteristics

Cell morphology was examined by light microscopy after Gram staining, and flagellum type was determined by transmission electron microscopy (JEM 1010, JEOL, Tokyo, Japan) using preparations negatively stained with 1% phosphotungstic acid. Phenotypic characterization was carried out using standard methods and API50CHB kits (bioMérieux, Montalieu Vercieu, France). The methods described by Cowan and Steel (14) were used for the following physiological tests: catalase and oxidase activity as well as hydrolyses of skim milk, gelatin, casein, cellulose, and starch. Growth in response to various concentrations of NaCl and temperatures was determined using TSB as a basal medium.

Chemotaxonomy and 16S rDNA analysis

The biomass for cellular fatty acid analysis was prepared from a culture grown on a TSA plate for 24 hr at 37^oC. Fatty acid methyl esters were prepared using the method described in the manual of the MIDI Microbial Identification System. The resultant esters were separated using a gas chromatograph fitted with a phenylmethyl silicone-fused silica capillary column (25 m ×0.2 mm; Hewlett Packard, Palo Alto, CA, USA).

The 16S rDNA of strain CSY191 and strain CSY388 were amplified by PCR. PCR amplification was performed as follows: 30 cycles of denaturation at 90^oC for 1 min, annealing at 55^oC for 30 sec, and extension at 72^oC for 1 min. The PCR primers used to amplify the 16S rDNA fragments were the bacterial-specific 5'-CGGAGAGTTTGATCCTGG-3' (1BF, forward) and 5'-TACGGCTACCTTGTTACGAC-3' (2BR, reverse) primers (13). Amplified 16S rDNA fragments were used as the sequencing templates. Nucleotide sequences were determined by the dideoxy-chain termination method using a PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer, Norwalk, CT, USA). Assembly of the nucleotide sequences was performed using the DNAMAN analysis system (Lynnon Biosoft, Quebec, Canada). All reference sequences were obtained from the National Center for Biotechnology Information and Ribosomal Database Project databases. The 16S rDNA similarity values were determined based on alignments, and the evolutionary distances were calculated. Phylogenetic analysis was performed using the neighbor-joining method (15). Bootstrap analysis was performed on the data (re-sampled 1,000 times) using the

DNAMAN analysis system (Lynnon Biosoft). Nucleotide sequence data reported for the 16S rDNA of the strains CSY191 and CSY388 are available in the GenBank database under the accession numbers HQ328857 and HQ328856, respectively.

Preparation of methanol extractions

Cells were grown in Number 3 medium (No. 3: 10 g polypeptone, 10 g glucose, 1 g KH_2PO_4, and 0.5 g $MgSO_4 \cdot 7H_2O$ per liter, pH 6.8) at $30^{\circ}C$. After 3 days of cultivation, the supernatant was collected by centrifugation and was adjusted to pH 2.0 using 2 N HCl. The resultant precipitate was collected by centrifugation and extracted three times with methanol (MeOH). The MeOH extractions (surfactin-like compound) were concentrated and then dissolved in MeOH.

Antimicrobial activity

Thirteen food-borne pathogenic bacteria, including *Escherichia coli* KCTC 1682, *Pseudomonas aeruginosa* KCTC 1750, *Salmonella enterica* KCTC 12456, *Salmonella* Enteritidis KCTC 12400, *Salmonella* Typhimurium KCTC 1925, *Shigella flexineri* KCTC 2008, *Shigella sonnei* KCTC 2518, *Bacillus cereus* KCTC 1012, *Listeria innocua* KCTC 3586, *Listeria ivanovii* KCTC 3444, *Listeria monocytogenes* KCTC 3569, *Staphylococcus aureus* KCTC 1916, and *Staphylococcus epidermidis* KCTC 1917, were used as test organisms. These test organisms were grown on the TSA plates at $37^{\circ}C$.

Fifty microliters of the MeOH extractions (100 μg/mL) were applied to 8-mm sterile paper disks, and the disk was placed on a TSA plate, which was spread with a test organism, for the antibacterial activity assay. After 48 hr of incubation at $37^{\circ}C$, antibacterial activity was measured as the diameter of the clear zone formed.

Immune activity

RAW264.7 murine macrophage-like cells (KCLB 40071) were obtained from the Korean Cell Line Bank in Seoul, Korea. Cells were incubated in Dulbecco's modified Eagle's medium (DMEM, Difico, Detroit, MI) supplemented with 10% (v/v) fetal bovine serum and penicillin (100 IU/mL) and streptomycin (100 μg/mL) at $37^{\circ}C$ in humidified air with 5% (v/v) CO_2. RAW264.7 cells were seeded at 2×10^5 cells/mL on a 96-well plate, incubated for 24 hr. After 24 hr, the cultured cancer cells were washed with phosphate buffered saline (PBS) buffer (pH 7.4), and then fresh medium was added on a 96-well plate. To examine nitric oxide production, this plate was treated with various concentrations of MeOH extractions (20 μL) from CS90, CSY191 and CSY388 strains or 1 μg/mL lipopolysaccharide (LPS; from *E. coli* Serotype O111:B4; Sigma-Aldrich) in PBS buffer (pH

7.4) for 24 hr. As control, cells were treated with only PBS buffer (pH 7.4) and mycoplasma for 24 hr. The cell supernatants (100 μL) were added to 96-well plates with the Griess reagent [1% (w/v) sulfanilamide in 5% H_3PO_4 : 0.1% (w/v) *N*-[1-naphthyl]-ethylenediamine in $H_2O = 1:1$] (100 μL), and absorbance was measured using a microplate reader (Bio-Rad, Philadelphia, PE, USA) at 570 nm. Sodium nitrite was used as a standard.

Statistical analysis

Data were expressed as means $\pm$ SD (standard deviation) of three replicates. The differences among all groups were analyzed by one-way analysis of variance (ANOVA) using the SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). In addition, when significant difference was indicated, Duncan's multiple range test was performed to separate difference; the level of significance was $p < 0.05$.

RESULTS AND DISCUSSION

Survival rate of isolated *Bacillus* strains under gastro-intestinal tract conditions

Survival rates of several *Bacillus* strains under acidic and artificial gastric conditions are shown in Table 1. In general, survival rates of the isolated *Bacillus* strains decreased with the lapse of time under acidic conditions. Among the strains isolated, CSY191 and CSY388 showed significantly high survival rate. Survival rate of CS90, as previously reported (13), and strain CSY191

Table 1. Survival rates of isolated strains from different soybean fermented foods under acidic and artificial gastric acidic conditions after 3 and 6 hr of incubation[1]

Isolated strain	Survival rate (%)			
	Acid (pH 3.0)		Artificial gastric acid (pH 3.0)	
	3	6	3	6
CS90[2]	67.0	43.0	57.4	28.1
CSY22	40.7	22.5	25.6	4.2
CSY49	45.8	34.6	35.6	10.9
CSY135	48.2	35.4	39.1	28.2
CSY191	68.8	65.1	58.3	30.0
CSY382	42.0	35.3	20.0	14.8
CSY383	10.2	1.0	4.6	0
CSY384	37.7	16.6	9.0	6.7
CSY385	38.5	16.7	3.3	2.5
CSY386	8.4	1.0	2.3	0
CSY387	13.7	1.9	0.6	0.2
CSY388	66.0	42.8	58.1	31.0
CSY391	38.9	21.5	6.2	1.4
CSY393	39.0	26.9	5.0	3.3
CSY398	44.7	23.6	23.8	16.8

[1]Each isolated *Bacillus* sp. was tested in triplicate for its tolerance in acidified and artificial gastric acidified TSBs.
[2]Cho (13) previously reported the potential probiotic *B. subtilis* CS90.

and strain CSY388 under acidic conditions of pH 3.0 after 3 hr were 67.0%, 68.8%, and 66.0%, respectively. *B. subtilis* CS90 and *Bacillus* sp. CSY191 and CSY388 showed significantly higher survival rates of 57.4%, 58.3% and 58.1%, respectively, under artificial gastric acidic conditions of pH 3.0 after 3 hr. All strains were able to withstand bile concentrations higher than 4.0% (data not shown).

Bacillus probiotics differ in many characteristics from those based on lactic acid bacteria (16,17). Whereas lactobacilli represent a normal resident GI microflora of humans, the saprophytic gastrointestinal tract (GIT) bacteria of *Bacillus* genera belong only to the transitory GI bacteria. Thus, the use of *Bacillus* products raised a number of questions, including their safety. Over the past three decades, this genus has expanded to include more than 100 species (19). However, only a few of these species are used as probiotics for humans, including the following: *B. subtilis*, *B. licheniformis*, *B. calusii*, *B. coagulans*, *B. cereus*, *B. pumilus*, *B. laterosporus*, as well as some invalid species called *B. toyoi* and *B. polyfermenticus* (2,3,18). On the contrary, Sorokulova et al. (19) reported that the *B. subtilis* strains may be considered non-pathogenic and safe for human consumption. Previously, Cho (13) suggested that the isolated *B. subtilis* from soybean paste was able to be used as probiotics for humans and foods.

Identification of strain CSY191 and strain CSY388

The morphological, biochemical, and physiological characteristics of CSY191 and CSY388 were analyzed (Table 2). After 24 hr of incubation at 37°C, these strains were Gram-positive and rod-shaped, measuring 0.6 to 0.8×2 to 3 μm (CS90 and CSY388 strains) and 0.6 to 0.8×2 to 3 μm (CSY191 strain). Three strains are facul-

tatively anaerobic and grew at 10 to 50°C, with an optimum growth temperature range of 30 to 37°C. These strains grew in the presence of 0 to 15% (w/v) NaCl and showed catalase and oxidase activities but no urease activity. Skim milk, gelatin, cellulose, and starch were hydrolyzed by the strains. Reactions for the oxidation and fermentation of carbohydrates as the sole carbon sources are shown in Table 2. Both CSY191 and CSY388 strains were able to use lactose as the sole carbon source, but strain CS90 was not. Also, strain CSY388 can use galactose and D-turanose, but the other two strains cannot use these carbon sources. The cellular fatty acid profiles of strain CSY191 and strain CSY388 include large amounts of saturated and branched fatty acids, with anteiso-$C_{15:0}$ (data not shown).

Morphological, biochemical, and physiological analyses clearly demonstrated that the strains were members of the genus *Bacillus*. From chemotaxonomic data, the fact that anteiso-$C_{15:0}$ was the major cellular fatty acid indicates these two strains were belong to the genus *Bacillus*. In addition, results of the physiological properties indicated that strain CSY191 and strain CSY388 differed from the other species of *Bacillus* in their carbohydrate utilization pattern.

A complete 16S rDNA sequence of the CSY191 (1,532 bp; accession number; HQ328857) and CSY388 (1,532 bp; accession number; HQ328856) were determined. A phylogenetic tree was constructed using the sequence data shown that included the CSY191 and CSY388 strains grouped within the evolutionary radiation, encompassing the genus *Bacillus* and occupying a distinct phylogenetic position within this genus. The levels of 16S rRNA similarity between the strains CSY191 and CSY388 and the *Bacillus* species ranged from 93.1 to 99.9%. The highest

Table 2. Comparison of phenotypic characteristics of probiotic *Bacillus subtilis*

Characteristics	Reaction		
	Strains		
	B. subtilis CS90[1]	*B. subtilis* CSY191	*B. subtilis* CSY191
Morphology			
Shape	Rod	Rod	Rod
Gram stain	+[2]	+	+
Cell dimension (mm)	0.6 to 0.8×2 to 3	0.5 to 0.7×2 to 3	0.6 to 0.8×2 to 3
Flagellation	+	+	+
Swarming on soft TS agar	+	+	+
Endospore formation	+	+	+
Physiological properties			
Aerobic growth	+	+	+
Growth at temperature (°C)	10 to 50	10 to 50	10 to 50
Growth in NaCl (%)	<15	<15	<15
Biochemical characteristics			
Oxidase activity	+	+	+
Catalase activity	+	+	+
Urease activity	−	−	−

Table 2. Continued

Characteristics	Reaction		
	Strains		
	B. subtilis CS90	*B. subtilis* CSY191	*B. subtilis* CSY191
Hydrolysis of			
Skim milk	+	+	+
Casein	−	−	−
Gelatin	+	+	+
Cellulose	+	+	+
Starch	+	+	+
Carbohyrates			
Glycerol	+	+	+
Ertythritol	−	−	−
D-arabinose	−	−	−
L-arabinose	+	+	+
Ribose	+	+	+
D-xylose	+	+	+
L-xylose	−	−	−
Adonitol	−	−	−
β methyl-xyloside	−	−	−
Galactose	−	−	+
D-glucose	+	+	+
D-fructose	+	+	+
D-mannose	+	+	+
L-sorbose	−	−	−
Rhamnose	−	−	−
Dulcitol	−	−	−
Inositol	+	+	+
Mannitol	+	+	+
Sorbitol	+	+	+
α methyl-D-mannoside	−	−	−
α methyl-D-glucoside	+	+	+
N acetyl glucosamine	−	−	−
Amygdaline	+	+	+
Arbutine	+	−	−
Esuline	+	+	+
Salicine	+	+	+
Cellobiose	+	+	+
Maltose	+	+	+
Lactose	−	+	+
Melibiose	+	+	+
Saccharose	+	+	+
Trehalose	+	+	+
Inuline	−	−	+
Melezitose	−	−	−
D-raffinose	+	+	+
Amidon	+	+	+
Glycogen	+	+	+
Xylitol	−	−	−
β gentiobiose	+	−	+
D-turanose	−	−	+
D-lyxose	−	−	−
D-tagatose	−	−	−
D-fucose	−	−	−
L-fucose	−	−	−
D-arabitol	−	−	−
L-arabitol	−	−	−
Gluconate	−	−	−
2 ceto-gluconate	−	−	−
2 ceto-gluconate	−	−	−

[1]Cho (13) previously reported the potential probiotic *B. subtilis* CS90.
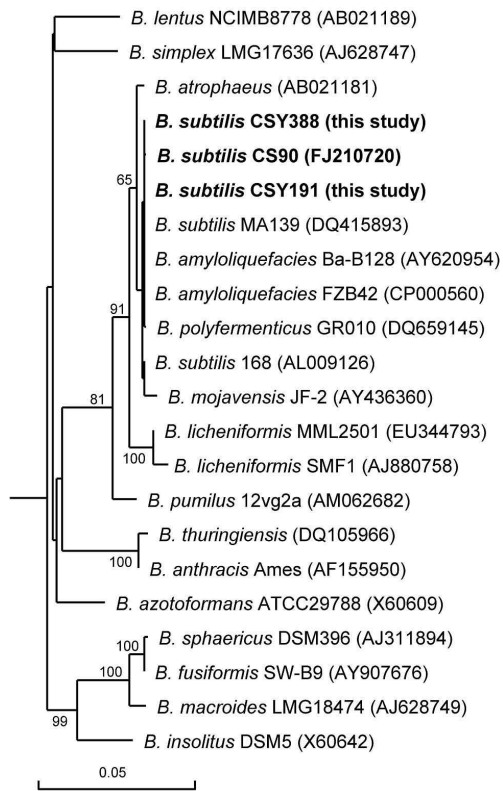[2]+, positive reaction; −, negative reaction.

Fig. 1. Phylogenetic relationships of the CS90, CSY191 and CSY388 strains and other closely related *Bacillus* sp. based on 16S rDNA. Number above each node us the confidence level (%) generated from 1,000 bootstrap trees. The scale bar is in fixed nucleotide substitutions per sequences positions.

16S rDNA sequence similarity (99.9%) was observed between CSY191 strain, CSY388 strain, and *B. subtilis*

MA139, respectively (Fig. 1). The present phylogenetic study clearly established that the CSY191 and CSY388 are closely related to *B. subtilis*.

Antimicrobial activity against food-borne pathogenic bacteria

The MeOH extractions by the potential probiotic three *B. subtilis* strains showed a broad range of antibacterial activities (Table 3). All three *B. subtilis* strains showed antibacterial activities against *S.* Enteritidis, *S.* Typhimurium, *S. sonnei*, *B. cereus*, *L. monocytogenes*, and *S. aureus*. In particular, *B. subtilis* CSY191 showed inhibitory activities against all Gram-negative bacteria tested, such as *E. coli*, *P. aeruginosa*, *S. enterica*, *S.* Enteritidis, *S.* Typhimurium, *S. flexineri*, and *S. sonnei*, known as the main pathogens causing diarrhea in humans (11,20,21). An antimicrobial activity is considered to be one of the major functions of probiotics; therefore, this function may be one of the principal criteria applied for the screening of potential probiotic strains (7,8,13, 16,22).

The antimicrobial agents from probiotic *Bacillus* strains are considered one of the principal mechanisms (microbial interference therapy) for inhibiting pathogenic microorganisms in GITs. *Bacillus* species produce a large number of antimicrobials (18), including bacteriocins and bacteriocin-like inhibitory substances (e.g., subtillin and coagulin) as well as antibiotics (e.g., surfactin, iturin, and bacilysin) (8). In particular, *B. subtilis* produces antimicrobial lipopeptides, such as surfactin,

Table 3. Antibacterial activity of probiotic *Bacillus subtilis* when incubated with 13 food-borne pathogenic bacteria

Food-borne pathogenic microorganisms	Inhibitory zone[1] (mm)		
	Strains		
	B. subtilis CS90[2]	*B. subtilis* CSY191	*B. subtilis* CSY388
Gram-negative bacteria			
E. coli KCTC[3] 1682	−[4]	8.4	8.7
P. aeruginosa KCTC 1750	13.2	15.4	−
S. enterica KCTC 12450	9.6	8.6	−
S. Enteritidis KCTC 12400	13.4	18.6	16.7
S. Typhimurium KCTC 1926	22.0	24.3	18.6
S. flexineri KCTC 2008	12.4	16.2	−
S. sonnei KCTC 2518	14.6	9.0	24.2
Gram-positive bacteria			
B. cereus KCTC 1012	24.9	25.4	23.6
L. innocua KCTC 3586	−	−	12.6
L. ivanovii KCTC 3444	12.2	21.1	−
L. monocytogenes KCTC 3569	21.7	24.3	23.2
S. aureus KCTC 1916	12.7	24.5	11.6
S. epidermidis KCTC 1917	11.8	8.5	−

[1]The antibacterial activity was estimated by measuring the diameter of the clear zone (including paper disks, 8 mm diameter) of growth inhibition.
[2]Cho (13) previously reported the potential probiotic *B. subtilis* CS90.
[3]KCTC, Korea Collection for Type Culture.
[4]−, no inhibition.

fengycin, iturin, bacillomycins, mycosubtilin, and plipastatin (23-25). Also, Teo and Tan (26) reported that the antimicrobial factor, bacteriocin, produced by a probiotic *B. subtilis* PB6. Recently, an antibiotic compound (non-proteolytic bacteriocin BP6) isolated from a probiotic *B. subtilis* BP6 strain was found to have antimicrobial activity against *Salmonella* Gallinarum (7).

Immune activity

The effect of MeOH extractions (surfactin-like compounds) on NO production in RAW264.7 macrophages was examined using a Griess reaction (27). After 24 hr of exposure (10, 50, 100, 250 or 500 µg/mL), RAW264.7 macrophages showed a concentration-dependent production of NO. In particular, a MeOH extraction of the CSY388 strain produced more NO than MeOH extractions of strain CS90 and strain CSY191 (Fig. 2). This result suggests that strain CSY191 more may have greater potential to affect the immune system than strain CS90 and strain CSY191.

Stimulation of the immune system or immunomodulation is considered an important mechanism to support probiosis (8). Surfactin exhibits diverse biological activities, including antitumoral (28), antiviral (29), antibacterial (30), hemolytic (31), and fibrinolytic activities (32). Previously, Hwang et al. (33,34) reported that surfactin inhibited mycoplasma-induced NO production in LPS-stimulated RAW264.7 cells. In addition, *natto*, a food made by fermenting cooked soybeans with *B. subtilis* (*natto*) or *B. subtilis* (var. *natto*), has been shown

to have probiotic properties, and the *B. subtilis* (var. *natto*) component is thought to stimulate the immune system and produce vitamin K2 (8). To the best of our knowledge, this is the first report on the immune activities by the isolation of the potential probiotic *B. subtilis* from *doenjang*.

In conclusion, the present study identified probiotic activities of *B. subtilis* CSY191 and *B. subtilis* CSY388 against food-borne pathogenic bacteria, such as *S. enteric, S.* Enteritidis, *S.* Typhimurium, *B. cereus, L. ivanovii, L. monocytogenes, S. aureus,* and *S. epidermidis.* In addition, methanol extractions (surfactin-like compounds) from strain CSY191 and strain CSY388 activated RAW264.7 microphages and induced the production of NO. In particular, *B. subtilis* CSY191 had stronger antimicrobial activity against all Gram-negative bacteria than other strains. Furthermore, *B. subtilis* CSY388 showed the highest the immune activity among the tested strains. From these results, we suggests that *B. subtilis* CSY191 and *B. subtilis* CSY388 was possible to use the potential probiotics.

ACKNOWLEDGMENTS

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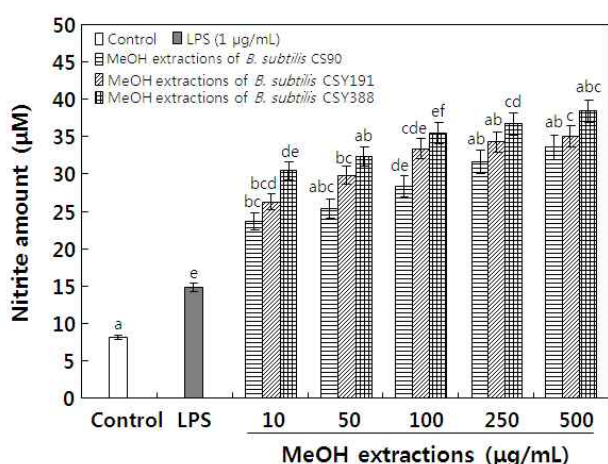

Fig. 2. Effect of MeOH extractions from *B. subtilis* CS90, CSY191 and CSY388 on nitric oxide (NO) production in RAW264.7 macrophages. As control, cells were treated with only PBS buffer (pH 7.4). RAW264.7 cells were treated with either LPS (0.1 µg/mL) or MeOH extractions (10, 50, 100, 250 or 500 µg/mL) in PBS buffer (pH 7.4). Results are expressed as mean±SD (n=3), and different letters are significantly different (p<0.05) by Duncan's multiple range test.

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